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Jiaqi Wu
Liaoning Normal University
Boting Lan
Liaoning Normal University
Peng Su
sp40462@163.com
Liaoning Normal University

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Identification, molecular evolution and synergistic mechanism of *TAK1* and *TAB* gene families in Lampreys

Jiaqi Wu[1, 2,#], Boting Lan[1, 2,#], Peng Su[1, 2, *]

1 College of Life Sciences, Liaoning Normal University, Dalian 116081, China

2 Lamprey Research Center, Liaoning Normal University, Dalian 116081, China

*Corresponding authors at: College of Life Sciences, Liaoning Normal University, Dalian, 116081, China. E-mail addresses: sp4046@163.com (Peng Su).

[#]Jiaqi Wu and Boting Lan contributed equally to this work.

Abstract

Transforming growth factor-β-activated kinase 1 (TAK1) and TAK1-binding proteins (TABs) are critical regulators of innate immune signaling pathways. In this study, *TAK1*, *TAB1*, and *TAB2* were identified from lampreys (*Lethenteron reissneri*), their evolutionary characteristics and immune functions were investigated. Multiple sequence alignment analysis, phylogenetic characterization, syntenic analysis, genetic structure analysis, and 3D structure analysis revealed that Lr-TAK1, Lr-TAB1, and Lr-TAB2 possess highly conserved functional domains and evolutionary features shared with vertebrate orthologs. Quantitative real-time PCR demonstrated that all three genes were significantly induced in immune-related tissues following poly(I) and lipopolysaccharide stimulation, indicating their involvement in antiviral and antibacterial immune responses. Furthermore, co-immunoprecipitation assays confirmes that Lr-TAK1 physically interacts with both Lr-TAB1 and Lr-TAB2 in HEK293T cells. These findings provide the first systematic characterization of the *TAK1* and *TAB* gene families in lampreys, highlight the evolutionary conservation of the TAK1-TAB signaling module in early vertebrates, and offer new insights into the origin and evolution of vertebrate innate immunity.

Keywords： TAK1 TAB Lamprey Evolution Immunity

1. Introduction

Transforming growth factor-β-activated kinase 1 (TAK1, also known as MAP3K7) is a pivotal serine/threonine kinase belonging to the mitogen-activated protein kinase kinase kinase (MAP3K) family and plays an essential role in immune and inflammatory signaling(Kajino et al., 2006). Structurally, TAK1 consists of an N-terminal kinase domain(PKD), a regulatory linker region(TAB1 BD), and a C-terminal coiled-coil domain(TAB2/3 BD), with the kinase domain mediating ATP binding and substrate phosphorylation and the coiled-coil domain facilitating interactions with regulatory adaptors(Zhang et al., 2024). These structural modules cooperate to enable precise activation of TAK1 through autophosphorylation and ubiquitin-dependent mechanisms, thereby allowing stable propagation of downstream signaling(Xia et al., 2009). Functionally, TAK1 integrates diverse upstream signals from Toll-like receptors (TLRs), interleukin-1 receptor (IL-1R), tumor necrosis factor receptor (TNFR), T-cell receptor (TCR), and B-cell receptor (BCR), inducing activation of the NF-κB and MAPK pathways and promoting the transcription of inflammatory cytokines and immune-responsive genes(Cai et al., 2022).

The activation of TAK1 strictly depends on TAK1-binding proteins (TABs), including TAB1, TAB2, and TAB3(Safina et al., 2011). TAB1 contains a PPD-like pseudophosphatase domain and a TAK1-binding region; although the pseudophosphatase domain resembles Mg^{2+}/Mn^{2+}-dependent protein phosphatases structurally, it lacks essential metal-binding and catalytic residues and therefore does not possess phosphatase activity (Conner et al., 2006). In fact, TAB1 promotes its own phosphorylation and kinase activation by inducing conformational changes in TAK1. In contrast, TAB2 and TAB3 are highly homologous signaling adaptors that exhibit a remarkably similar linear, modular domain organization(Besse et al., 2007). Both proteins consist of three key functional domains: an N-terminal CUE domain, a central interaction region(TAK1BD), and a C-terminal NZF (Npl4 Zinc Finger) domain(Besse *et al.*, 2007,Kanayama et al., 2004). Together, these domains govern the ubiquitin recognition properties, protein–protein interaction capabilities, and regulatory functions of TAB2 and TAB3 in modulating TAK1 activation(Kanayama *et al.*, 2004,Ali et al., 2024,Sato et al., 2009).

Transforming growth factor-β-activated kinase 1 (TAK1) operates as a central signaling hub that integrates diverse receptor-derived cues into coherent inflammatory responses (Xu and Lei, 2020,Mukhopadhyay and Lee, 2020). Despite the variability of adaptor molecules used by different receptor systems, TAK1 activation typically follows a conserved ubiquitin-dependent mechanism (Wang et al., 2001,Adhikari et al., 2007). In the Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) pathways, E3 ubiquitin ligases such as TRAF2 and TRAF6 catalyze the assembly of K63-linked polyubiquitin chains (Conze et al., 2008,Kawasaki and Kawai, 2014,Yang et al., 2009,Bai et al., 2022), which are specifically recognized by the C-terminal NZF domains of *TAB2* and *TAB3*(Kanayama *et al.*, 2004). This ubiquitin-sensing process recruits the TAK1–TAB complex to receptor-proximal signaling platforms(Hu and Sun, 2016). Once activated, TAK1 phosphorylates both the IKK complex and MAPK kinases, thereby triggering NF-κB and MAPK cascades that collectively drive the transcription of inflammatory genes, such as IL-6, TNF-α, and IL-1β(Xu and Lei, 2020,Janssens and Beyaert, 2002,Ninomiya-Tsuji et al., 1999,Chen, 2005). Although TAB1, TAB2, and TAB3 all participate in TAK1 regulation, their roles are functionally distinct. TAB1 directly binds the TAK1 kinase domain and promotes its autophosphorylation, whereas TAB2 and TAB3 act as ubiquitin sensors that recruit TAK1 to receptor-associated signaling complexes, enabling spatially controlled activation(Xia *et al.*, 2009,Xu and Lei, 2020,Hirata et al., 2017,Weng and Koh, 2017).

Lampreys, among the most ancient extant vertebrates, belong to the class cyclostomata and represent the earliest diverging lineage of jawless vertebrates in vertebrate evolution(Zhu et al., 2020). Owing to their unique phylogenetic position, lampreys are widely regarded as an ideal model for investigating the origin and evolution of core immune regulatory pathways in early vertebrates(Xu et al., 2016). With the continuous improvement of genomic resources, we identified *TAK1* and two homologous *TAB* genes (*TAB1* and *TAB2*) in the genome of lampreys (*Lethenteron reissneri*), providing a valuable foundation for exploring how key components of ubiquitin-dependent inflammatory signaling emerged and diversified during vertebrate evolution(Zhu et al., 2021). To further investigate this, we conducted comprehensive

bioinformatic analyses of Lr-TAK1, Lr-TAB1, and Lr-TAB2 across vertebrates, including phylogenetic reconstruction, domain and motif characterization, sequence homology assessment, synteny analysis, gene structure comparison, and three-dimensional (3D) protein modeling. Furthermore, we examined the tissue-specific expression patterns of these genes using quantitative real-time PCR and evaluated their transcriptional responses to pathogen stimulation, further elucidating the roles of *Lr-TAK1, Lr-TAB1*, and *Lr-TAB2* in innate immune regulation.

2. Methods and materials

2.1. Animals, immunization and cells

Lampreys (*Lethenteron reissneri*) were collected from the Tongjiang section of the Songhua River in Heilongjiang Province, China, and maintained in a circulating-water culture flume at 4 °C. All procedures involving lamprey husbandry and experimental operations were approved by the Animal Welfare and Research Ethics Committee of Liaoning Normal University (approval no: LL2024007). During immune stimulation, lampreys were injected with Poly I:C (a synthetic viral analog of double-stranded RNA, polyinosinic–polycytidylic acid) or lipopolysaccharide (LPS) at a dosage of 100 μg per individual. The control group received phosphate-buffered saline (PBS) at a volume of 0.1 mL per fish. Sampling time points were set at 0h, 2h, 4h, 8h, 12h, and 24h. HEK293T cells, sourced from the Cell Resource Center of Peking Union Medical College in Beijing, China, were cultured in DMEM enriched with 10% fetal bovine serum and 5% penicillin-streptomycin, and maintained at 37 ℃ under 5% CO_2 conditions.

2.2. Identification of the open reading frame (ORF) of the Lr-TAK1, Lr-TAB1, and Lr-TAB2 and plasmids synthesis

The cDNA sequences of the *TAK1, TAB1,* and *TAB2* genes were derived from a database developed by our laboratory(Zhu *et al.*, 2021). The sequence was validated using the Basic Local Alignment Search Tool X (BLASTX) software. Subsequently, the ORFs of Lr-TAK1 and Lr-TAB1, 2 were synthesized and subcloned into the pcDNA3.1 vector (GenScript) with a 3′ His tag or 3′ HA tags, respectively, named pcDNA3.1-HA-Lr-TAK1, pcDNA3.1-His-Lr-TAB1, and pcDNA3.1-His-Lr-TAB2.

2.3. Phylogeny analysis, conserved motifs, and domains analysis

The representative vertebrate species (Table 1) were selected to study the phylogenetic relationships of TAK1 and TAB family members in vertebrate evolution. Amino acid sequences for every species, excluding lampreys, were sourced from the NCBI database (https://www.ncbi.nlm.nih.gov/). The BioEdit sequence alignmenteditor was utilized to align all protein sequences. Phylogenetic trees were generated using MEGA-X software based on the neighbor-joining (NJ) method with 1000 bootstrap replicates. Conserved motifs of TAK1 and TAB proteins were identified using MEME version 5.4.1 (http://meme-suite.org/tools/meme), with the number of motifs set to 20. Protein domain architectures were predicted using the SMART online tool (http://smart.embl-heidelberg.de/)(Letunic and Bork, 2018).

Table 1 Protein sequences data of TAK1 and TAB members from different species.

Accession number	Gene name	Organism	Abridge
NP_003289.2	*TAK1*	*Homo sapiens*	Hs
XP_026367584.1	*TAK1*	*Ursus arctos*	Ua
XP_049753415.1	*TAK1*	*Elephas maximus indicus*	Emi
KAB0355831.1	*TAK1*	*Muntiacus reevesi*	Mr
NP_001101390.2	*TAK1*	*Rattus norvegicus*	Rn
KFP02094.1	*TAK1*	*Calypte anna*	Ca
XP_062464389.1	*TAK1*	*Pezoporus occidentalis*	Po
XP_046770548.1	*TAK1*	*Gallus gallus*	Gg
XP_025055161.1	*TAK1*	*Alligator sinensis*	As
XP_039385048.1	*TAK1*	*Mauremys reevesii*	Mr
AAC14008.1	*TAK1*	*Xenopus laevis*	Xl
XP_043923137.1	*TAK1*	*Protopterus annectens*	Pa
QCQ77673.1	*TAK1*	*Larimichthys crocea*	Lc
AAT07829.1	*TAK1*	*Danio rerio*	Dr
XP_032829516.1	*TAK1*	*Petromyzon marinus*	Pm
XP_061424488.1	*TAK1*	*Lethenteron reissneri*	Lr
QHR63342.1	*TAK1*	*Branchiostoma belcheri*	Bb
CAH1273356.1	*TAB1*	*Branchiostoma lanceolatum*	Bl
XP_061416785.1	*TAB1*	*Lethenteron reissneri*	Lr
XP_040215436.1	*TAB1*	*Rana temporaria*	Rt
XP_037737873.1	*TAB1*	*Chelonia mydas*	Cm
NP_006107.1	*TAB1*	*Homo sapiens*	Hs
NP_055908.1	*TAB2*	*Homo sapiens*	Hs
XP_032750888.1	*TAB2*	*Rattus rattus*	Rr

XP_046770524.1	*TAB2*	*Gallus gallus*	Gg
XP_043399053.1	*TAB2*	*Chelonia mydas*	Cm
XP_040206526.1	*TAB2*	*Rana temporaria*	Rt
NP_001303622.2	*TAB2*	*Danio rerio*	Dr
XP_019108869.1	*TAB2*	*Larimichthys crocea*	Lc
XP_061423559.1	*TAB2*	*Lethenteron reissneri*	Lr
CAH1233512.1	*TAB3*	*Branchiostoma lanceolatum*	Bl
QCQ77672.1	*TAB3*	*Larimichthys crocea*	Lc
XP_040192431.1	*TAB3*	*Rana temporaria*	Rt
XP_037757969.1	*TAB3*	*Chelonia mydas*	Cm
XP_046765313.1	*TAB3*	*Gallus gallus*	Gg
XP_032750888.1	*TAB3*	*Rattus rattus*	Rr
XP_054182792.1	*TAB3*	*Homo sapiens*	Hs

2.4. Synteny construction, gene structure analysis and tertiary structure prediction

The evolutionary origin of the *TAK1* and *TAB* families can be inferred through the identification of homologous gene relationships. To further elucidate the evolutionary characteristics of the *TAK1* and *TAB* gene families, we conducted comparative synteny analyses of their genomic neighborhoods across jawless and jawed vertebrates. Homologous gene information was retrieved from the Genomicus online platform (https://www.genomicus.bio.ens.psl.eu/genomicus), the Ensembl database (https://asia.ensembl.org/index.html), as well as the *Lethenteron reissneri* genome database established in our laboratory. The exon–intron architectures of *TAK1* and *TAB* genes were annotated using the NCBI database. Subsequently, gene structure visualization for *TAK1* and *TAB* genes across multiple species was performed using TBtools software (version: TBtools-II v2.310) in combination with the Gene Structure Display Server (GSDS) (https://gsds.gao-lab.org/Gsds_help.php). The tertiary structures were predicted using SWISS-MODEL (http://swissmodel.expasy.org/interactive).

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from normal lamprey tissues, including leukocyte, gill, liver, and supraneural body, as well as from the corresponding tissues following stimulation. RNA extraction was carried out using RNA isolater Total RNA Extraction Reagent (Vazyme, Nanjing, China) in strict accordance with the manufacturer's

protocol. Tissue samples were homogenized thoroughly and centrifuged at 12, 000 rpm for 5 min at 4 °C. After removal of the supernatant, 200 μL of chloroform was added, followed by vigorous mixing and centrifugation at 12, 000 rpm for 15 min at 4 °C. Subsequently, 500 μL of the aqueous phase was transferred to a new tube and mixed with an equal volume of isopropanol, then incubated at -20 °C for 20 min to precipitate RNA. After discarding the supernatant, the RNA pellet was washed twice with 75% ethanol. Each wash was followed by centrifugation at 12, 000 rpm for 5 min at 4 °C. The pellet was then air-dried briefly and resuspended in 20 μL of RNase-free water. RNA concentration and purity were determined by spectrophotometric analysis, and samples with A260/A280 ratios between 1.8 and 2.0 were used for subsequent experiments. First-strand cDNA synthesis was performed using the HiScript III All-in-one RT Super Mix Perfect for qPCR (Vazyme, Nanjing, China).

2.6. Quantitative reverse transcription PCR (qRT-PCR)

Specific primers for the *Lr-TAK1, Lr-TAB1,* and *Lr-TAB2* genes were designed using PrimerPremier 5.0 software (Table 2). cDNA was used as the template to determine the mRNA expression levels of *TAK1, TAB1,* and *TAB2*. Quantitative reverse transcription PCR (qRT-PCR) was performed with SupRealQ Purple Universal SYBR qPCR Master Mix (U+) (Vazyme, Nanjing, China). Each 20 μL PCR reaction contained 2 μL of cDNA (50 ng/μL), 0.4 μL of 10 mM forward primer, 0.4 μL of 10 mM reverse primer, 10 μL of 2×SupRealQ Purple Universal SYBR qPCR Master Mix (U+) (Vazyme, Nanjing, China), and 7.2 μL of ddH_2O. The real-time PCR program consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5s, 55°C for 30s, and 72°C for 30s. All reactions were conducted in triplicate (n=3), with Lr-GAPDH used as the internal reference gene. Relative transcription levels of TAK1, TAB1, and TAB2 were calculated using the 2-ΔΔCt method, and bar graphs representing expression differences were generated with GraphPad Prism.

Table 2 List of qRT-PCR primers in this study

Primers	Sequence	Size，bp
Lr-TAK1 R	5′-TGCCTCGATGCTGATGTGG-3′	19bp
Lr-TAK1 F	5′-CCGACTTTAGCACCCTCACC-3′	20bp
Lr-TAB1 R	5′-TCCGGGCCCTGCGAG-3′	15bp

Lr-TAB1 F	5′-CGAACGGGGAAGGGACTTTT-3′	20bp
Lr-TAB2 R	5′-CCGGCATCGTCAAACCTGA-3′	19bp
Lr-TAB2 F	5′-CAGGTGATGTGGCACCGATT-3′	20bp
Lr-GAPDH R	5′-AGGCTGCCAACGGACCAATG-3′	20bp
Lr-GAPDH F	5′-CGAAGATGGAGGAGCGAGTGTC-3′	22bp
Hs-TAK1 R	5′-GGGATAACTGCCGAAGCTCT-3′	20bp
Hs-TAK1 F	5′-GATCGCCGGGTAGTAGGACT-3′	20bp
Hs-TAB1 R	5′-GTCGGGCTTTGGTTGGTGA-3′	19bp
Hs-TAB1 F	5′-TGAGGAACTTTGGCTACCCG-3′	20bp
Hs-TAB2 R	5′-GGTGGCACAGGACCTACAAA-3′	20bp
Hs-TAB2 F	5′-TTTTTCAAGCCCGAGGACCA-3′	20bp
Hs-GAPDH R	5′-ACCACCCTGTTGCTGTAGCCAA-3′	22bp
Hs-GAPDH F	5′-GTCTCCTCTGACTTCAACAGCG-3′	22bp

2.7. Coimmunoprecipitation

HEK293T cells were co-transfected in a six-well plate with pcDNA3.1-HA-Lr-TAK1, pcDNA3.1-His-Lr-TAB1, and pcDNA3.1-His-Lr-TAB2 (2.5 μg per construct) plasmids. After 48 hours of transfection, whole-cell lysates were collected using an IP lysis buffer containing 50 mM Tris (pH 7.4), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, and a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). These lysates were incubated with primary antibodies overnight at 4°C, followed by incubation with Protein G Sepharose (Sigma-Aldrich, St. Louis, MO) for 3 hours at the same temperature. The next day, the immunoprecipitates were washed three times with the lysis buffer and analyzed by SDS-PAGE and Western blotting using the Enhanced Chemiluminescence (ECL) kit from AbbKine (China). The antibodies included polyclonal anti-His (diluted 1:2500), monoclonal anti-HA (1:2500), anti-mouse secondary antibody (1:5000), and anti-goat secondary antibody (1:5000), all purchased from Thermo Fisher Scientific.

2.8. Statistical analysis

All statistical analyses were carried out using GraphPad Prism 9 (GraphPad Software, Inc., USA). Each experiment was independently repeated three times, and the data are presented as the mean ± standard error of the mean (SEM). Differences between group means were evaluated using Microsoft Excel. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3. Result

3.1. Multiple sequence alignment analysis of TAK1, TAB1, and TAB2

Multiple sequence alignment (MSA) revealed that lamprey TAK1, TAB1, and TAB2 share significant sequence conservation with their human orthologs, exhibiting sequence identities of 72.34%, 58.71%, and 69.70%, respectively. These results underscore the pivotal roles of these genes in both agnathans and mammals, reflecting their evolutionary proximity and the functional constraints imposed on their gene products. Structural analysis indicated that the lamprey TAK1 protein comprises three core functional domains: a protein kinase domain (PKD), a TAB1-binding domain (TAB1BD), and a TAB2/3-binding domain (TAB2/3BD). Similarly, the TAB1 protein contains a pseudophosphatase domain (PPD) and a TAK1-binding domain (TAK1BD), while the TAB2 protein is composed of a CUE domain, a TAK1-binding domain (TAK1BD), and a zinc-finger domain (NZF) (Fig. 1).

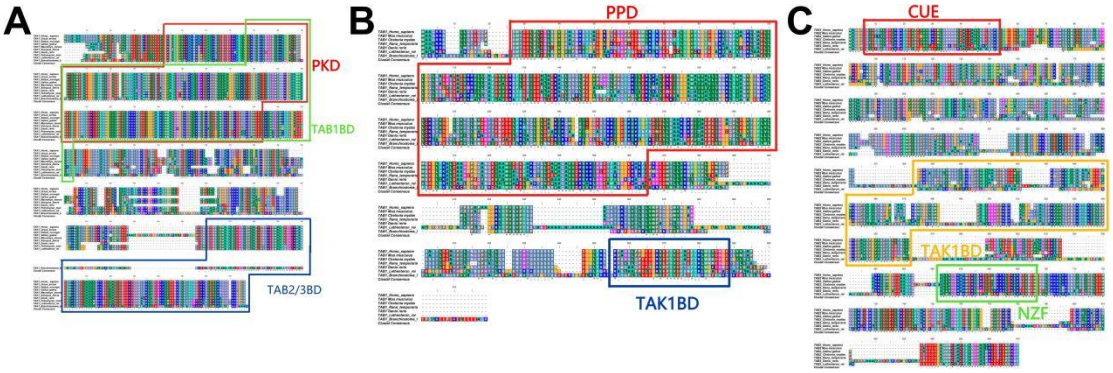


Fig 1. Comparative analysis of *TAK1, TAB1,* and *TAB2* homologous sequences using BioEdit software. (A) Multiple sequence alignment of *TAK1*: red boxes indicate PKD domains, green boxes indicate TAB1BD, blue boxes indicate TAB2/3BD domains. (B) Multiple sequence alignment of *TAB1*: red boxes indicate PPD domains, blue boxes indicate TAK1BD domains. (C) Multiple sequence alignment of *TAB2*： red boxes indicate CUE domains, yellow boxes indicate TAK1BD domains, green boxes indicate NZF domains.

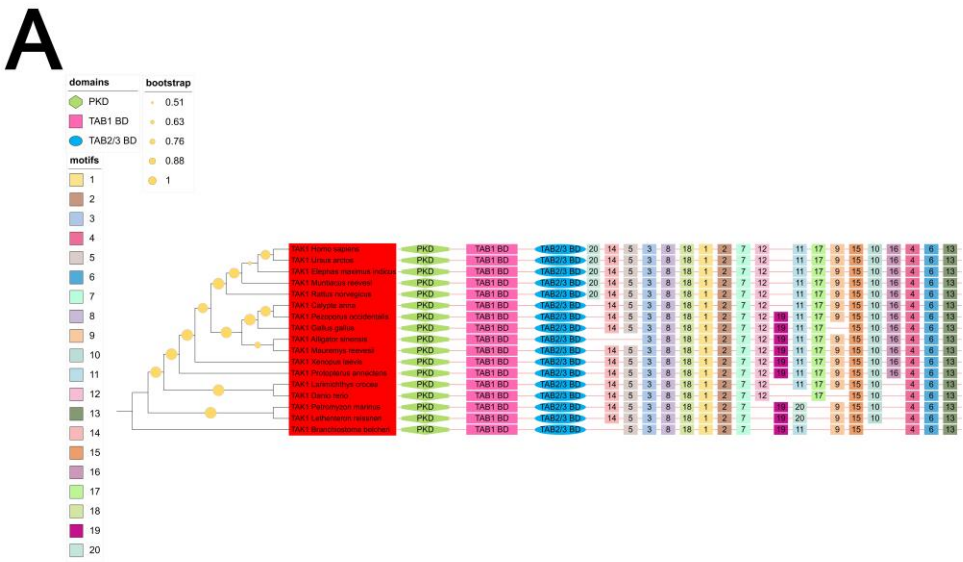
3.2. Phylogenetic characterization of the *TAK1, TAB1,* and *TAB2* genes

To investigate the evolutionary relationships of lamprey *TAK1, TAB1,* and *TAB2* genes, phylogenetic trees were constructed using the amino acid sequences of Lr-TAK1, Lr-TAB1, and Lr-TAB2 together with their homologs from other vertebrates (Fig. 2). The species included in the analysis were *Homo sapiens, Rattus rattus, Gallus gallus,*

Chelonia mydas, Rana temporaria, Danio rerio, Lethenteron reissneri, Larimichthys crocea, and *Branchiostoma lanceolatum*. The phylogenetic trees showed that TAK1, TAB1, and TAB2 proteins clustered into distinct and well-supported clades, consistent with their known functional classifications. TAK1 proteins from different species clustered into a single major branch with high bootstrap support, reflecting strong evolutionary conservation. The analysis indicated a close evolutionary relationship between Lr-TAK1 and Pm-TAK1. In the TAB family, phylogenetic analysis revealed three distinct monophyletic clades: TAB1, TAB2, and TAB3. Among them, TAB2 and TAB3 form sister clades that together constitute a sister group to TAB1. Furthermore, the highly similar species composition and distribution patterns observed in the TAB2 and TAB3 clades suggest that these two genes may have originated from a gene duplication event, and their structural and functional conservation during evolution implies potential cooperative or redundant roles.

All TAK1 orthologs contained the conserved PKD (kinase domain), TAB1 BD(regulatory linker region) and TAB2/3BD (TAB2/3-binding domain), confirming their essential role in kinase activity and complex formation, while MEME motif analysis identified 20 conserved motifs in TAK1, with motifs 1, 2, 3, 4, 6, 7, 8, 13, 15 and 18 universally present across all species and predicted to constitute the structural core of the PKD domain, and primitive vertebrates such as *Lethenteron reissneri* and *Petromyzon marinus* possess unique Motif 19 and Motif 20, which are absent in higher vertebrates. Further analysis of protein domains showed obvious differences in the domain composition among different subfamilies: the TAB1 subfamily only contains two domains, PPD and TAK1BD, while the TAB2 and TAB3 subfamilies share three common domains, including CUE, TAK1BD, and NZF. The differentiation of these domains completely corresponds to the classification of subfamilies, which directly confirms the functional division of labor among different subfamilies: TAB1 mainly binds to TAK1 through the PPD domain to regulate its kinase activity, whereas TAB2/3 rely on the CUE and NZF domains to participate in the recognition and transduction of ubiquitination signals. Meanwhile, all three subfamilies form complexes with TAK1 through the conserved TAK1BD domain, and collectively participate in the regulation

of key signaling pathways such as TGF-β and NF-κB(Xu and Lei, 2020,Chen, 2005,Hirata *et al.*, 2017).



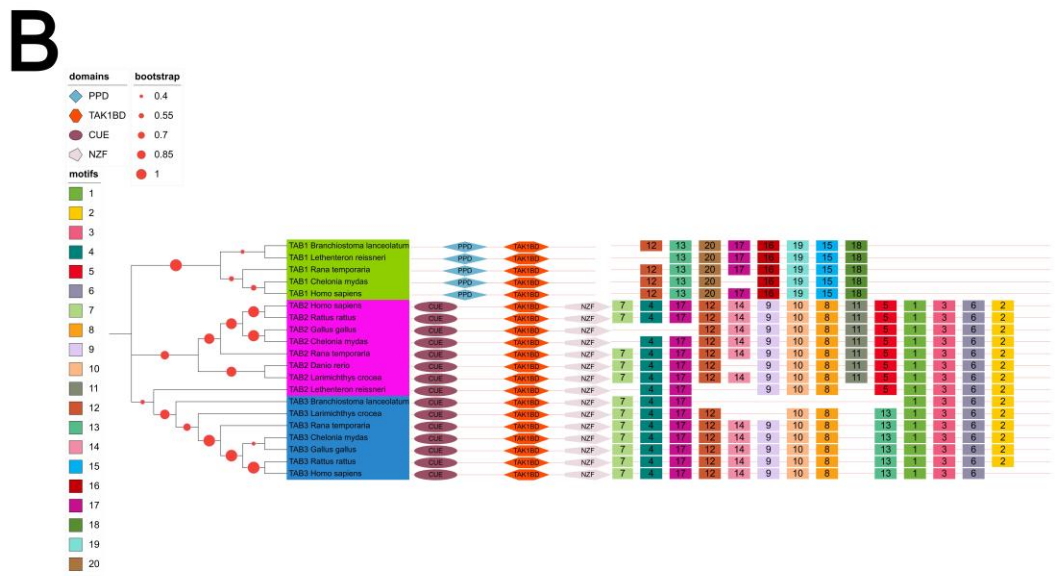


Fig 2. Phylogenetic relationships, conserved protein motifs, and domains of *TAK1, TAB1,* and *TAB3* genes in the lamprey. MEGA-X software was used. *TAK1* is highlighted in red. *TAB1* is highlighted in blue, *TAB2* in pink, and *TAB3* in green.

3.3 Syntenic analysis of TAK1, TAB1, and TAB2

Divergent evolutionary demands for gene function and regulation across different lineages have led to the diversification of chromosomal structures and gene organization patterns. These variations not only document the evolutionary history of species but also reflect the dynamic plasticity of genomes in adapting to environmental changes(Carroll, 2000). In this study, we obtained information on 10 protein-coding genes flanking the *TAK1, TAB1,* and *TAB2* genes from various species in the NCBI Gene Database. By constructing chromosomal collinearity maps, we precisely mapped the genomic distribution of these genes and conducted comparative analyses across species including humans, chickens, frogs, zebrafish, and lamprey (Fig. 3). The results demonstrated that *TAK1, TAB1,* and *TAB2*, along with their neighboring genes, exhibit conserved homologous relationships across higher vertebrates. Several adjacent genes formed continuous syntenic connections among multiple species, suggesting that this genomic region has remained relatively conserved during vertebrate evolution. Notably, *TAK1* was consistently co-localized with the *BACH2* gene, while *TAB1* showed a stable association with the *ADSL* gene across the examined species. The persistence of these conserved gene arrangements across multiple lineages indicates strong syntenic stability and suggests that these genomic regions may be subject to functional constraints during vertebrate evolution. Such conservation may reflect coordinated functional relationships among these genes, potentially related to their roles in immune regulatory pathways.

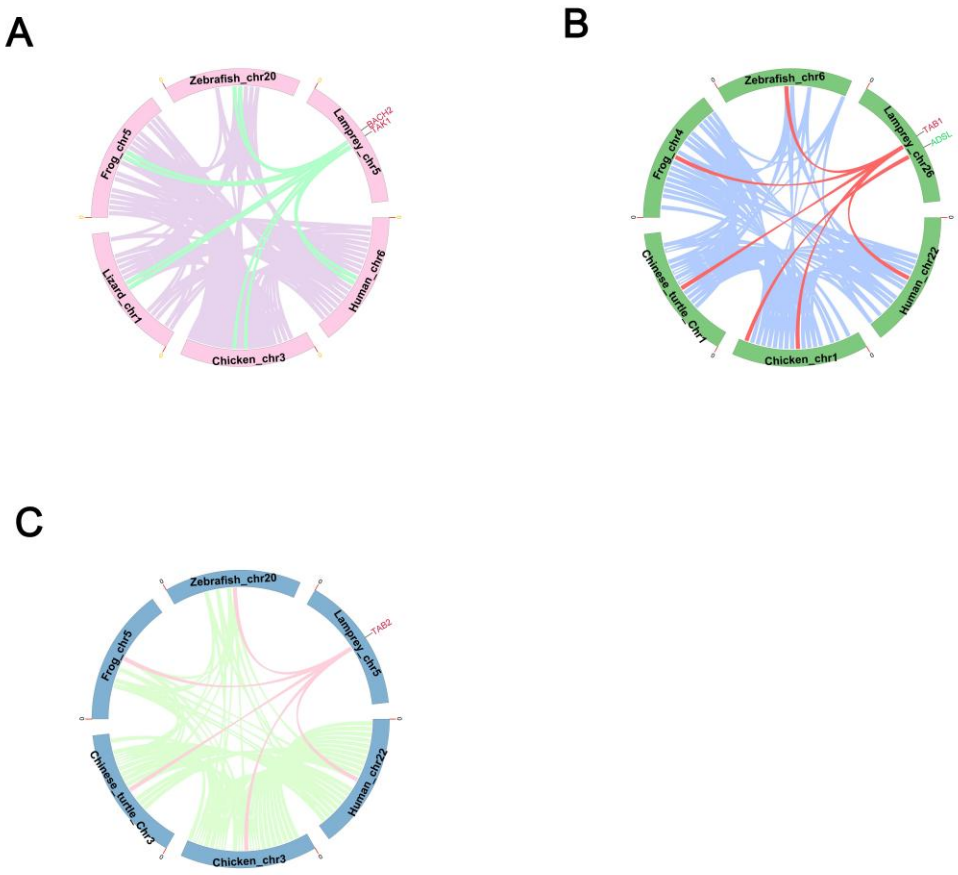


Fig 3. Homology analysis of *TAK1, TAB1,* and *TAB2* gene loci across species. (A) Homology analysis of *TAK1* across different species. (B) Homology analysis of *TAB1* across different species. (C) Homology analysis of *TAB2* across different species.

3.4. Genetic structure analysis of the *TAK1, TAB1,* and *TAB2* genes

Gene structure analysis revealed that *Lr-TAK1* consists of multiple exons, with exon number and arrangement generally conserved across vertebrates, although its overall gene length is relatively short. For instance, *the Lr-TAK1* gene is approximately 31.10 kb, substantially shorter than the human *TAK1* gene (73.49 kb), indicating a more compact gene structure (Fig. 4A). In representative vertebrate species, the TAB1 genes contain 11-13 exons (Fig. 4B). Moreover, the *Lr-TAB2* gene is considerably more streamlined, with a total length of approximately 6.0 kb, markedly shorter than human (93.90 kb) and zebrafish (20.79 kb) (Fig. 4C). In other vertebrates, *TAB2* typically comprises a few large exons separated by long introns, whereas in Lr-TAB2, the overall

structure is more compact but still retains the fundamental architecture observed in higher vertebrates. Overall, from lamprey to higher vertebrates, the intron–exon organization of *TAK1, TAB1,* and *TAB2* genes is highly conserved across vertebrates.

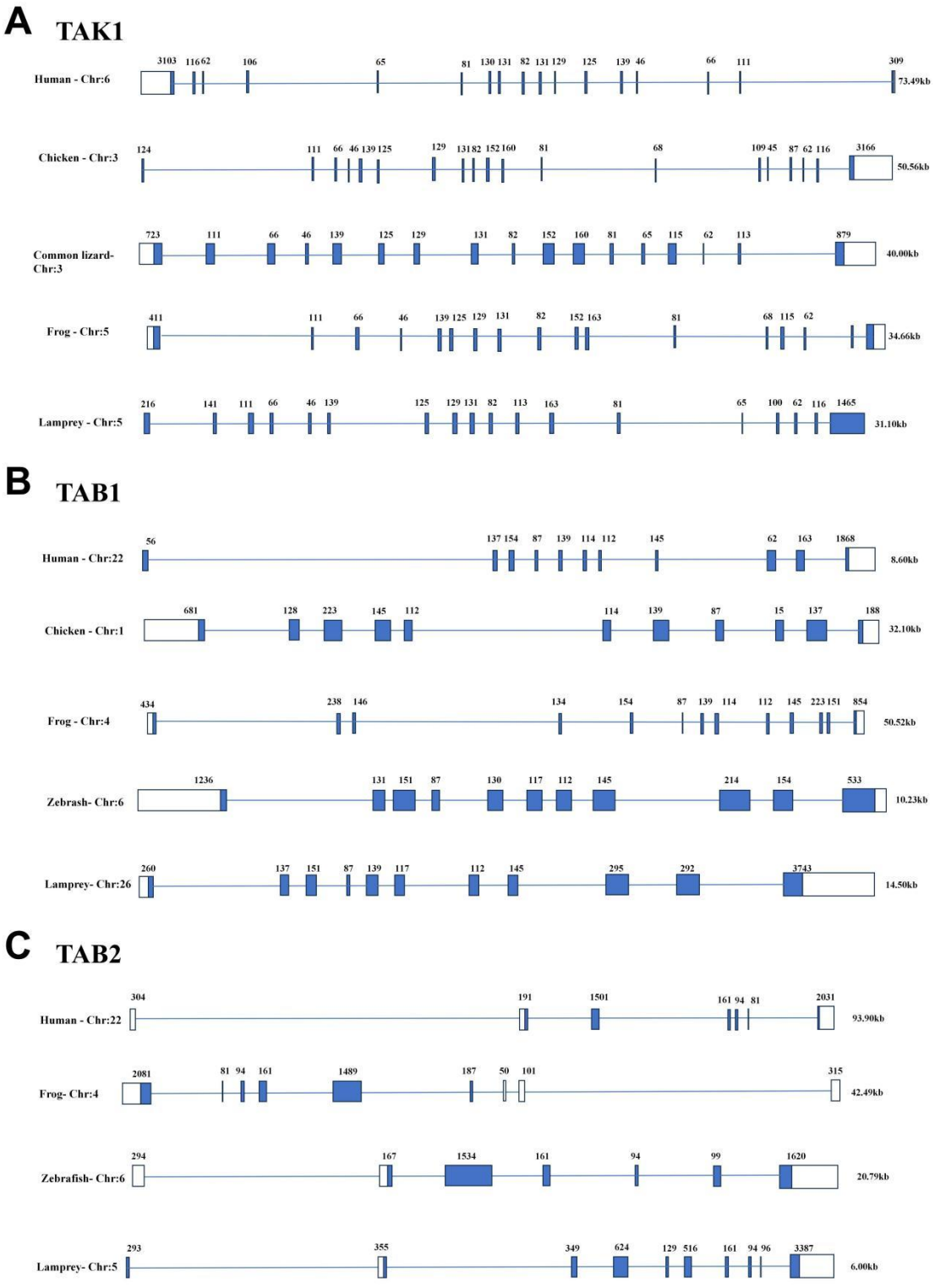


Fig 4. Schematic diagram of the structural and overall framework of *TAK1, TAB1,* and *TAB2* genes in different biological groups. (A) Comparison of the structural

organization of vertebrate *TAK1* gene. (B) Comparison of the structural organization of vertebrate *TAB1* gene. (C) Comparison of the structural organization of vertebrate *TAB2* gene. Rectangular boxes indicate exons, with numerical labels above the boxes indicating exon length.

3.5. 3D structure analysis of the TAK1, TAB1, and TAB2

Comparative models of Lr-TAK1, Lr-TAB1 and Lr-TAB2 were constructed based on the structures of Hs-TAK1, Hs-TAB1 and Hs-TAB2 (human TAK1, TAB1, and TAB2) (Fig. 5). Lr-TAK1 possesses a typical protein kinase architecture, primarily composed of PKD (residues 30–303), TAB1BD (residues 480–530), and TAB2/3BD (residues 531–579). The core PKD exhibits a characteristic bilayered fold, with its catalytic pocket formed by an N-lobe rich in β-sheets and a C-lobe dominated by α-helices. Adjacent to this core, TAB1BD constitutes an elongated stalk-like α-helix responsible for physical anchoring to the scaffold protein TAB1; whereas the TAB2/3BD at the C-terminus serves as a flexible terminal extension, providing the structural basis for ubiquitin-mediated complex assembly. Structural superimposition with Hs-TAK1 reveals that its catalytic core is highly conserved, with only minor variations observed in the surface loop region(Xu and Lei, 2020,Brown et al., 2005).

Lr-TAB1 possesses a modular architecture consistent with its role as a scaffold protein, primarily comprising a pseudophosphatase domain (residues 1-368) and a TBD (residues 480-504). The core region exhibits a globular fold characterized by a central β-sheet sandwich flanked by multiple α-helices, while the C-terminal TBD manifests as a characteristic distal α-helix that facilitates physical anchoring to the kinase domain of TAK1(Conner *et al.*, 2006). Homology modeling reveals that Lr-TAB2 adopts a highly elongated scaffold architecture, primarily comprising three functional modules: an N-terminal CUE domain (residues 8-51), a central TAK1BD (residues 450-520), and a C-terminal NZF (residues 660–693). The structural core is dominated by a prominent α-helical coiled-coil stalk, which serves as the primary scaffold facilitating the assembly of the TAK1 complex(Sato *et al.*, 2009).

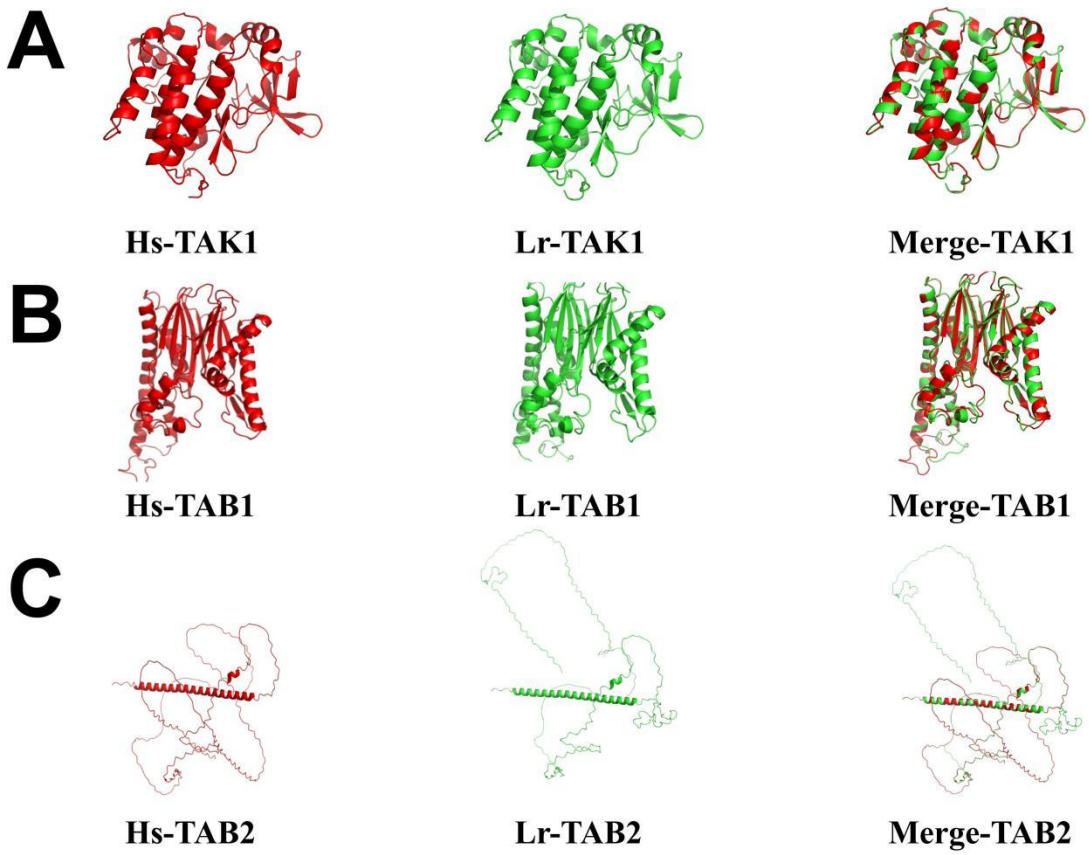


Fig 5. Three-dimensional structure prediction of TAK1, TAB1, and TAB2. (A) Three-dimensional structures of Hs-TAK1, Lr-TAK1, and Merge-TAK1 (a merged structure of Hs-TAK1 and Lr-TAK1). (B) Three-dimensional structures of Hs-TAB1, Lr-TAB1, and Merge-TAB1 (a merged structure of Hs-TAB1 and Lr-TAB1). (C) Three-dimensional structures of Hs-TAB2, Lr-TAB2, and Merge-TAB2 (a merged structure of Hs-TAB2 and Lr-TAB2).

3.6. Expression profiles of *Lr-TAK1, Lr-TAB1,* and *Lr-TAB2* after pathogen stimulation

To investigate the expression characteristics of *TAK1, TAB1,* and *TAB2* in the immune response of the lamprey, real-time quantitative PCR (qRT-PCR) was employed to detect the transcription levels of these three genes in leukocytes, liver, gills, and supraneural bodyafter polyI:C and LPS stimulation (Fig. 6). The results demonstrated that in the liver, both polyI:C and LPS significantly induced upregulation of *TAK1, TAB1,* and *TAB2* expression. In gill tissue, all three genes exhibited significant responses to both stimuli and maintained high expression levels during the late stages of stimulation. In the supraneural body, polyI:C stimulation led to a robust upregulation

of *TAK1*, *TAB1*, and *TAB2*, whereas LPS treatment only elicited transient changes in *TAK1* expression at specific time points. Conversely, in leukocytes, *TAK1* and *TAB1* were significantly induced by LPS stimulation, while the transcriptional responses to polyI:C were markedly attenuated.

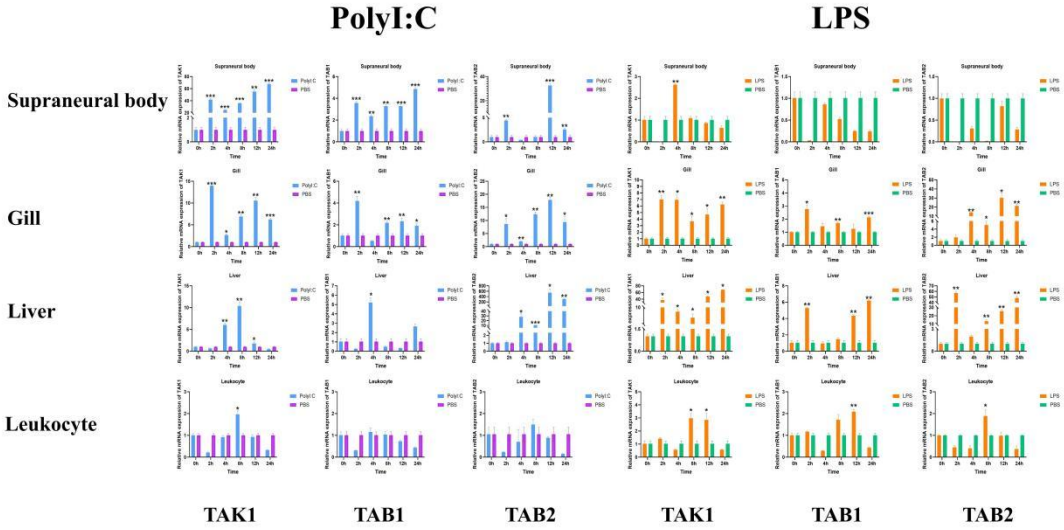


Fig 6. In vitro experiment. The expression levels of *Lr-TAK1, Lr-TAB1,* and *Lr-TAB2* in the leukocytes, liver, gills, and supraneural body tissues of eel were detected by qRT-PCR at 0, 2, 4, 8, 12, and 24 hours after stimulation with LPS and poly(I:C). Data were normalized using Lr-GAPDH expression as an internal reference, with results derived from three technical replicates (n=3; *P<0.05, **P<0.01, ***P<0.001).

3.7. Validation of the exogenous interaction between *Lr-TAK1, Lr-TAB1,* and *Lr-TAB2* in HEK293T cells

To investigate the physical interaction between *Lr-TAK1* and its adaptor proteins, *Lr-TAB1* and *Lr-TAB2*, derived from lamprey, the recombinant plasmids pcDNA3.1-HA-Lr-TAK1 and pcDNA3.1-His-Lr-TAB1 (or pcDNA3.1-His-Lr-TAB2) were co-transfected into HEK293T cells for co-immunoprecipitation (Co-IP) analysis. Western blot results (Fig. 7) revealed that Lr-TAK1 was specifically detected by the anti-HA antibody in the complexes immunoprecipitated by anti-His magnetic beads targeting Lr-TAB1 or Lr-TAB2. Conversely, no corresponding bands were observed in the IgG negative control group, ruling out non-specific binding. Additionally, the input groups

confirmed that both HA-tagged and His-tagged proteins were effectively and uniformly expressed in the cells. These results clearly demonstrate that Lr-TAK1 can form stable protein complexes with both Lr-TAB1 and Lr-TAB2 in a mammalian overexpression system, providing direct physical evidence for the further exploration of this complex's activation mechanism in the innate immune pathways of lampreys.

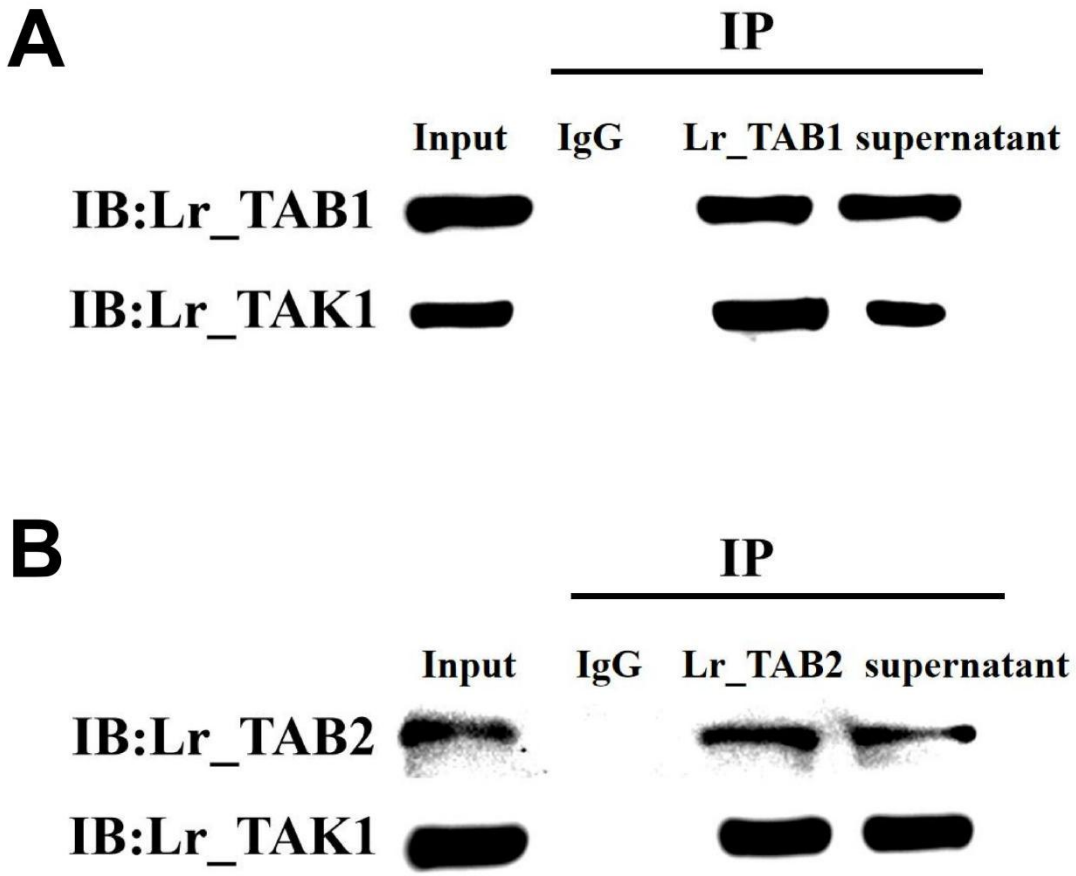


Fig 7. Coimmunoprecipitation (Co-IP) assays confirm the direct interaction between Lr-TAK1and Lr-TAB1(A) and Lr-TAK1 and Lr-TAB2 (B).

4. Discussion

As a representative jawless vertebrate, the lamprey occupies a key evolutionary position in vertebrate phylogeny and represents one of the earliest diverging lineages of extant vertebrates(Green and Bronner, 2014). Owing to its unique evolutionary status and relatively primitive immune system, the lamprey has long been regarded as an ideal model for investigating the origin and early evolution of vertebrate immune signaling pathways(Zhu *et al.*, 2020,Xu *et al.*, 2016). Therefore, characterization of *TAK1* and its regulatory adaptors TAB1 and TAB2 in lamprey provides important insights into the

ancestral features and early functional organization of the TAK1–TAB signaling module(Zhu *et al.*, 2021).

In this study, *TAK1* and two *TAB* family members, *TAB1* and *TAB2*, were identified in the genome of *Lethenteron reissneri*, whereas the additional *TAB* paralog *TAB3*, which is widely present in jawed vertebrates, was absent. This simplified composition of the *TAB* family in lamprey suggests that the diversification and expansion of *TAB* genes likely occurred after the divergence of jawless and jawed vertebrates. Such lineage-specific expansion may reflect increasing regulatory complexity and functional specialization of *TAK1* signaling in higher vertebrates, whereas the lamprey TAK1–TAB system likely represents an ancestral configuration sufficient for early immune regulation(Safina *et al.*, 2011,Kanayama *et al.*, 2004,Xu and Lei, 2020,Zhu *et al.*, 2021).

Phylogenetic analyses placed *Lr-TAK1, Lr-TAB1,* and *Lr-TAB2* at basal positions within their respective vertebrate clades, supporting their ancestral status in vertebrate evolution. In addition, comparative gene structure analyses revealed conserved exon–intron organization for *TAK1* across vertebrates, indicating strong evolutionary constraints on its genomic architecture(Ninomiya-Tsuji *et al.*, 1999). Protein domain and motif analyses demonstrated that all three lamprey proteins retain essential functional domains required for *TAK1* complex formation and signal transduction(Kanayama *et al.*, 2004,Xu and Lei, 2020,Brown *et al.*, 2005). Together, these conserved structural features suggest that *Lr-TAK1, Lr-TAB1,* and *Lr-TAB2* likely preserve fundamental biological functions shared across vertebrates.

Comparative synteny analysis revealed that the genomic regions flanking *TAK1*, *TAB1*, and *TAB2* are well conserved among jawed vertebrates but show pronounced disruption in the lamprey genome(York and McCauley, 2020). In lamprey, many neighboring homologous genes present in jawed vertebrates have undergone chromosomal relocation or deletion, resulting in fragmented syntenic blocks. This lack of conserved synteny is likely attributable to extensive chromosomal rearrangements and gene loss events during early vertebrate evolution(Xu and Lei, 2020,Bradshaw and Allison, 2022). Such large-scale genomic reorganization has been proposed as a

common feature shaping immune-related gene architecture in jawless vertebrates(Smith et al., 2018,Bajoghli, 2013).

The innate immune system relies on conserved signaling pathways, such as the NF-κB and MAPK pathways, to mount rapid responses against pathogenic challenges(Ninomiya-Tsuji *et al.*, 1999,Bajoghli, 2013). *TAK1* functions as a central signaling hub in these pathways by integrating receptor-derived signals and transmitting them to downstream effectors(Wang *et al.*, 2001,Ninomiya-Tsuji *et al.*, 1999,Brown *et al.*, 2005). In the present study, Lr-TAK1, Lr-TAB1, and Lr-TAB2 were significantly upregulated following poly(I:C) and LPS stimulation, suggesting that these genes participate in both antiviral and antibacterial innate immune responses in lamprey. The distinct tissue- and stimulus-specific expression patterns further imply potential functional specialization of the TAK1–TAB signaling module in different immune-related tissues(Safina *et al.*, 2011,Xu and Lei, 2020). Importantly, co-immunoprecipitation analysis demonstrated that Lr-TAK1 could interact with both Lr-TAB1 and Lr-TAB2, providing direct evidence for the formation of conserved TAK1–TAB complexes in lamprey. Since TAK1 is a key upstream kinase regulating NF-κB and MAPK signaling pathways, the coordinated transcriptional induction and protein interactions observed in this study suggest that the Lr-TAK1/TAB complex may play an essential role in immune signal transduction and inflammatory regulation. These findings further support the evolutionary conservation of the TAK1–TAB signaling machinery in early vertebrates(Safina *et al.*, 2011,Kanayama *et al.*, 2004,Xu and Lei, 2020,Mukhopadhyay and Lee, 2020,Wang *et al.*, 2001,Ninomiya-Tsuji *et al.*, 1999).

Our findings support the view that the TAK1–TAB module represents an ancient and fundamental component of vertebrate immune systems and offer valuable insights into the evolutionary origins of inflammatory signaling pathways. However, the precise molecular mechanisms governing the TAK1–TAB signaling remain to be fully elucidated.

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