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Shenghui Chen[a,b], Huiping Fan[a], Chenrui Ran[a], Yun Hong[a], Huixiong Feng[a], Zirui Yue[a], Hao Zhang[a], Pierre Pontarotti[c], Anlong Xu[a,d,*] and Shengfeng Huang[a,b,*]

[a]State Key Laboratory of Biocontrol, Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Guangdong Key Laboratory of Pharmaceutical Functional Genes, School of Life Sciences, Sun Yat-sen University, Guangdong, China
[b]Laboratory for Marine Biology and Biotechnology, Qingdao Marine Science and Technology Center, Qingdao, China.
[c]Aix Marseille Université, IRD, APHM, MEPHI, IHU Méditerranée Infection, Marseille, France 2 CNRS, SNC 5039, Marseille, France
[d]School of Life Sciences, Beijing University of Chinese Medicine, Beijing, China

[*]Corresponding author. School of Life Sciences, Sun Yat-sen University, 135 Xingang West Street, Haizhu District, Guangzhou 510275, Guangdong Province, People's Republic of China.

Email: lssxal@mail.sysu.edu.cn (AX)
Email: hshengf2@mail.sysu.edu.cn (SH)

Abstract

The IL-17 pathway displays remarkably diverse functional modes between different subphyla, classes and even orders, yet its driving factors remain elusive. Here, we demonstrate that the IL-17 pathway originated through domain shuffling between a TLR/IL-1R pathway and a neurotrophin-RTK (receptor-tyrosine-kinase) pathway (a Trunk-Torso pathway). Unlike other new pathways that evolve independently, the IL-17 pathway remains intertwined with its donor pathways throughout later evolution. This intertwining not only influenced the gains and losses of domains and components in the pathway but also drove the diversification of the pathway's functional modes among animal lineages. For instance, we reveal that the crustacean female sex hormone (CFSH), a neurotrophin inducing sex differentiation, could interact with IL-17Rs and thus be classified as true IL-17s. Additionally, the insect prothoracicotropic hormone (PTTH), a neurotrophin initiating ecdysis in *Drosophila* by binding to Torso, could bind to IL-17Rs in other insects. Furthermore, IL-17R and TLR/IL-1R pathways maintain crosstalk in amphioxus and zebrafish. Moreover, the loss of the Death domain in the pathway adaptor CIKSs dramatically reduced their abilities to activate NF-κB and AP-1 in amphioxus and zebrafish. Reinstating this Death domain not only enhanced NF-κB/AP-1 activation but also strengthened anti-bacterial immunity in zebrafish larvae. This could explain why the mammalian IL-17 pathway, whose CIKS also lacks Death, is considered a weak signaling activator, relying on synergies with other pathways. Our findings provide new insights into the functional diversity of the IL-17 pathway and unveil evolutionary principles that could govern the pathway and be used to redesign and manipulate it.

Significance statement

The IL-17 pathway exhibits diverse functions between animal subphyla, classes and even orders, yet what drives this remain elusive. Here, we demonstrate that this pathway originated at the dawn of eumetazoans through domain shuffling between a neurotrophin pathway and a TLR/IL-1R pathway. Unlike most new pathways that evolve independently, the IL-17 pathway remains intertwined with its donor pathways throughout subsequent evolution. We demonstrate that this intertwining has resulted in distinct functional modes for the pathways in crustaceans, insects, amphioxus and vertebrates. Our study not only provides an interesting example of cytokine origination and evolution, but also unveils crucial evolutionary principles that govern the functional plasticity of the IL-17 pathway. These principles may be applied to redesign and manipulate the pathway.

Introduction

In mammals, interleukin-17s (IL-17) were initially identified as the major effectors of T helper 17 (Th17) cells, but it is now evident that IL-17s act as versatile pro-inflammatory cytokines (1-4). Together with their cognate receptors (IL-17R) and adapter (CIKS), IL-17s form a signaling pathway crucial for various processes, including host defense, tissue repair, and the pathogenesis of cancer, inflammation, autoimmunity, bone diseases and nervous system diseases (3-5).

The mammalian IL-17 pathway typically comprises six ligands (IL-17A-F), five receptors (IL-17RA-E) and one cytoplasmic adapter (CIKS). IL-17 ligands have a C-terminal cystine knot domain for binding to IL-17Rs, while canonical IL-17Rs have a pair of extracellular fibronectin-3 domains (hereafter termed as double-FN3) for binding ligands and an intracellular SEFIR domain for binding adapters. The adapter CIKS contains a long N-terminal portion and a C-terminal SEFIR domain. While some IL-17Rs can activate signaling on their own, CIKS is considered the multifunctional "hub" of IL-17R signaling (6-9). CIKS contains various functional motifs, especially in its long N-terminal portion, including RNA-binding sites, TRAF binding sites, and a U-box with E3 ubiquitin ligase activity for TRAFs. During signaling, IL-17Rs recruit CIKS through SEFIR-SEFIR interactions, and CIKS recruits TRAF6, leading to the K63-ubiquitination of TRAF6 and subsequent activation of NF-κB, AP-1 and C/EBPs (7-9). Besides, CIKS promotes mRNA stability by directly interacting with target mRNAs or by recruiting TRAF2/5 to mobilize other RNA-binding proteins (9-11).

Despite playing prominent roles at the physiological level, the mammalian IL-17 pathway has been considered a weak signaling activator on its own (12-14). Its effects *in vivo* are believed to be mainly achieved through synergy with other cytokines (TNFα, IFNγ, TGFβ, IL-1β and IL-13), as well as through crosstalk with other pathways, including TLRs/IL-1Rs, NOTCH1, TNFRs, EGFRs and FGFRs (14-16). Due to this unique functional mode, IL-17 has been proposed to be a "chief orchestrator of immunity" (17). The mechanisms behind the evolution of the IL-17 pathway into this functional mode remain largely unresolved.

IL-17 is the only interleukin family conserved in both vertebrates and invertebrates (18-21). However, there is limited research on the functional aspects of the IL-17 pathways in lower vertebrates and invertebrates (22-33), and it is unlikely that they will be explored to the same extent as in mammals in the near future. Therefore, it is challenging to determine whether the IL-17 pathways in different phyla follow similar principles, especially if they operate in the same mode as in mammals.

There have been long-standing suggestions of structural connections between IL-17, TLR/IL-1R and neurotrophin/growth factor pathways. For example, both IL-17s and classic growth factors such as nerve growth factors (NGFs), beta transforming growth factors (TGFβs), and platelet-derived growth factors (PDGFs) have a C-terminal cystine knot structure (34). Additionally, the SEFIR domains of IL-17Rs and CIKS, as well as the TIR domain of TLRs/IL-1Rs and their adapters, belong to the same STIR domain superfamily (35). However, further exploration is needed to determine whether these pathways contributed to each other's origination.

In this study, we investigated the influence of the neurotrophin and TLR/IL-1R pathways on the IL-17 pathway. Firstly, we conducted a comprehensive phylogenomic analysis of IL-17-related proteins across major metazoan subphyla. Next, we selected representative IL-17 pathway components from crustaceans, insects, amphioxus and zebrafish for functional studies. Our findings suggest that the IL-17 pathway emerged at the dawn of eumetazoans through domain shuffling between a neurotrophin pathway and a TLR/IL-1R pathway, and subsequently, it remains intertwined with these pathways. Furthermore, our study indicates that this origin and intertwining influenced the structural and compositional evolution of the IL-17 pathway, and led to the diversification of its functional modes among different animal subphyla, classes and orders.

Results

1. Identification and annotation of IL-17-related sequences

The IL-17/IL-17R/CIKS sequences were identified and annotated using a procedure established in our previous studies (36, 37). However, we noticed a substantial number of sequences and domain structures were too divergent to be discovered by this standard procedure. To uncover them, we employed a sensitive *de novo* method originally developed to identify novel domains (**see Methods**) (38, 39). The obtained sequences were grouped into non-redundant clusters and inspected manually. We curated a collection of representative sequences (**Supplementary Dataset 1**), mainly focusing on the well-annotated species representing thirteen eumetazoan subphyla (**Fig. 1A**).

Our data suggest that the IL-17 pathway is present in all thirteen eumetazoan subphyla but absent in poriferans (the most basal living metazoans) and choanoflagellates (the closest living relatives of metazoans) (**Fig. 1A)**. We also counted the numbers of non-redundant proteins in each examined species (**Fig. 1A**). The result shows that most eumetazoan species have two to four IL-17s and IL-17Rs, but there are exceptions. For instance, amphioxus and some mollusks have over ten IL-17s, while some arthropods like *Drosophila* have one IL-17 and no IL-17R. Regarding CIKS, most species have one or two genes. However, in echinoderms, arthropods and annelids, CIKS has not been discovered, even using a sensitive method (**see Methods**).

2. Sequence divergence and structure variations of the IL-17 pathway

The protein structures of the IL-17 pathway components are highly diversified. The annotation data allowed us to trace their structural variations along the species tree (**Fig. 1**). Phylogenetic analyses confirm that the protein sequences of the IL-17 pathway are highly diversified between and within subphyla, with no observed IL-17 or IL-17R orthologs between subphyla **(Fig. S2)**, except for CIKS, which remains a single-copy gene in many species **(Fig. 2A and 2B; Fig. S3)**.

3. Appearance and disappearance of disulfide patterns in IL-17 ligands

IL-17s exhibit little sequence homology, but they all share the cystine knot structure, a feature also present in many neurotrophins and growth factors (34, 40). This structure comprises two pairs of β-strands "knotted" by three disulfide bonds from six unusually positioned cysteines, providing high structural stability and tolerance for sequence diversity in the β-strands (40-42). Our annotation data reveal that IL-17 cystine knots can be classified into five structural types (T1A/B/C, T2, and T3), based on their disulfide patterns and 3D structures (**Fig. 1B-C**; **Fig. S1**). T1A is the ancestral type, possessing all three disulfides (cysteines 1/4, 2/5, and 3/6) that covalently knot four β-strands together. T1B and T1C were derived from T1A by losing one disulfide (cysteines 2/5 and 3/6, respectively), but the remaining two disulfides still link four β-strands together. T1A/B/C constitute a super-type and represent the most prevalent cystine knot type, existing in all metazoan taxa but lost in the olfactores (tunicates and vertebrates) (**Fig. 1A**). In the chordate amphioxus, the T1A/B/C types still exist but the T2 type first emerges, whereare in tunicates and vertebrates, T1A/B/C have all been lost and T2 becomes the only existing type (**Fig. 1A**). T2 was derived from T1A by losing an important disulfide (cysteines 1/4), which leaves two β-strands unbound (**Fig. 1B-C**). Therefore, all tunicate and vertebrate IL-17s lack two cysteines to complete the cystine knot, which, as previously suggested (34), requires compensatory non-covalent interactions to stabilize the structural conformation. However, T2 is not the only type of incomplete cystine knots. The T3 type of IL-17s, discovered in cnidarians, also leaves two β-strands unbound due to the lack of cysteines 5/6 (**Fig. 1B-C**).

4. Recurrent domain degeneration and acquisition in IL-17 receptors

Typically, IL-17R is defined as a type-I transmembrane receptor with two extracellular FN3 domains (double-FN3) and an intracellular SEFIR domain. However, many IL-17Rs exhibit little sequence similarity in these domains. Consistent with this observation, detecting double-FN3 and SEFIR in many IL-17Rs proves challenging, even using conserved domain models. To understand the extent of the divergence of

IL-17R ectodomains, we defined a double-FN3 as “degenerated” when its RPS-BLAST bit-score is below 25 (where false positives significantly increase). We then counted the occurrence of "degenerated" double-FN3 in different subphyla. The results indicated that about half of IL-17Rs in each subphylum, except for amphioxus, could be considered to carry a “degenerated” double-FN3 (**Fig. 1A and 1D**). Likewise, sequence “degeneration” was also frequently observed in the SEFIR domain of IL-17Rs, though to a lesser extent than the double-FN3. Our analysis also indicated that domain “degeneration” in IL-17Rs occurred independently in different subphyla, which may be explained by the subphylum-specific rapid coevolution between IL-17Rs and their ligands and adapters. Finally, it has been observed that some IL-17Rs of cnidarians and some IL-17Rs of ambulacrarians (echinoderms and hemichordates) incorporate EGF and IGSF domains into their FN3-containing ectodomains, respectively (**Fig. 1A and 1D**). For comparison, EGFs and IGSFs are also found adjacent to the FN3-containing ectodomains in many receptor-tyrosine kinases (RTKs).

5. Repeated domain losses in the adapter CIKSs

We showed that CIKS is a single-copy gene in most species, and its evolution largely recapitulated the species tree (**Fig. 1A and Fig. 2A**). The prevalent architecture of CIKS comprises an N-terminal Death domain, a C-terminal SEFIR domain, and a long middle region with U-box E3 ligase and TRAF binding sites (the T1 type in **Fig. 1E**). This architecture is conserved from the basal eumetazoan cnidarians to the chordate amphioxus (**Fig. 1A**), suggesting that it represents the ancestral state. However, we observed domain losses in CIKS proteins from several animal clades (**Fig. 1A and Fig. 2A**; **Fig. S3**). First, all vertebrate and tunicate CIKSs lost the N-terminal Death domain (the T2 type in **Fig. 1E**). Second, one of the two amphioxus CIKSs lost not only the Death domain but also a large portion of the middle region (the T3 type in **Fig. 1E**). Third, some CIKS in cnidarians, like hydra, also lost a large part of the middle region (the T4 type in **Fig. 1E**). Finally, as a unique but most extreme case of domain loss,

CIKS is entirely absent in three subphyla, including annelids, arthropods, and echinoderms.

6. Structural and phylogenetic evidence for the domain-shuffling origin of IL-17 pathways

To investigate the origin of the IL-17 pathway, we conducted a comprehensive analysis of the domain architectures of IL-17 pathways, TLR/IL-1R pathways, RTK pathways, and cystine-knot neurotrophin/growth factor pathways (**Fig. 3**). In brief, we propose that after the split of poriferans and eumetazoans and before the radiation of eumetazoans, the IL-17 pathway originated from an event of domain shuffling that occurred between an ancient TLR/IL-1R pathway and an ancient cystine-knot neurotrophin-RTK pathway (most likely a Trunk-Torso pathway) (**Fig. 3A**). We described the evidence for this hypothesis below.

We showed that among those neurotrophin/growth factor families, five of them adopt the cystine knot structure, including Trunk, Spätzle, NGF, PDGF (including VEGF), and TGFβ (**Fig. 3B**). As for the RTK families, five of them have extracellular double-FN3 domains (occasionally triple-FN3), including Torso, Eph receptor, Insulin receptor, Tie receptor, and AXL receptor. As for the TIR domain, which has a common origin with SEFIR (35), it is present in TLRs, IL-1Rs, and their cognate cytoplasmic adapters, including MyD88, TRIF, TRAM, Mal and SARM.

It has been known that IL-17 signaling relies on the interaction between the cystine knot domain of the ligands and the double-FN3 region of the receptors. Here we found that Trunk is the only cystine-knot neurotrophin that binds with the double-FN3-containing RTK (**Fig. 3B**). According to sequence and phylogenetic analysis, Trunk is an invertebrate-specific neurotrophin, which is conserved from cnidarians to the chordate amphioxus and is the closest relative of the IL-17 family (**Fig. 2C**; **Fig. S5**). Trunk's cognate receptor, Torso, is an RTK containing no other domains but a double-FN3 in its ectodomain. Therefore, the Trunk-Torso pathway, which has been well-known for mediating terminal patterning in *Drosophila* (43-45),

exhibits the highest similarity to the IL-17 ligand and receptor ectodomains (**Fig. 3B**).

There are other indications of domain shuffling between the TLR/IL-1R and neurotrophin/growth factor pathways. For instance, the fibroblast growth factor (FGF) pathway and the IL-1/IL-1R pathway share a beta-trefoil domain in the ligands and double/triple-IGSF domains in the receptor ectodomains but differ in the receptor cytoplasmic tails (tyrosine kinase versus TIR domain) (**Fig. 3B**). Another example is the NGF-RTK pathway and the Spätzle-TLR pathway, which both feature a cystine knot structure in the ligands and N-terminal LRR repeats in the receptor ectodomains but differ in the receptor cytoplasmic tails (tyrosine kinase versus TIR domain) (**Fig. 3B**).

Regarding the IL-17 pathway, all its components (IL-17/IL-17R/CIKS) first appeared in cnidarians (**Fig. 3A**), whereas IL-1R homologs (i.e., IgTIRs), MyD88, IRAKs, cystine-knot neurotrophins, and FN3-containing RTKs could be traced even further back to the poriferans. Therefore, following the principle of maximum parsimony, we hypothesize that the IL-17 pathway originated after poriferans but before cnidarians, by splicing the receptor ectodomain of a Trunk-Torso pathway to the receptor cytoplasmic tail of a TLR/IL-1R pathway (**Fig. 3A**). This hypothesis is further supported by functional evidence from our group and others (discussed below).

7. IL-17 and neurotrophin pathways functionally intertwined in arthropods

Sequence and phylogenetic analysis suggest that two families of arthropod neurotrophins, the crustacean female sex hormones (CFSHs) and the prothoracicotropic hormones (PTTHs), are closer to IL-17 proteins than to Trunk proteins (**Fig. 2C**). However, the statistical support from phylogenetics is insufficient to convincingly classify CFSHs and PTTHs as IL-17 members. Therefore, we sought functional evidence to support such classifications.

In crustaceans, CFSHs were reported as neurotrophins in crabs and shrimps, released by neurosecretory cells in the eyestalk ganglia to induce sex differentiation and female reproductive organ development (46, 47). However, so far, CFSHs have not

been recognized as IL-17 members, nor have their receptors been identified due to high sequence divergence. In this study, we discovered IL-17Rs in decapods, and found no other IL-17 homologs except CFSHs in decapods (**Fig. 4**). To verify if IL-17Rs are receptors for CFSHs, we investigated the interactions between four CFSHs and two IL-17Rs from white shrimps. Co-IP assays showed that CFSHs physically interacted with IL-17Rs when co-expressed in mammalian HEK293T cells (**Fig. 5A**). However, Co-IP assays may produce false positives due to overexpression and protein denaturation. Therefore, we conducted a controlled Co-IP assay, in which the IL-17 from amphioxus failed to bind to the shrimp IL-17Rs (**Fig. 5A**). We then performed immunofluorescence subcellular localization assays. When expressed separately in mammalian HeLa cells, CFSHs were only present in the cytosol, whereas IL-17Rs were concentrated on the cell surface. When co-expressed, CFSHs were colocalized with IL-17Rs not only in the cytosol but also clearly on the cell surface, suggesting that CFSHs can bind to the cell surface IL-17Rs (**Fig. 5C; Fig. S7**). However, these assays might also create artifacts because both ligands and receptors were co-expressed within the same cells in these assays. We therefore proceeded to carry out flow cytometry assays to further verify the ligand-receptor interactions. Recombinant HA-tagged CFSH1 proteins were purified from bacteria and examined using Western blot (**Fig. S8**). The purified CFSH1 proteins were incubated with the 293T cells transfected with GFP-tagged IL-17R proteins, and then analyzed by flow cytometry after being stained with anti-HA antibodies (**Fig. 5E**). The results demonstrated that recombinant CFSH1 specifically bound to their surface IL-17R1 and 2. Controlled assays were performed in parallel, including replacing shrimp CFSH1 with insect PTTH (from the lygus bug), but PTTH could not bind to the surface shrimp IL-17R1 (**Fig. 5E**). Taken together, these findings confirm the interactions between shrimp CFSHs and IL-17Rs.

In insects like *Drosophila*, Trunk mediates embryonic terminal patterning and molting, whereas PTTH mediates light avoidance, molting and metamorphosis (43, 45, 48). Trunk and PTTH are considered related neurotrophins because they both exert their roles by binding to the same RTK called Torso (43-45, 49). However, unlike

Trunk, which is an ancient RTK and conserved in different invertebrate phyla, PTTH is specific to insects and shown to be clustered with IL-17/CFSH rather than with Trunk (**Fig. 2C**). Curiously, we identified IL-17Rs in many insect orders but not in dipterans (e.g., *Drosophila*) and lepidopterans (e.g., silkworms). This observation prompted us to hypothesize that the loss of IL-17Rs in dipterans and lepidopterans caused their PTTH to use Torso as the receptor. However, other insects, such as the hemipteran lygus bug (*Lygus hesperus*), possess IL-17Rs. We, therefore, hypothesized that PTTH may interact with IL-17Rs in these insects. To test this hypothesis, we selected PTTH and two IL-17Rs from the lygus bug to carry out Co-IP assays and subcellular localization assays. With proper controls, these assays indicated lygus PTTH, but not amphioxus IL-17L1, could bind with both lygus IL-17R1 and 2 (**Fig. 5B and 5D**). Flow cytometry assays further confirmed that purified recombinant lygus PTTH (Fig. S10) could specifically bind to the surface lygus IL-17R1 and 2, while recombinant shrimp CFSH1 could not bind lygus IL-17R1 (**Fig. 5F**). Taken together, these findings are the evidence that in insects, the PTTH-IL-17R pathway remains intertwined with the Trunk-Torso pathway.

8. Crosstalk between amphioxus IL-17R and TLR/IL-1R pathways

Amphioxus, recognized as the most basal living chordate, possesses thirteen IL-17s (twice the number in humans), five IL-17Rs and two CIKSs. The T2 structural type of IL-17s, which includes all vertebrate IL-17s, first emerged in amphioxus (7 genes) (**Fig. 1**). Meanwhile, the ancestral structural type (T1A) and two other derived types (T1B/C) of IL-17s, commonly found in invertebrates but lost in vertebrates, are all preserved in amphioxus (1/3/2 genes for T1A/B/C, respectively) (**Fig. 1 and Fig. S1**). Additionally, amphioxus has two CIKSs: one similar to other invertebrate CIKSs, preserving the Death domain, and the other similar to vertebrate CIKSs, lacking the Death domain (**Fig. 1**).

In terms of molecular functions, Co-IP assays using HEK293T cells suggested interaction between amphioxus IL-17 ligand IL-17L1 and receptor IL-17R1 (**Fig. S6**).

Co-IP assays and luciferase reporter assays also demonstrated that amphioxus IL-17R1 interacted with both CIKS1 and CIKS2, and both CIKS1 and CIKS2 could activate NF-κB and AP-1 (**Fig. 6A-D**). Moreover, both CIKS1 and CIKS2 could interact with TRAF6 and enhance subsequent NF-κB activation (**Fig. 6E-G**). These findings suggest that amphioxus IL-17R1 recruited TRAF6 to activate NF-κB and AP-1 through CIKS1/2, thus recapitulating the signaling cascade of the mammalian IL-17R pathway (6, 14).

Given that the TLR/IL-1R pathway contributed the receptor cytoplasmic tail and adapters to the IL-17R pathway, the two pathways could maintain crosstalk in amphioxus. Through Co-IP assays, we detected physical interactions between TLR1 and CIKS1 (**Fig. 6H**), as well as between IL-17R1 and MyD88 (**Fig. 6I**), indicating that the receptors of two pathways retain the abilities to recruit each other's cognate adapters. Furthermore, Co-IP assays and subcellular localization assays revealed interactions between MyD88 and CIKS1 as well as CIKS2 (**Fig. 6J and Fig. S10**), meaning that the cognate adapters of the two pathways are capable of direct crosstalk. Notably, the reporter assays indicated that CIKS1 enhanced the activating effect of MyD88 on NF-κB, while conversely, CIKS2, lacking a Death domain, exerted an inhibitory function in this process (**Fig. 6K**).

Moreover, IL-1R associated kinases (IRAK) are also key downstream signal transducers for the TLR/IL-1R-MyD88 pathway (50). Amphioxus preserves two ancient lineages of IRAKs, Pelle and IRAK4, both shown to be parts of the amphioxus MyD88-TRAF6 signaling complex (51). We suspected that these IRAKs are also recruited by CIKS1, through the interactions between their closely-related Death domains (**Fig. 2B**; **Fig. S4**). Indeed, Co-IP assays showed that CIKS1 was associated with Pelle and IRAK4 (**Fig. 6L**). Reporter assays further showed that Pelle and IRAK4 could weaken the activity of CIKS1 when co-expressed in HEK293T cells (**Fig. 6M**), which agrees with an early observation that amphioxus Pelle and IRAK4 mildly suppressed MyD88-mediated NF-κB activation (51). Taken together, these findings showed that TLR, MyD88, Pelle and IRAK4, the cognate signaling components of the

TLR/IL-1R pathway, could interact with the amphioxus IL-17R pathway.

9. Crosstalk between zebrafish IL-17R and TLR/IL-1R pathways

We further investigated the crosstalk between the IL-17R and TLR/IL-1R pathways in zebrafish (**Fig. 6N**). Zebrafish possess three CIKSs (CIKS1/2a/2b), all of which are conserved in other cyprinids such as grass carp. Through Co-IP assays, we detected interactions between TLR4 and three CIKSs (CIKS1/2a/2b) (**Fig. 6O**), as well as between three IL-17Rs (IL-17R1a/AL/C) and MyD88 (**Fig. 6P**), indicating that the two zebrafish pathways maintain the capacity to recruit each other's key adapters. Reporter assays suggested that IL-17RAL had a significant inhibitory effect on MyD88-mediated NF-κB activation in HEK293T cells (**Fig. 6Q**). This is reminiscent of an early discovery that mouse IL-17RD could negatively regulate TLR signaling by interacting with the TLR adapters MyD88, TRIF and Mal (15). Moreover, Co-IP assays and subcellular localization assays showed that, like amphioxus CIKSs and MyD88 (**Fig. 6J**), zebrafish CIKSs and MyD88 could also directly interact with each other (**Fig. 6R and Fig. S11**). Because all zebrafish CIKSs lack the N-terminal Death domain like amphioxus CIKS2 does, we tested whether zebrafish CIKSs could inhibit MyD88 activity as amphioxus CIKS2 did. Zebrafish CIKS2a/2b showed some inhibitory effect (**Fig. 6S**), but not as strong as amphioxus CIKS2 did (**Fig. 6K**).

10. Loss of the Death domain in CIKS attenuated the direct signaling ability of the IL-17R pathway in both amphioxus and zebrafish

Reporter assays indicated that in amphioxus, CIKS2, which lost the N-terminal Death domain, was much less potent in activating NF-κB and AP-1 compared to CIKS1 and MyD88 (**Fig. 6C-D**). In fact, amphioxus CIKS1 appeared to be more potent than MyD88 (**Fig. 6C-D**). We reasoned that amphioxus CIKS1 may be as strong, if not stronger, a signaling activator than MyD88 because its Death domain can recruit IRAKs to enhance downstream signaling. Indeed, our previous study demonstrated that in amphioxus, when MyD88 lost its Death domain, its ability to activate NF-κB was

abolished (52). To verify whether the Death domain is essential for the activity of amphioxus CIKS1 and CIKS2, we created a mutant CIKS1 without the Death domain and a mutant CIKS2 with the Death domain restored (**Fig. 7A**). Subsequently, we carried out reporter assays to compare their activity with wild-type CIKS1 and CIKS2. It revealed that the Death-less CIKS1 lost the ability to activate NF-κB and AP-1. Conversely, by fusing the Death domain (from CIKS1) to CIKS2, the activity of CIKS2 in inducing NF-κB and AP-1 could be enhanced by tens to hundreds of times (**Fig. 7B-C**).

The mammalian IL-17 pathway has long been considered a weak activator on its own (12-14). As a result, it has been proposed as a "chief orchestrator of immunity" through synergy and crosstalk with many other pathways (14-17). We hypothesized that the loss of the Death domain in CIKS is one of the main factors contributing to the attenuation of the direct activity of the IL-17 pathway in mammals and other vertebrates. In line with this hypothesis, reporter assays conducted in HEK293T cells revealed that all three zebrafish CIKSs were much less potent than MyD88 in terms of NF-κB and AP-1 activation (**Fig. 7D-F**). On the other hand, when restoring the Death domain (from zebrafish MyD88) to these CIKSs (DD-CIKSs), their activating abilities could be restored and reached an even higher level than MyD88 (**Fig. 7D-F**). However, these assays were conducted with mammalian cell lines and might produce artifacts. Therefore, we injected these DD-CIKS constructs into zebrafish embryos and selected those GFP-labeled, DD-CIKS-expressing embryos for reporter assays (**Fig. 7G**). The results indicated that in zebrafish embryos, DD-CIKSs exhibited significantly higher activating abilities than wild-type CIKSs (**Fig. 7H-J**). Finally, we injected CIKS1 and DD-CIKS1 into zebrafish eggs to see if they could enhance immunity. DD-CIKS1 was chosed given that among three CIKS genes, it exhibited the most consistent high effect on NF-κB in both HEK293T cells and embryos. Fourty-eight hours later, we infected the embryos by puncturing the egg yolk with live bacteria (*Vibrio anguillarum*). Subsequent observation indicated that GFP-labeled, DD-CIKS1-expressing larvae exhibited consistently higher survival rates than those expressing wild-type CIKS1

(**Fig. 7K; Fig. S12**). These results reveal that reinstating the Death domain to CIKS1 enhanced not only the signaling ability but also the anti-bacterial immunity in zebrafish larvae. Taken together, our findings suggest that the loss of the Death domain in CIKS attenuated the direct activating ability of the vertebrate IL-17 pathway.

Discussion

1. The origin and evolution of the IL-17 pathway influenced by neurotrophin and TLR/IL-1R pathways

In this study, we presented structural, phylogenomic and functional evidence supporting the idea that the IL-17 pathway originated at the dawn of eumetazoans through domain shuffling between a TLR/IL-1R pathway and a Trunk-Torso-like cystine-knot neurotrophin-RTK pathway (**Fig. 3A**). In line with this, multicellularity at this era is believed to be accompanied by the assembly of many novel intercellular signaling proteins through domain shuffling (53-55). Besides, there are two other possible instances of domain shuffling related to TLRs/IL-1Rs and cystine knot neurotrophins, one involving the NGF-RTK and Spätzle-TLR pathways, and the other involving the FGF-FGFR and IL-1-IL-1R pathways (**Fig. 3B**). These four derived pathways appear to have evolved independently after their emergence. In contrast, the evolution of the IL-17 pathway appears to have been continuously intertwined with its donor pathways. For example, here we showed that in arthropods, CFSH and PTTH were closer to IL-17s and capable of binding with IL-17Rs. In *Drosophila*, however, PTTH actually signals through binding with Torso, the cognate RTK receptor of the Trunk-Torso pathway (45). Moreover, in both amphioxus and zebrafish, the IL-17R pathway has been shown to maintain crosstalk with the TLR/IL-1R pathway. In line with this, there have been other studies suggesting crosstalk between the IL-17 pathway and its donor pathways: in *C. elegans*, IL-17s signal through IRAKs, the downstream signal transducers of TLRs/IL-1Rs (30, 31); in mammals, IL-17RD inhibits TLR-induced inflammation by interacting with the TLR adapters MyD88, TRIF and Mal (15).

2. Neurotrophin and TLR/IL-1R pathways contribute to the structural and functional plasticity of the IL-17 pathway

The IL-17 pathway exhibits significant structural diversity both between and within subphyla (**Fig. 1**). In addition to recurrent domain degeneration, losses and gains, key

components have been lost. For instance, the T1A/B/C structural types of IL-17s are entirely lost in vertebrates and urochordates, CIKS adapters are absent in echinoderms, annelids and arthropods, and both IL-17Rs and CIKSs are missing in certain insect orders such as *Dipetera* and *Lepidoptera*. Viewed from a different perspective, this structural diversity may reflect functional plasticity. First, the cystine-knot structure is known for its ability to tolerate significant diversity in its β-strands (40-42), enabling cystine-knot neurotrophins to bind with very different receptor structures, such as NGF signaling through both the RTK TrkA and the tumor necrosis factor receptor NGFR. In this sense, it may not be surprising to observe that insect PTTHs could interact with both IL-17R and RTK Torso, especially given that both Torso and IL-17R adopt a similar double-FN3 ectodomain structure, and the Trunk-Torso pathway is presumed to be a donor pathway for the IL-17 pathway (**Fig. 3**). Second, the sharing of intracellular adapters between the IL-17 pathway and the TLR/IL-1R pathway could relax purifying selection, enabling the IL-17 pathway to tolerate domain losses or even component losses. For example, the direct signaling activity of the vertebrate IL-17R pathway was significantly reduced by the loss of the Death domain in CIKS (**Fig. 7**). This reduction could cause the pathway to rely on synergy with other pathways (14-16), rendering the pathway into a chief immune "orchestrator" rather than a strong immune "activator" (17). Another example concerns those animal subphyla (echinoderms, annelids, and arthropods) absent with CIKS; in these cases, their TLR/IL-1R adapters might play a crucial role in the IL-17R pathway, but it requires verification. Taken together, we propose that the intertwining with its donor pathways contribute to the structural and functional plasticity of the IL-17 pathway.

3. The diverse physiology of the IL-17 pathway related to neurotrophin and TLR/IL-1R pathways

The intertwining between the IL-17 pathway and its donor pathways may also contribute to the diverse physiology of the IL-17 pathway. It is known that classic cystine knot growth factors, such as NGFs, PDGFs, TGFβs, Spätzles and Trunks, can

function as important neurotrophins. In line with this, in ecdysozoans, IL-17s appear to primarily function as neurotrophins. For example, Crustacean CFSHs are released by neurosecretory cells in the eyestalk ganglia to induce sex differentiation and female productive phenotypes (46, 47). *Drosophila* PTTH is produced by the brain, promotes light avoidance, and mediates molting and metamorphosis (43, 45, 48). *C. elegans* IL-17s are secreted by neurons and modulate sensory responses, escape behaviors, associative learning, longevity and immunity (30, 31). On the other hand, in mammals, the IL-17 pathway is mainly considered a key regulator in immunity, including regulating the activity of its donor pathway, the TLR/IL-1R pathway. However, the mammalian IL-17 pathway also has diverse non-immune functions in cancer, bone diseases and the nervous system diseases, such as neurodegeneration, depression and autism (3-9). In mammals, the IL-17R pathway also promotes other growth factor pathways, such as EGFR, FGFR, and NOTCH1, through crosstalk with them (9).

4. Concluding remarks

Currently, the diverse canonical and non-canonical functions of the IL-17 pathway are under intense study both in mammals and various non-mammal species. In this study, we have unveiled crucial evolutionary principles that could govern the functional plasticity and diversity of the IL-17 pathway, as observed among different animal subphyla, classes, and even orders. In addition to offering a new framework for understanding and investigating the functions of the IL-17 pathway, these evolutionary principles could be employed to control and redesign the pathway for potential applications.

Materials and Methods

A comprehensive search for metazoan IL-17-related sequences was conducted on NCBI NR and TSA_NR databases, using the standard homology-based procedure as described (36, 37), with modifications. A more sensitive, in-house *de novo* method developed for identifying novel domains (38, 39) was employed to recover extremely divergent sequences. Retrieved sequences were manually curated before phylogenetic inference, structural prediction and other bioinformatic analyses. Sequences encoding IL-17-related proteins were cloned or synthesized from lygus bug *Lygus Hesperus*, shrimp *Penaeus vannamei*, amphioxus *Branchiostoma belcheri* and zebrafish *Danio rerio*. Interactions between recombinant ligands, receptors and adaptors were analyzed using human cell lines with Co-immunoprecipitation assays, subcellular co-localization immunofluorescence assays and flow cytometry assays. Activities between interacting receptors and adaptors were assessed by luciferase reporter assays in either human cell lines or zebrafish embryos. The effects of zebrafish CIKS1 with the reinstated Death domain on antibacterial immunity were evaluated using transient transgenic zebrafish larvae. For details on materials and methods, see ***SI Materials and Methods***.

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Author contributions: S.H., A.X. and P.P. designed research. S.C. performed experiments and analyzed data. H.F., C.R., Y.H., H.F., Z.Y. and H.Z. also conducted some experiments and analyzed the data. S.H. and S.C. drafted the manuscript. A.X. and P.P. proofread the manuscript. All authors discussed and agreed on the results and approved the manuscript.

Competing interests: The authors declare no competing interest.

Figures

Figure 1

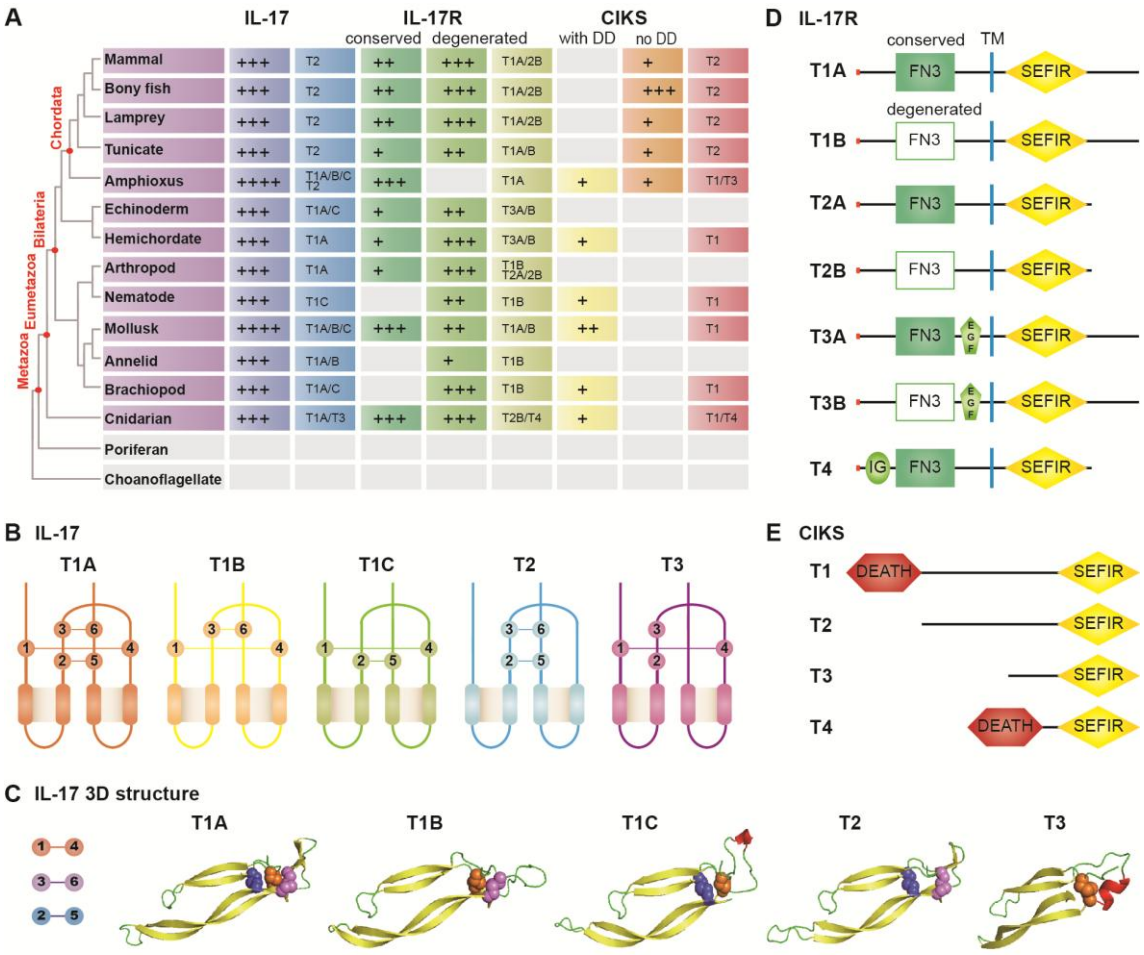

Figure 1. Compositional and structural diversity of the IL-17 pathway components among different metazoans.

(A) Distribution of the IL-17 pathway components in different species. Symbols +, ++, +++, ++++ represent 1, 2, 3-9 and 10+ genes per species, respectively. “Conserved” and “degenerated” IL-17Rs contain conserved and extremely diverged FN3 domains, respectively. Grey boxes represent no genes found in the species. DD: Death domains.

(B) The 2D structures of the cystine-knot domains of different IL-17 types. The balls numbered 1-6 represented conserved cysteines in the cystine-knot domains. The thin lines indicate the disulfide bonds between these cysteines. More details of the structural features are presented in the multiple sequence alignment in **Fig. S1**.

(C) The 3D structures of different IL-17 types. Three disulfide bonds are shown by balls in different colors. Yellow: β-sheet, red: α-helix, green: random coils.

(D) Different architectures of IL-17Rs.

(E) Different architectures of adaptor CIKSs.

Figure 2

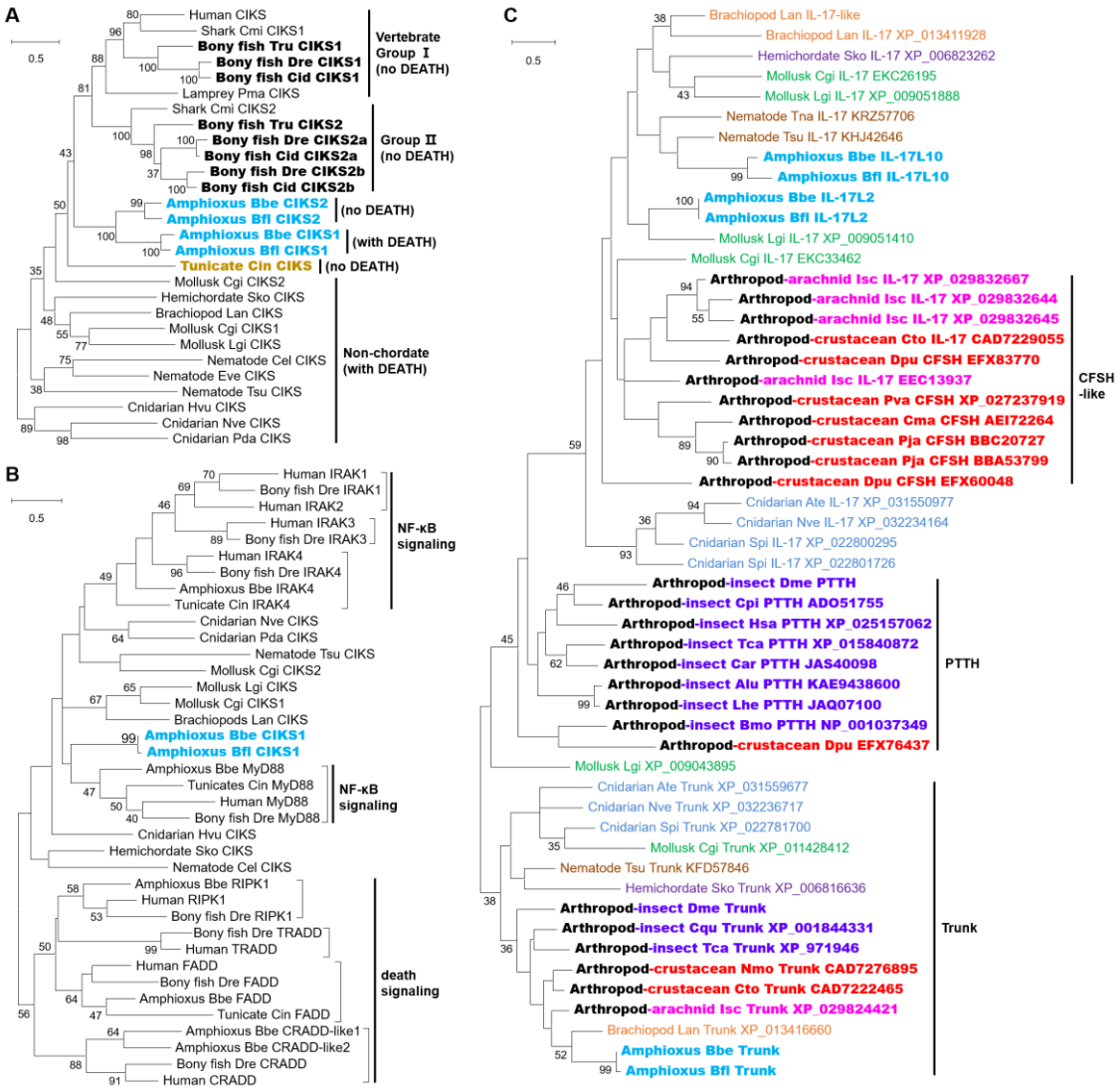

Figure 2. Phylogenetic analysis of IL-17 and CIKS proteins.

(A) Phylogenetic analysis of CIKSs based on the SEFIR domains.

(B) Phylogenetic analysis of CIKSs based on the Death domains.

(C) Phylogenetic analysis of invertebrate Trunks, PTTHs, CFSHs and IL-17s.

These trees were constructed using the Maximum Likelihood method (**see Methods**). The bootstrap values lower than 30 are omitted in the tree. Trees constructed using the Minimum Evolution method are provided in **Fig. S3-5**. The full species names are given in **Tables S3**.

Figure 3

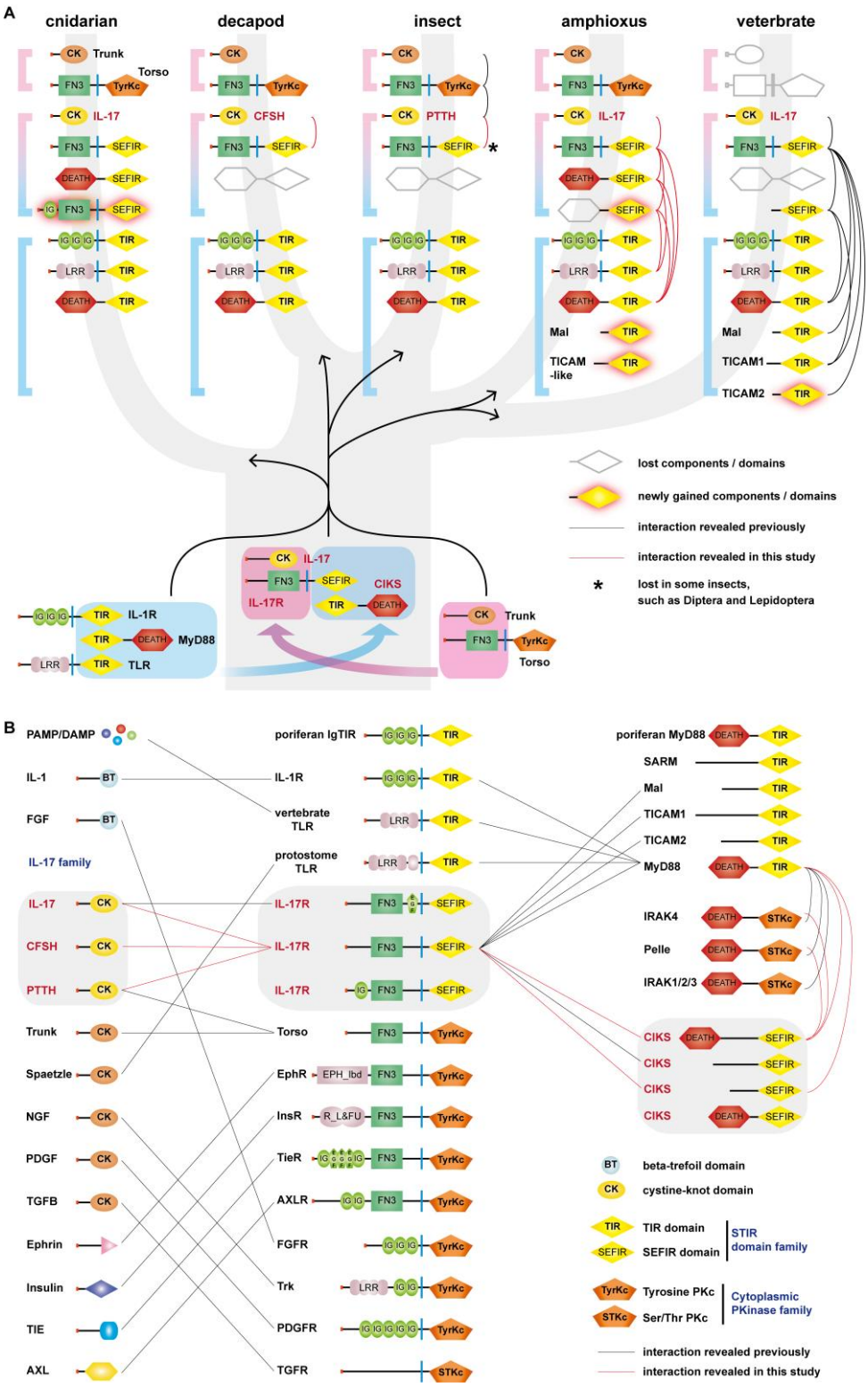

Figure 3. The relations between the IL-17 pathway, the TLR/IL-1R pathways and the neurotrophin-RTK pathways

(A) A schematic summarizes the domain-shuffling origin of the IL-17 pathway, the subsequent gains and losses of domains and components, and the crosstalk (interactions) with its donor pathways. Five animal taxa were chosen as representatives.

(B) A schematic shows the similar domain architectures, interactions and crosstalk among IL-17

699 pathways, TLR/IL-1R pathways, the neurotrophin-RTK pathways and other related RTK pathways.
700 The cystine-knot superfamily of neurotrophins and growth factors include NGFs, TGFs, PDGFs,
701 Spätzles, Trunks, and IL-17s. The ligands, receptors and adapters of the IL-17 pathway are boxed and
702 highlighted in red color. Validated interactions are indicated by lines.
703

Figure 4

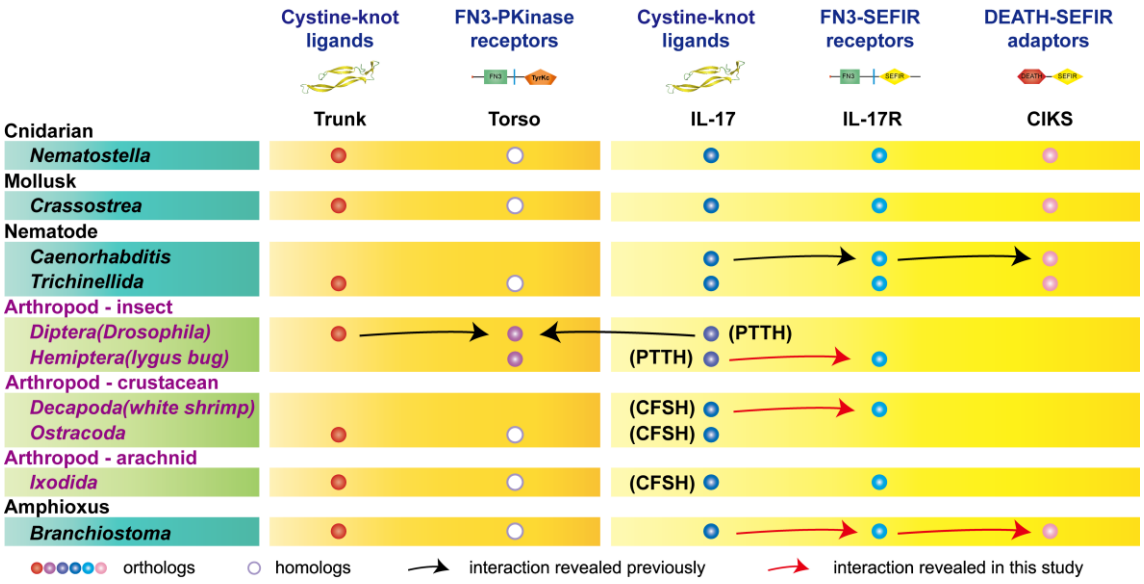

Figure 4. An overview of the presence and absence of the IL-17 pathway components in cnidarians, mollusks, nematodes, arthropods and amphioxus.

For comparison, Trunk and Torso, the ligand and receptor of an ancient cysteine-knot neurotrophin pathway, and the presumed contributors to the extracellular components of the IL-17 pathway, are included. The functionally verified interactions between components are either taken from early literature (black arrows) or revealed in this study (red arrows). The evidence for the interactions between arthropod PTTH, CFSHs and IL-17Rs is presented in **Fig. 5** and **Fig. S7-9**. The evidence for the interactions between amphioxus IL-17, IL-17R and CIKS is presented in **Fig. 6** and **Fig. S6**.

Figure 5

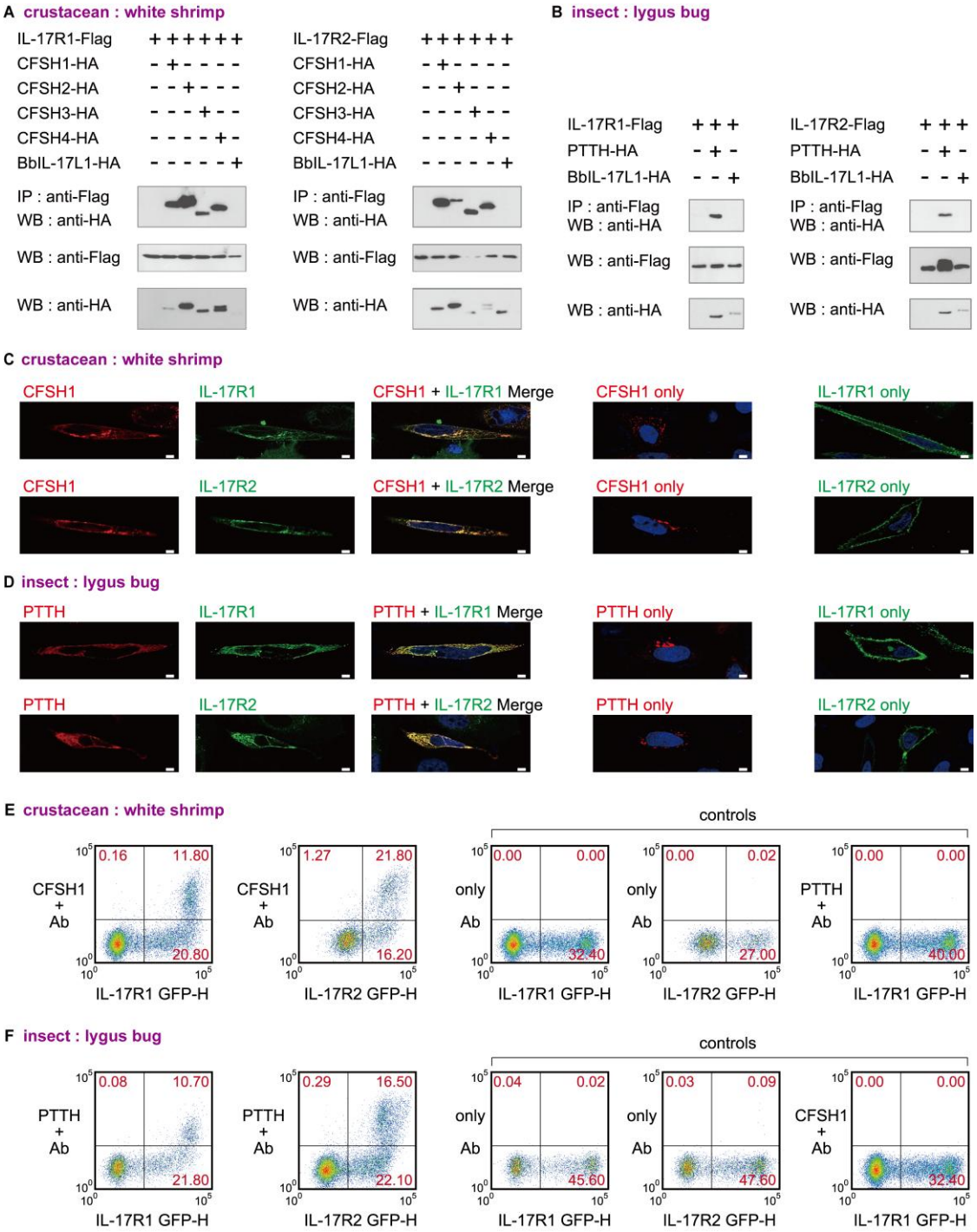

Figure 5. The interactions between IL-17 ligands and receptors from shrimps and lygus bugs.

(A-B) Co-IP assays with HEK293T cells were used to assessed the interactions between crustacean white shrimp CFSHs and IL-17Rs, and between insect lygus bug PTTH and IL-17Rs**.** Amphioxus BbIL-17L1 ws used as the negative control.

(C-D) Subcellular col-localization immunofluorescence assays with HeLa cells were used to evaluated the interactions between shrimp CFSH1-mcherry and IL-17R-GFPs, and between lygus bug PTTH-mcherry and IL-17Rs-GFPs. Transfection with only ligands and receptors served as controls. Additional results for shrimp CFSH2/3/4 were provided in **Fig. S7**. The scale bar indicated 10 μm.

(E-F) Biaxial flow cytometry assays were used to verify the interactions between shrimp CFSH1 and its surface receptors IL-17R1/R2 expressed on HEK293T cells, as well as between lygus bug PTTH

and its surface receptors IL-17R1/R2 on HEK293T cells. HA-tagged recombinant CFSH1 and PTTH proteins were extracted from bacteria and examined using Western blot (**Fig. S8-9**). The receptors were GFP-tagged. Primary antibodies (Ab) are anti-HA monoclonal antibodies. Two types of controls were conducted: one using the anti-HA antibodies (Ab) only, the other using PTTH and shrimp CFSH1 as negative controls for shrimp IL-17Rs and lygus IL-17Rs, respectively. The red number represents the percentage of cells in each gate.

Figure 6

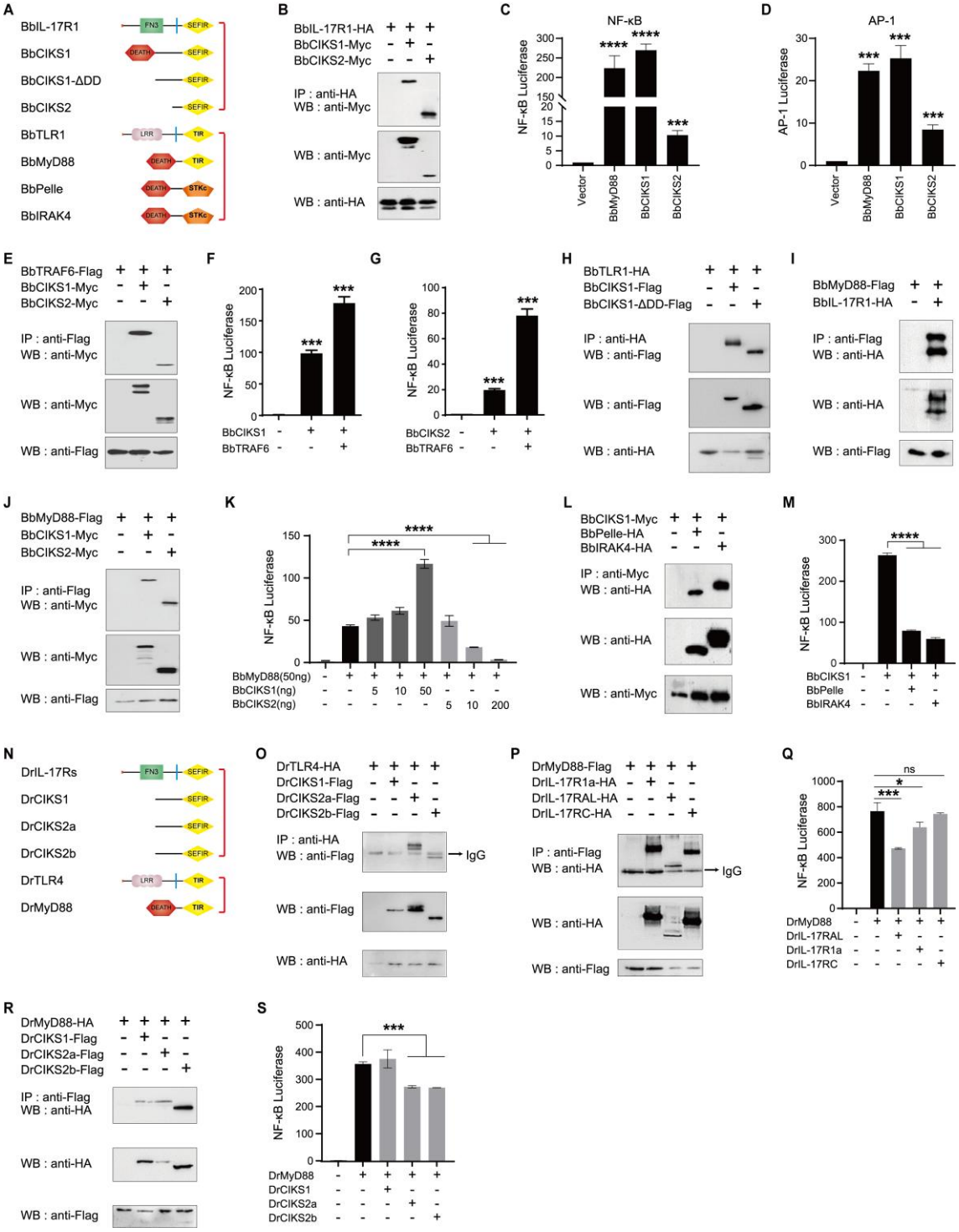

Figure 6. Crosstalk between the IL-17R and TLR/IL-1R pathways in amphioxus and zebrafish.

(A) The constructs used for amphioxus (Bb) proteins.

(B) Co-IP assays with HEK293T cells were used to analyze the interactions between BbIL-17R1 and BbCIKSs.

(C-D) Luciferase reporter assays with HEK293T cells were used to assess the activities of BbCIKSs on NF-κB and AP-1 activation.

(E) The interactions between BbTRAF6 and BbCIKSs.

(F-G) The activity of BbTRAF6 on the NF-κB activation mediated by BbCIKS1 and by BbCIKS2.

(H) The interactions between BbCIKS1/BbCIKS1-ΔDD and BbTLR1.
(I) The interactions between BbIL-17R1 and BbMyD88.
(J) The interactions between BbCIKS1/BbCIKS2 and BbMyD88.
(K) The activities of BbCIKS1 on NF-κB activation mediated by BbMyD88.
(L) The interaction between BbCIKS1 with BbPelle and BbIRAK4.
(M) The effects of BbPelle and BbIRAK4 on inhibiting BbCIKS1-mediated NF-κB activation.
(N) The constructs used for zebrafish (Dr) proteins.
(O) Co-IP assays with HEK293T cells were used to analyze the interactions between DrCIKSs and DrTLR4.
(P) The interactions between DrIL-17Rs and DrMyD88.
(Q) Luciferase reporter assays with HEK293T cells were employed to evaluate the the role of DrIL-17Rs in NF-κB activation mediated by DrMyD88.
(R) The interactions between DrCIKSs and DrMyD88.
(S) The effects of DrCIKSs on NF-κB activation mediated by DrMyD88.
The protein-protein interactions and NF-κB/AP-1 activation were analyzed by Co-IP and reporter assays, respectively. Both assays were conducted with HEK293T cells, and each assay was confirmed by at least three independent experiments. In the reporter assays, 300 ng of the respective plasmids were transfected into cells if not specified. The reporter results were expressed as means ± SD (n = 3), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns ($p>0.05$).

Figure 7

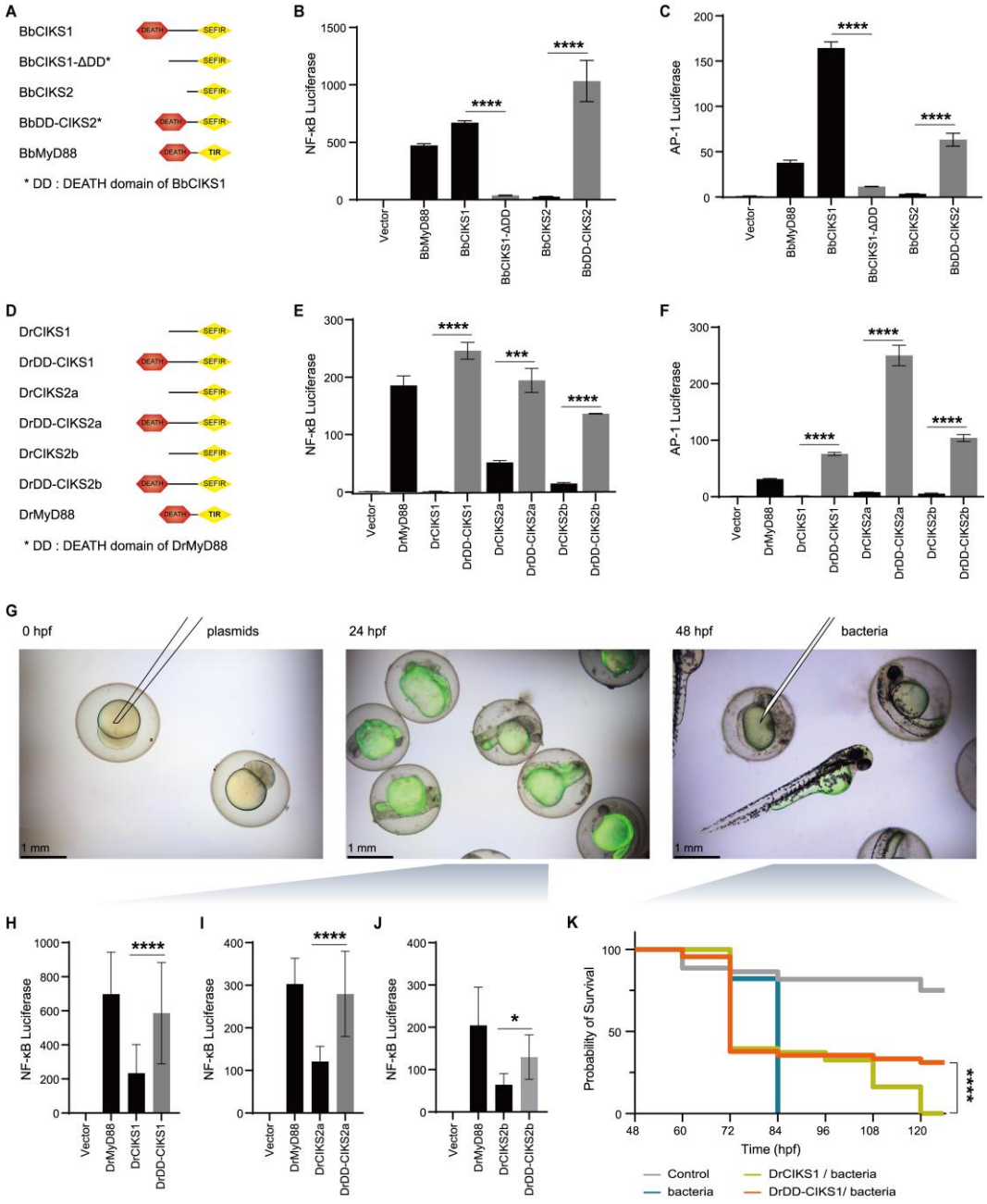

Figure 7. The loss of the Death domain in CIKSs attenuated the direct activating activity of the IL-17R pathways in both amphioxus and zebrafish.

(A-F) The constructs for amphioxus (Bb) proteins (**A-C**) and zebrafish (Dr) proteins (**D-F**) and their activities on NF-κB and AP-1 revealed by luciferasse reporter assays in HEK293T cells. BbDD-CIKS2 was created by fused the Death domain of BbCIKS1 to BbCIKS2. DrDD-CIKSs were created by fused the Death domain of DrMyD88 to DrCIKSs. For each construct, 300 ng of plasmids were transfected into cells. At least three independent experiments were conducted.

(G) GFP-tagged adaptor proteins shown in (D) were introduced into zebrafish embyos by microinjection at 0 hour post-fertilization (hpf). The 24 hpf GFP-labeled embryos were collected for luciferase reporter assays. The 48 hpf GFP-labeled embryos were transfered to a medium containing live bacteria *Vibrio anguillarum* (OD600=0.5), punctured with a fine needle in the yolk, and monitored of their subsequent survival rates.

(H-J) The activities of DrCIKSs and DrDD-CIKSs on NF-κB activation were evaluated by using luciferase reporter assays in zebrafish embryos The composition of the microinjection systems were presented in **Table S4**. At least three independent experiments were conducted.

(K) The survival rates of zebrafish larvae expressing DrCIKS1 and DrDD-CIKS1 after bacterial infection were monitored until 120 hpf. Two other independent experiments with slightly different designs were shown in **Fig. S12**. The specific composition of the injection systems were listed in **Table S4**.

The significance were expressed as: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns (p>0.05).