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JB Review

Diverse physiological functions of MKK4 and MKK7 during early embryogenesis

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Mitogen-activated protein kinase kinases (MAPKKs) are important components of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signalling pathway. Two MAPKKs that are crucial transducers upstream of JNK signalling are MKK4 and MKK7. These two MAPKKs directly phosphorylate specific Tyr and Thr residues located in the activation loop of the JNK protein and activate this kinase in response to environmental stress, pro-inflammatory cytokines or developmental cues. Although much is known about the biochemical and structural bases of the catalytic mechanism of the MAPKKs, the regulation and physiological functions of these enzymes during early embryogenesis have remained a mystery until relatively recently. Studies employing a range of animal models have now revealed the essential roles that MAPKKs play in diverse developmental contexts, including in dorsoventral patterning, convergent extension and somitogenesis. Focusing primarily on extensive work done in mouse and zebrafish models, this review summarizes the functional properties of MKK4 and MKK7 during vertebrate and invertebrate development, and the mechanisms by which these kinases regulate multiple steps in the establishment of the body plan of an organism.

Keywords: Body plan/early embryogenesis/JNK signalling/MKK4/MKK7.

Abbreviation: CE, convergent extension; Dpp, Decapentaplegic; JIP, JNK-interacting protein; JLP, JNK associated leucine-zipper protein; JNK, c-Jun N-terminal kinase; MKK4, Mitogen-activated protein kinase kinase 4; MKK7, Mitogen-activated protein kinase kinase 7; MO, morpholino; POSH, plenty of Src homology 3; SAPK, stress-activated protein kinase.

Mitogen-activated protein kinase kinase (MKK) 4 and MKK7 are the only molecules known to directly activate the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). Both MKK4 and MKK7 are activated in response to a variety of cellular stresses, including UV and γ-irradiation, heat shock, hyperosmolarity, T cell receptor stimulation, peroxide and inflammatory cytokines. Interestingly, these stress-related enzymes are also activated by developmental cues. In mammals, the JNK family consists of three related genes, *Jnk1*, *Jnk2* and *Jnk3*, which encode 10 protein isoforms. These JNK enzymes phosphorylate a number of transcription factors, including c-Jun, ATF-2, Elk-1, p53 and c-Myc, as well as other proteins such as Bcl-2, Bcl-xL, paxillin and MAP2 (*1–4*). Thus, MKK4 and MKK7 are critical upstream activators of JNK signalling required for developmental programmes and responses to various extracellular stimuli. This review will present the state of our current knowledge on the physiological roles of MKK4 and MKK7 during early embryogenesis in widely divergent species, focusing on the biochemistry and signalling functions of these enzymes in mice and zebrafish.

Biochemical Characteristics of MKK4 and MKK7

MKK4 was first cloned in screens for novel members of the MAPKK family in *Xenopus laevis*, and thus termed XMEK2 (*5*). Subsequently, the homologues of this enzyme were cloned in mouse and human and termed MKK4 (also called SEK1 or JNKK1) (*6–8*). Murine MKK4 is a 397 amino acid protein that contains in its catalytic domain the 11 subdomains found in other protein kinases (Fig. 1A).

Mammalian MKK7 (also called SEK2 or JNKK2) was first identified in the mouse in 1997 (*9–11*). Murine *Mkk7* is most similar to the *Drosophila* JNK activator Hemipterous and mammalian MKK4, sharing 70% and 55% amino acid identity, respectively, within the kinase domain. Mouse *Mkk7* contains 14 exons that can be alternatively spliced to generate a group of protein kinases with three different NH_2-termini (the α-, β- and γ-isoforms) and two different COOH-termini (the 1 and 2 isoforms) (Fig. 1B) (*12*). Comparison of the activities of MKK7 isoforms towards JNK have demonstrated that MKK7α, which lacks the NH_2-terminal extension, exhibits a lower basal activity than the MKK7β- and γ-isoforms (*12*). The physiological relevance of the different MKK7 isoforms remains unclear.

The activities of MKK4 and MKK7 are increased following phosphorylation at Ser and Thr residues within a Ser-X-Ala-Lys-Thr motif in their activation loops. This phosphorylation is mediated by various MAPKKKs, including mixed lineage protein kinases

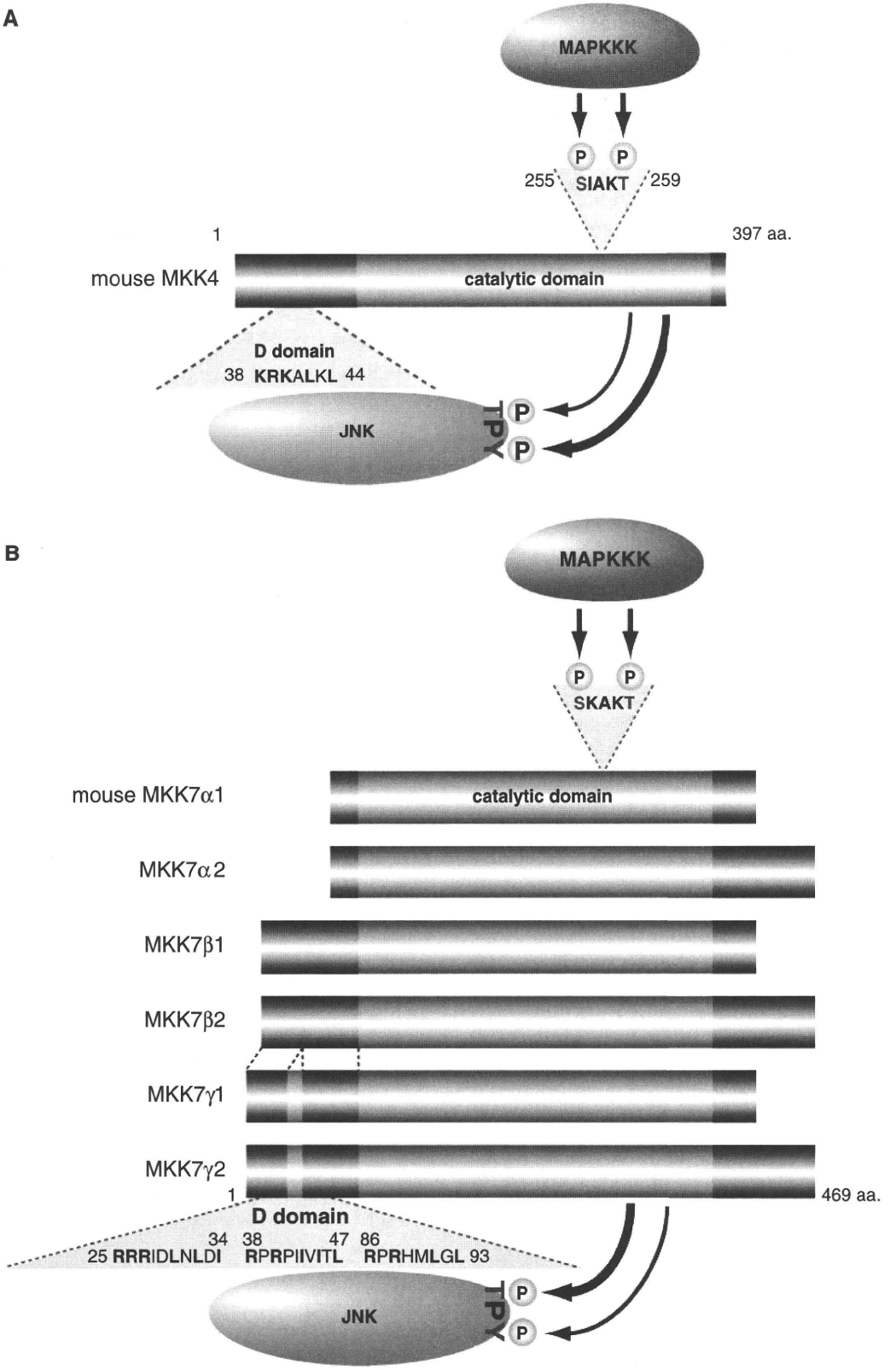

Fig. 1 Function and structure of murine MKK4 and MKK7 isoforms. A specific MAPKKK phosphorylates (A) MKK4 and (B) MKK7 at Ser (S) and Thr (T) residues within the Ser-X-Ala-Lys-Thr (SXAKT) motif of the catalytic domain. Activated dual specificity kinases, MKK4 and MKK7, in turn activate JNK by preferentially phosphorylating the Tyr and Thr residues within a Thr-Pro-Tyr (TPY) motif in JNK's activation loop, respectively (bold arrows indicate preferential phosphorylation). In (B), alternative splicing leads to the inclusion or exclusion of exons located in the 5′- and 3′-regions of the murine *Mkk7* gene, resulting in the generation of the indicated six different MKK7 isoforms that differ in their NH_2- and COOH-termini. The D domain is a JNK docking site in MAPKKs that permits the stable formation of a JNK signalling complex. Residues in the D domains of MKK4 and MKK7 that match the established consensus sequence (*19*, *20*) are depicted in bold.

(MLKs) and MAPK/ERK kinase (MEK) kinase (MEKK1) (*2*, *3*). Activated MKK4 and MKK7 in turn activate JNK by dual phosphorylation of the Thr-Pro-Tyr motif located in JNK's activation loop (Fig. 1A and B). Although MKK4 and MKK7 are dual specificity kinases (Thr and Tyr kinases), previous studies of JNK activation have shown that MKK4 preferentially phosphorylates the Tyr residue, whereas MKK7 phosphorylates the Thr residue. *In vitro* studies have confirmed that phosphorylation of these Tyr and Thr residues results in synergistic activation of JNK (*13–15*). Strong *in vivo* support for this activation mechanism has emerged from studies in our laboratory of mouse embryonic stem (ES) cells bearing targeted disruptions of the *Mkk4* and/or *Mkk7* genes (*16*, *17*). Biochemical analyses of JNK signalling in living ES cells from these animals have demonstrated that Tyr-phosphorylation by MKK4, followed by Thr-phosphorylation by MKK7, leads to synergistic JNK activation in response to stress (*18*).

Scaffold Proteins that Confer Specificity to MKK4 and MKK7 Activities

Multiple mechanisms exist to ensure specificity and prevent cross-talk between components of the MAPK signalling cascade. The specificity of signal transduction by JNK is mediated, in part, by the formation of distinct JNK signalling complexes. These complexes result from interactions between JNK and particular docking sites present on JNK-interacting proteins. The best characterized of these docking sites is the D domain present in MAPKKs. The D domains of MKK4 and MKK7 consist of a cluster of two to three basic residues, followed by a short spacer of 1–2 residues, and a hydrophobic-X-hydrophobic motif (Fig. 1A and B) (*19*, *20*). These MKK docking sites are evolutionarily conserved, and serve to regulate the specificity and enhance the strength of JNK pathway signal transduction. JNK also interacts with various scaffold proteins that can assemble functional signalling modules involving a MAPKKK, a MAPKK and a MAPK (Fig. 2A) (*21*). These scaffold proteins bind specifically to different JNK isoforms and different MAPK and MAPKKs, linking these kinases into a multienzyme complex that provides an insulated physical conduit for signal transduction. Using this conduit, signalling emanating from a particular MAPKK can be transmitted to the appropriate spatiotemporal cellular loci. In this way, MKK4 and MKK7 are responsible for distinct biological functions *in vivo* despite their similarities in sequence and *in vitro* activity.

Several scaffold proteins involved in mammalian JNK signalling modules have been identified, including JNK-interacting protein (JIP) 1, JIP2, JNK/SAPK associated protein 1 (JSAP1)/JIP3, JNK associated leucine-zipper protein (JLP) and plenty of Src homology 3 (POSH) and their various splice variants (*22–29*). JIP1, JIP2 and JSAP1 bind to JNK, MKK7 and various MLKs; JSAP1 associates with JNK, MKK4 and MEKK1; and JLP links Max with

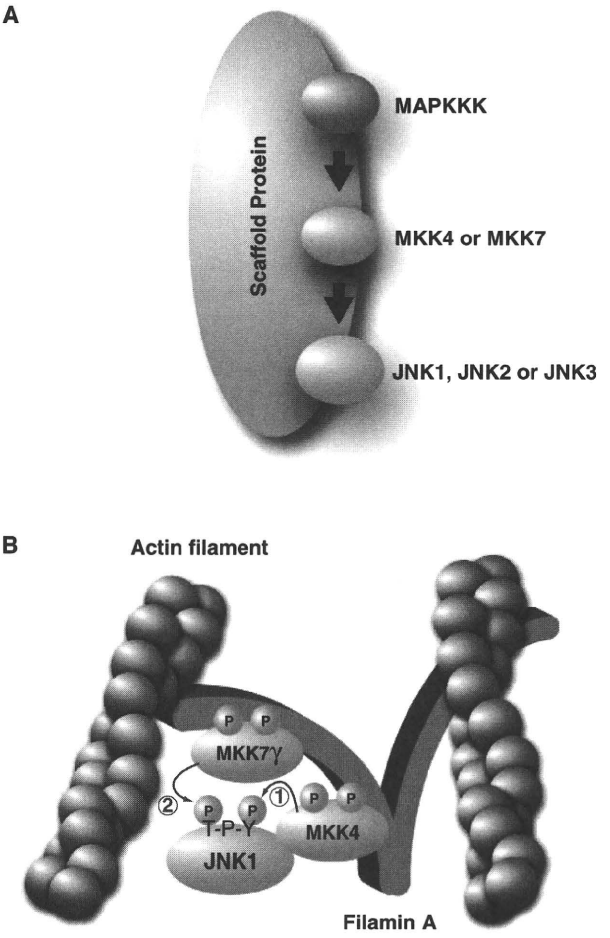

Fig. 2 Scaffold proteins mediating the structural and functional organization of the three-tier JNK signalling module. (A) Model of how a typical scaffold protein supports the assembly of a three-tier JNK signalling module consisting of a MAPKKK, a MAPKK (MKK4 or MKK7) and a JNK. Such scaffold proteins may play a catalytic role as well as an anchoring role depending on the nature of the scaffold protein and the cellular context. (B) Model of how Filamin A acts as a scaffold protein supporting the sequential phosphorylation of JNK by MKK4 and MKK7γ. Filamin A is routinely associated with actin filaments comprising the cytoskeleton. Filamin A also has distinct binding sites for MKK4 and MKK7γ, and can interact simultaneously with both MAPKKs. The interaction of all three proteins with JNK1 leads to synergistic activation of JNK1 in a sequential manner, in which activated (phosphorylated) MKK4 mediates the phosphorylation of the Tyr residue of the Thr-Pro-Tyr motif of JNK (Step 1), followed by Thr-phosphorylation of the same JNK molecule by activated (phosphorylated) MKK7 (Step 2). Filamin A may be the prototype of a novel type of scaffold protein whose function is to link two MAPKKs together and promote synergistic activation of JNK.

c-Myc, and JNK with p38, MKK4 or MEKK3. In addition, multiple upstream MAPKKKs can act as scaffold proteins as well as exert their intrinsic kinase activities. For example, MEKK1 binds to and regulates MKK4. Despite this flexibility, theoretical considerations have dictated that a single JIP-based MAPK module containing MKK4 and MKK7 physically cannot catalyse the sequential phosphorylation of JNK by these kinases. Furthermore, scaffold proteins, such as JIP1, JIP2 and JSAP1, can form homo- and hetero-oligomers (*23*, *24*). Therefore, these scaffolds

could connect two distinct sets of signalling modules, one containing MKK4 and the other containing MKK7. Recently, we identified Filamin A, which interacts with MKK4 (*30*), as a predicted 'binder' protein that can also interact with MKK7 (*31*). Filamin A binds to an NH_2-terminal region present in the MKK7γ and MKK7β splice isoforms but cannot bind to MKK7α, which lacks these amino acids. Experiments using Filamin A deletion mutants revealed that MKK7γ (but not MKK7α) can form a complex with Filamin A and MKK4. This work established a novel model in which MKK4 and MKK7γ utilize Filamin A as a scaffold protein to support their sequential Tyr- and Thr-phosphorylation of JNK and thus its synergistic activation (Fig. 2B) (*31*).

Roles of MKK4 and MKK7 During Early Embryogenesis in Various Species

Phylogenetic analyses of *Mkk4* and *Mkk7* genes have revealed some interesting relationships among species (Fig. 3). Mammals, avians and amphibians appear to have only one gene encoding the MKK4 protein, and these genes are closely clustered in terms of evolutionary distance. In contrast, teleosts such as zebrafish, medakafish and fugu have two Mkk4 genes, *Mkk4a* and *Mkk4b*, and the teleost *Mkk4b* genes are more closely related to each other than to their *Mkk4a* counterparts. These phylogenetic relationships suggest that the duplication of the *Mkk4* gene occurred in the common ancestors of teleosts and tetrapods. With respect to MKK7, all *Mkk7* genes examined to date form a group that includes not only the single *Mkk7* genes from mammalian, avian, amphibian and teleost species but also the one *Mkk7* gene of *Drosophila* and the two *Mkk7* paralogues of nematoda. Neither MKK4 nor MKK7 has been identified in yeast.

Invertebrates

Caenorhabditis elegans. In the nematode *Caenorhabditis elegans*, two homologues of mammalian *Mkk7* have been cloned and are named *mek-1* and *jkk-1* (*32*, *33*). Animals with *mek-1* mutations are hypersensitive to heavy metals and starvation (*33*), and *jkk-1* disruption alters the coordination of body

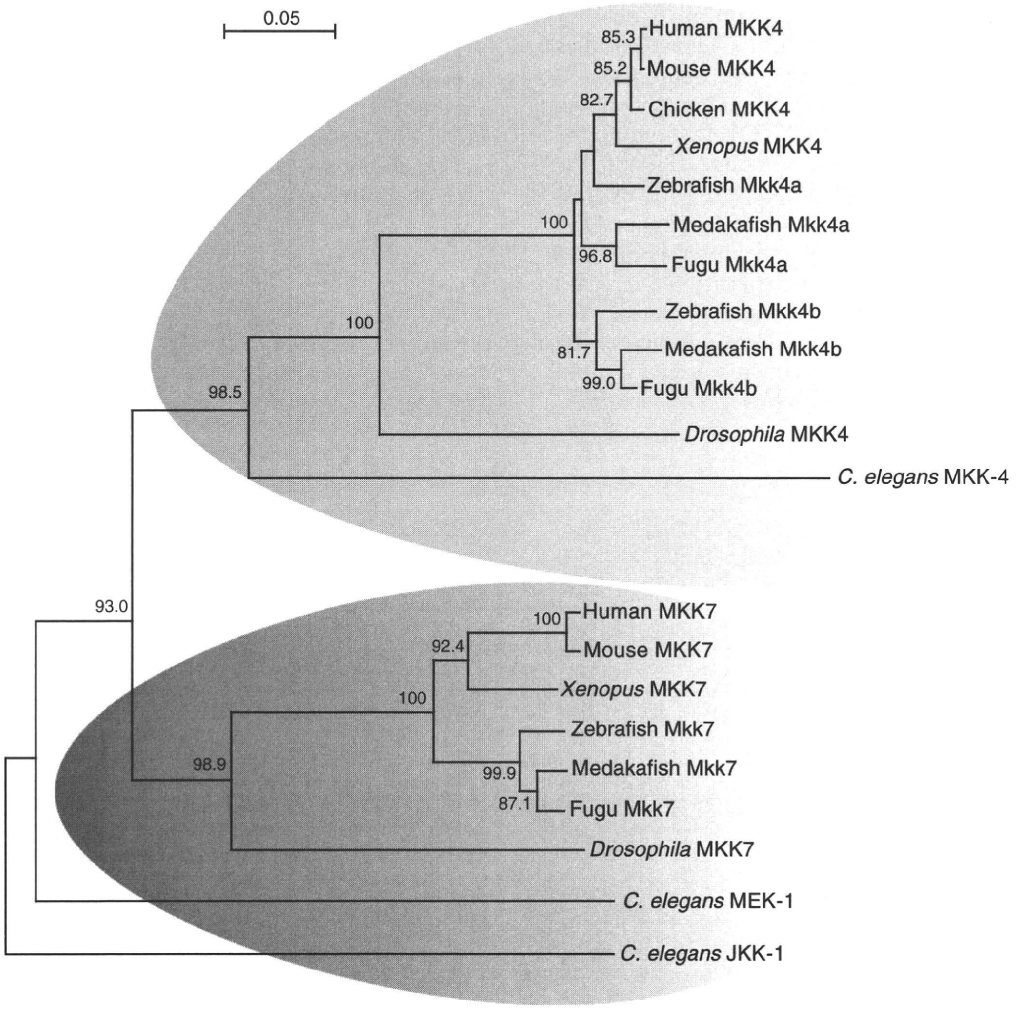

Fig. 3 Molecular phylogenetic tree relating the MKK4 and MKK7 proteins of nine species. The tree was constructed using the neighbour-joining method on the basis of the amino acid identity. The estimated bootstrap probabilities (percent) of local topologies are shown on each node. The length of the scale bar corresponds to an evolutionary distance of 0.05 amino acid substitution per site.

movement via type-D GABAergic motor neurons (*32*). However, neither mutant shows obvious developmental defects. The *C. elegans* genome also contains *mkk-4*, which is highly homologous to mammalian *Mkk4*. The inactivation of *mkk-4* caused an egg-laying defect in hermaphrodites, although there is to date no genetic evidence for MKK4 signalling through JNK in *C. elegans* (*34*). These studies suggest that *mek-1*, *jkk-1* and *mkk-4* are not essential for early embryogenesis in *C. elegans*, but that these genes are important for the regulation of stress responses, locomotion and egg laying.

Drosophila. Genetic studies in *Drosophila* have demonstrated that the JNK pathway is required for early embryonic development in this organism. dJNK (Basket) is activated by dJNKK (Hemipterous), a homologue of vertebrate MKK7 (*35–37*). Basket and Hemipterous are important for morphogenetic processes that involve epithelial cell sheet movement. In the absence of function of either Basket or Hemipterous, lateral epithelial cells fail to stretch and the embryo develops a hole in the dorsal cuticle. The involvement of the JNK pathway in *Drosophila* embryogenesis is further highlighted by the observation that mutants lacking *Drosophila* Jun (dJun) fail to complete dorsal closure (*38–40*). Detailed studies of the process of dorsal closure have demonstrated that dJNK activation is required for dJun phosphorylation and expression of the TGF-β homologue Decapentaplegic (Dpp) in the leading edge of the dorsal epidermis. dFos is also required for Dpp expression (*41*, *42*), indicating that dJNK may trigger Dpp expression by activating an AP-1 complex composed of dFos–dJun heterodimers. Dpp then acts as a secreted signal to control the elongation of lateral epidermis in a paracrine fashion (*43*). These data clearly indicate that the MKK7-JNK signalling pathway has essential functions during early morphogenesis in *Drosophila*.

Although a *Drosophila* orthologue of *Mkk4* has been isolated (*44*), it cannot substitute for Hemipterous (MKK7) function during fly embryonic development because *hemipterous* mutants are embryonic lethal (*35*). Recent genetic and biochemical studies have shown that dMKK4 is dispensable for normal fly development, but that this kinase plays a non-redundant role as a MAPKK acting in parallel to Hemipterous in dTAK1-mediated dJNK activation triggered by Eiger and Imd pathway activation (*45*).

Vertebrates

Mouse. Analyses of various knockout mice have demonstrated the importance of MKK4, MKK7 and JNK signalling in mammalian embryogenesis. $Mkk4^{-/-}$ and $Mkk7^{-/-}$ mice die on embryonic day 10.5 (E10.5) and E11.5, respectively, with severely disorganized livers and reduced hepatoblast numbers (*46–51*). $Jnk1^{-/-}$ $Jnk2^{-/-}$ double mutant mice die at about E11 with defective neural tube morphogenesis and reduced apoptosis in the lateral edges of the hindbrain (*52*, *53*). In contrast, increased apoptosis and caspase activation were found in the forebrain of these double mutants. Thus, the JNK pathway has both pro- and anti-apoptotic effects on the developing mammalian brain.

To determine whether JNK activation is required for the earliest embryonic stages when the vertebrate body plan is first laid down, we recently investigated the effect of combined disruption of the murine *Mkk4* and *Mkk7* genes. $Mkk4^{-/-}$ $Mkk7^{-/-}$ double mutant mice die at about E9.5 (Fig. 4). We examined the progeny of $Mkk4^{+/-}$ $Mkk7^{+/-}$ intercrosses at various developmental stages and found that the expected Mendelian ratio of $Mkk4^{-/-}$ $Mkk7^{-/-}$ embryos (1 : 16) was present at E8.5 but not beyond this point. Intriguingly, $Mkk4^{-/-}$ $Mkk7^{+/+}$ and $Mkk4^{-/-}$ $Mkk7^{+/-}$ mice died earlier than $Mkk4^{+/+}$ $Mkk7^{-/-}$ and $Mkk4^{+/-}$ $Mkk7^{-/-}$ mice, respectively. In addition,

	Genotype									
Mkk4	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	total
Mkk7	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	
E8.5	6	19	10	18	47	17	7	23	10*	157
E9.5	7	10	5	12	21	9	5	11 (2)	3 (2)	83
E10.5	4	6	4	6	10	4 (1)	4 (1)	6 (2)	1 (1)	45

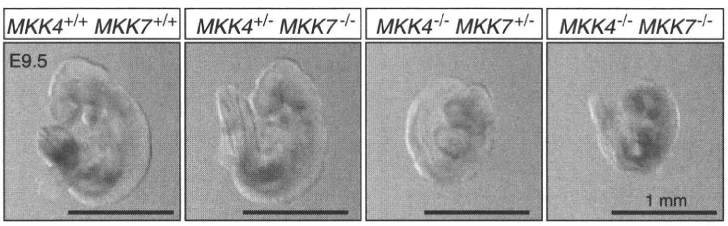

Fig. 4 Embryonic lethality in progeny of $Mkk4^{+/-}$ $Mkk7^{+/-}$ intercrosses. $Mkk4^{+/-}$ $Mkk7^{+/-}$ mice were intercrossed and the genotypes and viability of the progeny embryos were determined at the indicated time points of gestation. Dead embryos (numbers in parentheses) were defined as those in which the heart had stopped beating, as assessed by inverted microscopy. Asterisk indicates severely growth retarded and dying embryos.

at E9.5, $Mkk4^{-/-}$ $Mkk7^{+/-}$ embryos were more severely affected than $Mkk4^{+/-}$ $Mkk7^{-/-}$ embryos. These observations indicate that MKK4 is critical for murine embryogenesis, and that the functions of both MKK4 and MKK7 are required for mammalian body plan organization.

Zebrafish. We originally turned to studying MKK4 and MKK7 in zebrafish because our $Mkk4^{-/-}$ $Mkk7^{-/-}$ mouse embryos exhibited retarded growth and extremely small body size at E8.5, making it very difficult to analyse the precise nature of MKK4 and MKK7's functions in organizing the vertebrate body plan. Fertilized zebrafish eggs develop *ex utero* into transparent embryos that can be directly observed and are highly amenable to manipulations such as tissue transplantation and molecular perturbation. There is a high degree of conservation between zebrafish and mammalian genes, and a shared developmental path that results in fundamental similarities in many tissues and organs (*54*, *55*). In addition, there exists a wide selection of mutant zebrafish lines with developmental abnormalities, including gastrulation defects. Thus, zebrafish provide a very attractive alternative to mice for studying the molecular and cellular bases of vertebrate morphogenesis.

As introduced above, the zebrafish has two *mkk4* genes, *mkk4a* and *mkk4b*, but only one *mkk7*

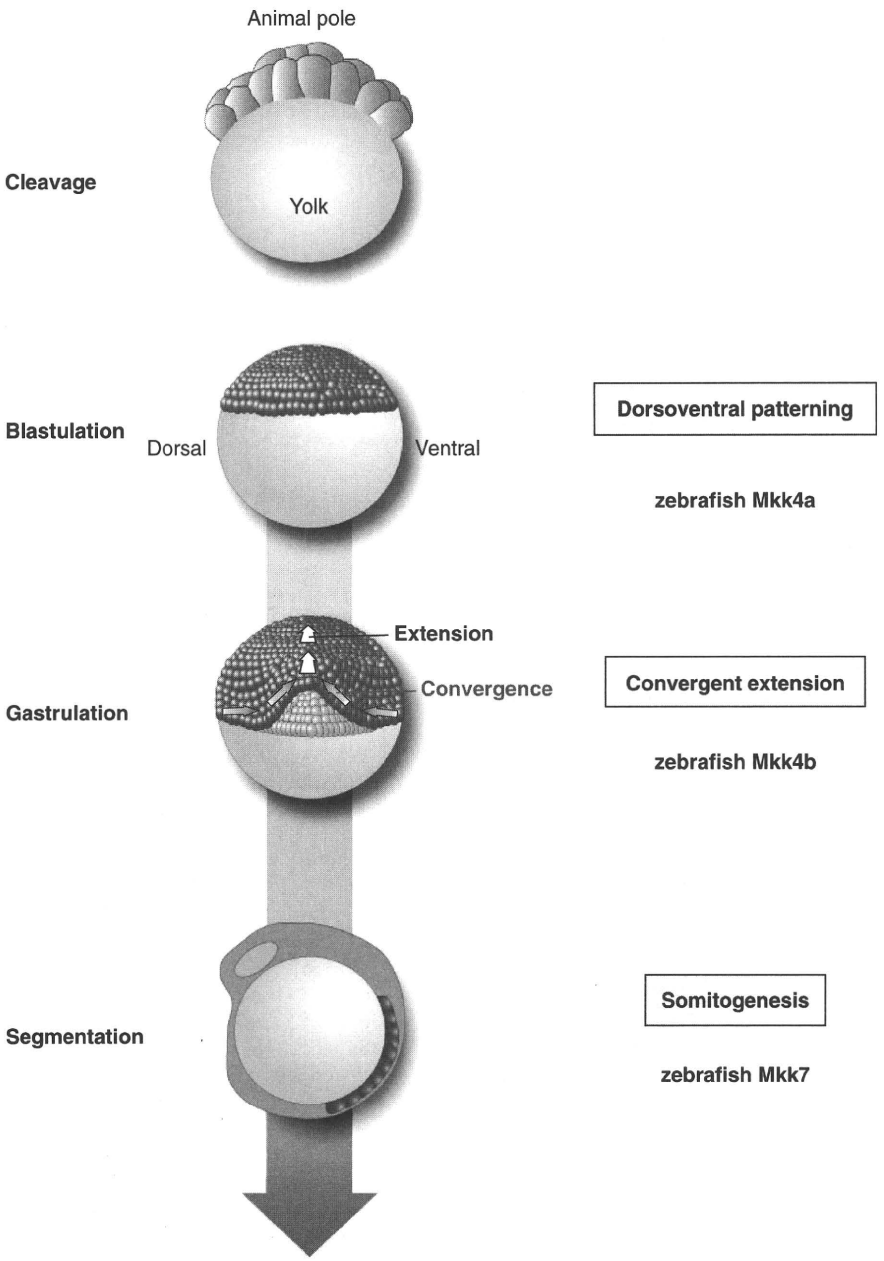

Fig. 5 Multiple roles of zebrafish Mkk4 and Mkk7 during body plan formation. The early embryonic development of the zebrafish occurs in four stages: cleavage, blastulation, gastrulation and segmentation. As indicated, Mkk4a is crucial for dorsoventral patterning, Mkk4b for convergent extension and Mkk7 for somitogenesis.

orthologue. When we used morpholino (MO)-mediated knockdown to examine zebrafish *mkk* gene functions, we found that *mkk4b* MO-injected embryos exhibited axial tissues, which were abnormally short and wide due to defective convergent extension (CE), a driving force of vertebrate gastrulation (Fig. 5). During CE, mesodermal cells migrate towards the future dorsal side of the embryo by means of highly directed and integrated movements, resulting in an overall medio-lateral narrowing (convergence) and anterior– posterior elongation (extension) of the embryo (*54*, *56*, *57*). Previous studies in *Xenopus* and zebrafish have shown that Wnt5 and Wnt11 ligands can signal through a non-canonical Wnt pathway via JNK to influence CE movements during gastrulation (*54*, *56*, *58*, *59*). Surprisingly, *mkk4b* morphants displayed marked up-regulation of *wnt11*, providing the first evidence that *wnt11* itself is a downstream target of the JNK cascade in the non-canonical Wnt pathway associated with early embryogenesis (*60*). More detailed studies revealed that Mkk4b-JNK signalling suppressed *wnt11* expression in a non-cell-autonomous fashion. Our findings suggest that the suppression of *wnt11* transcription by Mkk4b-JNK activation is important for precise regulation of CE.

When we examined our *mkk7* morphants, we found that they had no phenotype during gastrulation but showed abnormal somite morphologies during segmentation (Fig. 5). Mkk7 is thus critical for a slightly later stage of development than is Mkk4b. With respect to Mkk4a, Rui *et al.* (*61*) demonstrated that this kinase participates in dorsoventral patterning in zebrafish blastulas. Mkk4a knockdown reduced the expression of dorsal markers but expanded the expression of ventral markers.

Table I. Physiological roles of MKK4 and MKK7 in development: insights from animal models.

Molecules	Functions	References
Mouse MKK4	Hepatogenesis	(*46*, *48*, *50*)
	Alignment of the Purkinje cells in the cerebellum, radial migration in the cerebral cortex	(*62*)
Mouse MKK7	Hepatogenesis	(*49*)
Chicken MKK4	Not determined	
Xenopus MKK4	Not determined	
Xenopus MKK7	Convergent extension	(*58*)
Zebrafish Mkk4a	Dorsoventral patterning	(*61*)
Zebrafish Mkk4b	Convergent extension	(*60*)
Zebrafish Mkk7	Somitogenesis	(*60*)
Drosophila MKK4	Not detected[a]	(*45*)
Drosophila MKK7	Dorsal closure	(*35–37*)
Caenorhabditis elegans MKK-4	Not detected	(*34*)
Caenorhabditis elegans MEK-1[b]	Not detected	(*33*, *34*)
Caenorhabditis elegans JKK-1[b]	Not detected	(*32*, *34*)

[a]Mutation of this gene results in no detectable abnormal phenotype.
[b]MKK7 paralogue.

Conclusion

JNK activation by MKK4 and MKK7 is a mechanism utilized in parallel morphogenetic events among widely divergent species (Table I). The available data raise the possibility that, in both vertebrates and invertebrates, JNK signalling involving MKK homologues regulates the expression of secreted signalling molecules capable of promoting concerted movements of neighbouring cells (*60*), such as are required in dorsal closure, and cell movements during gastrulation. In *Drosophila*, MKK7 is the principal activator of JNK in the process of dorsal closure. In contrast, our recent researches show that both MKK4 and MKK7 are essential in the multiple processes of body plan formation in mice and zebrafish. The functional properties of MKK4-JNK and MKK7-JNK signalling modules may be governed in part by scaffold proteins that confer specificity to kinase actions. In the future, studies of MAPKKs in other species may reveal more about the evolutionary process by which the partitioning of functions between MKK4 and MKK7 has developed.

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Conflict of interest
None declared.

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The LIM protein Ajuba is required for ciliogenesis and left–right axis determination in medaka

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ABSTRACT

Cilia are microtubule-based organelles that are present on the surfaces of almost all vertebrate cells. Most cilia function as sensory or molecular transport structures. Malfunctions of cilia have been implicated in several diseases of human development. The assembly of cilia is initiated by the centriole (or basal body), and several centrosomal proteins are involved in this process. The mammalian LIM protein Ajuba is a well-studied centrosomal protein that regulates cell division but its role in ciliogenesis is unknown. In this study, we isolated the medaka homolog of Ajuba and showed that Ajuba localizes to basal bodies of cilia in growth-arrested cells. Knockdown of Ajuba resulted in randomized left–right organ asymmetries and altered expression of early genes responsible for left–right body axis determination. At the cellular level, we found that Ajuba function was essential for ciliogenesis in the cells lining Kupffer's vesicle; it is these cells that induce the asymmetric fluid flow required for left–right axis determination. Taken together, our findings identify a novel role for Ajuba in the regulation of vertebrate ciliogenesis and left–right axis determination.

1. Introduction

Cilia are microtubule-based hair-like organelles that extend from the surfaces of almost all vertebrate cells. Studies over the past decade have revealed that cilia, which can be motile or immotile, play pivotal roles in a wide variety of cellular functions [1]. For example, immotile cilia typically function as specialized sensory structures, such as the olfactory cilia and retinal photoreceptor outer segments. In contrast, the motile cilia located on epithelial cells, including respiratory epithelial cells and brain ependymal cells, transport extracellular fluid along the epithelial surface. In addition, motile cilia at the embryonic node generate an extraembryonic fluid flow that is required to establish embryonic left–right asymmetry [2]. Defects in genes involved in cilia assembly or function have been associated with many diseases of human development, including Bardet Biedl syndrome, hydrocephalus, and *situs inversus* (reversal of normal visceral asymmetry) [3]. These disorders reflect the crucial role of cilia in regulating vertebrate developmental processes.

The assembly of a cilium requires a basal body that is derived from one of the two centrioles that constitute the centrosome [4,5]. During ciliogenesis, the centriole migrates to the plasma membrane and ciliary microtubules elongate from its distal end. Once a centriole has docked with the plasma membrane, it is known as a basal body. Recently, it was reported that certain centrosomal proteins are required for cilium formation [6,7]. We therefore, hypothesized that additional centrosomal proteins might also be involved in regulating vertebrate ciliogenesis.

Ajuba is a conserved centrosomal protein that was originally identified in mice and contains three LIM domains in its C-terminal region [8]. Ajuba contributes to the formation and maintenance of cell–cell junctions [9,10] and plays a role in cell migration [11,12]. Ajuba is also important for mammalian cell division. In humans, the association of Ajuba with the LATS2 protein and microtubules at the centrosome regulates mitotic spindle organization [13–15]. To investigate whether Ajuba is required for the assembly of cilia and vertebrate embryonic development, we exploited a gene knockdown system in the ricefish medaka (*Oryzias latipes*). Medaka embryos develop outside the mother's body, making it easy to inspect them visually and to manipulate their tissues and cells [16]. Here, we provide evidence that Ajuba is required for the assembly of Kupffer's vesicle (KV) cilia and thus for left–right axis determination in developing medaka embryos.

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2. Materials and methods

2.1. Fish maintenance

Embryos and adults of the medaka Cab inbred strain were used for all experiments. Embryos were raised at 30 °C and embryonic stages were determined based on morphological features, as previously described [16].

2.2. Cloning and RT-PCR

Partial or full-length cDNAs of the *ajuba* (GenBank Accession No. AB523732) and *pitx2* (Ensembl ID ENSORLG00000020587) genes were generated by RT-PCR of mRNAs purified from medaka embryos at various stages of development. The primers used in the RT-PCR reactions are shown in Supplemental Table 1 (see Supplemental information online).

2.3. Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as previously described [17], using antisense digoxigenin (DIG)-labeled riboprobes generated from medaka *ajuba, pitx2, shh,* or *spaw* partial cDNAs. Probes for *spaw* and *shh* were used as previously described [18,19].

2.4. Cell culture and immunofluorescence

cDNA encoding medaka Ajuba was cloned into the expression vector pEGFP-C1; this recombinant EGFP vector was termed GFP-Ajuba. Medaka hepatoma (DIT) cells were grown to 50% confluency on 15-mm diameter glass coverslips in Leibovitz L-15 medium (Sigma) supplemented with 10% FBS (GIBCO) [20]. DIT cells were transfected with 1 μg of GFP-Ajuba plus Fugene HD (Roche) according to the manufacturer's instructions. Transfected cells were grown for 24 h and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min at room temperature (RT). Cells were treated with methanol at −20 °C for at least another 3 min, followed by permeabilizing with 0.2% Triton X-100 in PBS. Permeabilized cells were incubated with blocking solution [5% bovine serum albumin (BSA) in TBS] for 30 min at RT, then for 2 h at 37 °C with blocking solution containing mouse anti-acetylated tubulin monoclonal antibody (1:1000; Sigma) and mouse anti-γ tubulin monoclonal antibody (1:1000; Sigma). Antibody-bound cells were washed with PBS and incubated with Alexa 488-conjugated secondary antibodies for 30 min. After several PBS washes, coverslips were mounted and viewed on a Carl Zeiss confocal microscope equipped with LSM510 software.

2.5. Gene knockdown by morpholinos

Morpholino antisense oligos (MOs) were synthesized by GeneTools, LLC (Philomath, OR). MOs were injected into the cytoplasm of one-cell stage medaka embryos. Sequences of MOs used were as follows:

- Ajuba spMO (splice-blocking), 5′-GCCTT GACCT CAGCT CTTAC CATGT-3′;
- Ajuba augMO (translation-blocking), 5′-GCTTT GTTAT TGGCT TTTCC ATGGT-3′;
- Standard control MO, 5′-CCTCT TACCT CAGTT ACAAT TTATA-3′.

2.6. Immunohistochemistry

Medaka embryos were fixed in 4% PFA/PBST (PBS at pH 7.5 containing 0.1% Tween 20) at 4 °C overnight. The chorion of the embryos was removed and the embryos were washed several times with PBST and stored in methanol at −20 °C until use. Embryos to be used were gradually rehydrated, washed several times with PBST, and blocked in 2% BSA plus 1% DMSO in PBST at RT for 2 h. Blocked embryos were incubated in blocking solution containing anti-mouse acetylated tubulin antibody (1:1000, Sigma T-6793) at 4 °C overnight. After several washes with PBST, embryos were blocked again at RT for 2 h and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:1000) at 4 °C overnight. After removal of the tail region, immunostained embryos were mounted in Fluor Save Reagent (Calbiochem) and imaged using a Zeiss LSM scanning laser confocal microscope with a 40× objective.

2.7. Cryosectioning and hematoxylin/eosin staining

Hatched medaka larvae were fixed in 4% PFA/PBS overnight. Fixed embryos were washed in PBS and incubated in 20% sucrose solution in PBS for several days. The infused embryos were then embedded in optimal cutting temperature compound (Tissue-Tek) and cryosectioned at 6 μm. Sections were stained with hematoxylin/eosin and viewed using a Leica DMRA fluorescence microscope.

3. Results

3.1. Expression pattern and localization of medaka Ajuba

We isolated the full-length cDNA of medaka *ajuba* by performing BLAST searches and subsequent RT-PCR. A phylogenetic analysis revealed that the medaka Ajuba shows greater sequence similarity to the mammalian and teleost Ajuba proteins than to other Ajuba-related LIM protein members, Wtip and Limd1 (Fig. 1A) [21]. The predicted amino acid (aa) sequence of medaka Ajuba is 41–43% identical to the aa sequences of the human and mouse Ajuba proteins, and the Ajuba nuclear export signal (NES) and LIM domains are well-conserved among species (Fig. S1). We then examined the expression pattern of *ajuba* during early medaka development using RT-PCR analysis and whole mount *in situ* hybridization. Medaka *ajuba* mRNA was detectable in embryos from stages 17 to 30 (Fig. 1B). At early somite stages (stages 19 and 21), *ajuba* transcripts were broadly expressed, with higher levels observed in the tailbud (Fig. 1C, Fig. S2). At later somite stages (stages 25 and 30), expression of *ajuba* mRNA became more localized in the developing retina and several brain areas. These observations suggest that medaka *ajuba* is expressed in multiple ciliated tissues during early development.

To determine the localization of Ajuba protein in cells, we transfected EGFP-tagged medaka Ajuba (GFP-Ajuba) into medaka DIT cells and performed immunofluorescence analysis. Because primary cilia grow only at interphase, cells were cultured under low serum (0.5%) conditions to limit proliferative growth. In growth-arrested DIT cells, GFP-Ajuba co-localized with the centrosome/basal body marker γ-tubulin, although additional GFP-Ajuba granules could be seen scattered in the cytoplasm (Fig. 1D). This co-localization of medaka Ajuba with γ-tubulin is consistent with results reported for mammalian cells [13,14].

3.2. Knockdown of medaka ajuba disrupts the laterality of internal organs

To identify the physiological function of Ajuba during medaka embryogenesis, we designed translation- and splice-blocking morpholinos (augMO and spMO, respectively) to knock down *ajuba* mRNA and block Ajuba protein expression (Fig. 2A). To confirm

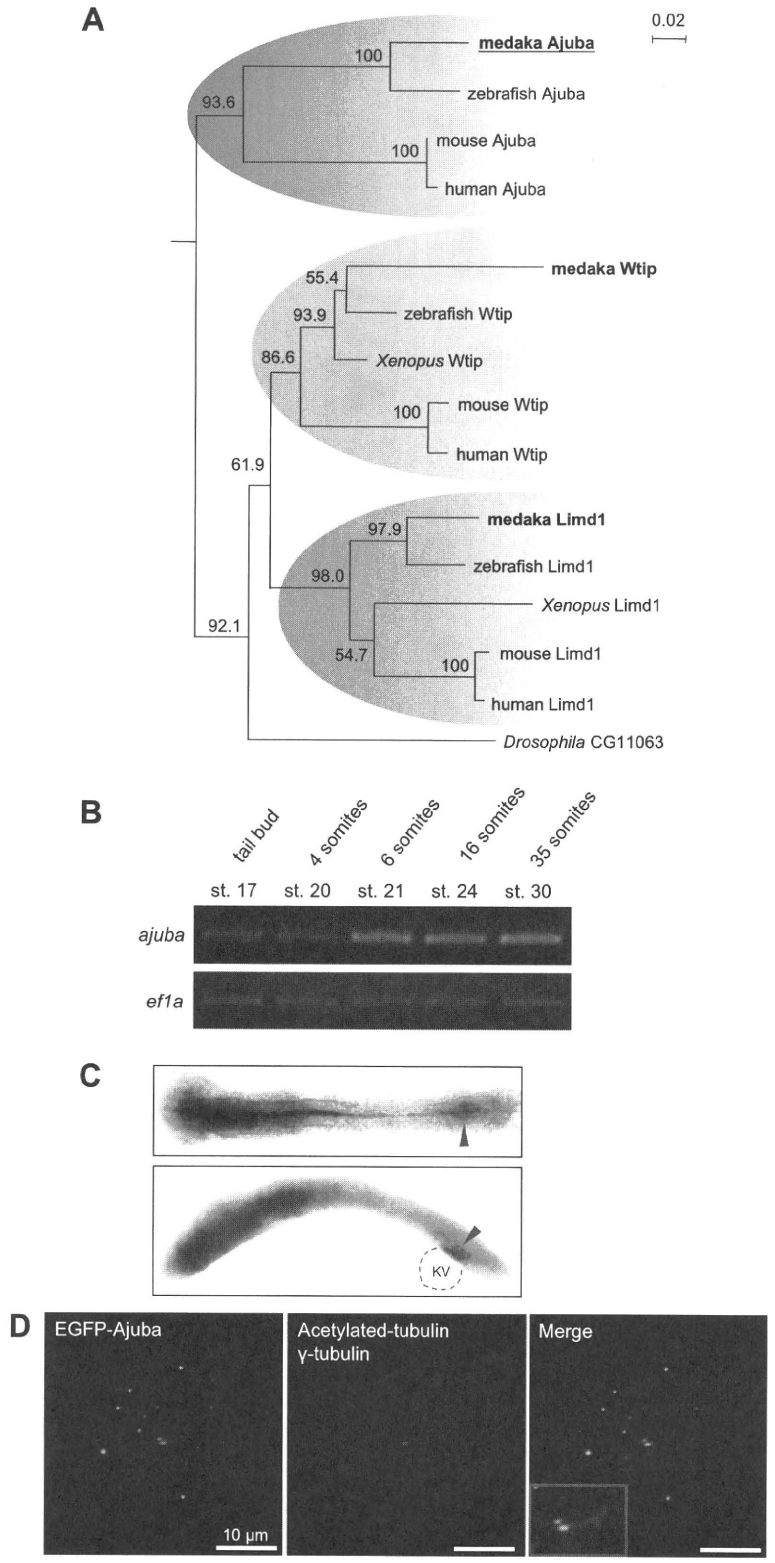

Fig. 1. Cloning of the medaka *ajuba* gene and localization of the Ajuba mRNA and protein in medaka embryos and cultured cells. (A) Phylogenetic classification of medaka Ajuba/Wtip/Limd1 proteins. Three medaka Ajuba/Wtip/Limd1 proteins were classified as members of the indicated vertebrate LIM families on the basis of amino acid (aa) sequence. The deepest roots of the trees were determined using the sequence of the *Drosophila* Zyxin homolog as an outgroup. The estimated bootstrap probabilities (percent) of local topologies are shown on each node. The length of the scale bar corresponds to an evolutionary distance of 0.02 aa substitution per site (2% sequence difference). (B) Broad expression of *ajuba* mRNA during early medaka development. RT-PCR analysis of *ajuba* expression was performed in WT medaka embryos at the indicated stages. *ef1a*, loading control. (C) Prominent Ajuba expression in the tailbud. A stage 21 medaka embryo was examined by whole mount *in situ* hybridization to detect *ajuba* mRNA. Upper panel, dorsal view; bottom panel, lateral view. Anterior of both embryos is to the left. Ajuba was broadly expressed but the higher levels were seen in cells (arrowheads) in the areas adjacent to the KV (outlined with dotted line). (D) Localization of Ajuba in the basal body of cilia. Medaka DIT cells were transfected with GFP-Ajuba and immunostained with anti-acetylated tubulin and anti-γ tubulin (red), which are the ciliary marker and the basal body marker, respectively. Localization of Ajuba protein was determined by immunofluorescence microscopy. A GFP-Ajuba granule was located at the basal body of cilia, but most were scattered in the cytoplasm. Inset, magnified image of co-stained basal body and cilium. For all Figures, results shown are representative of six trials.

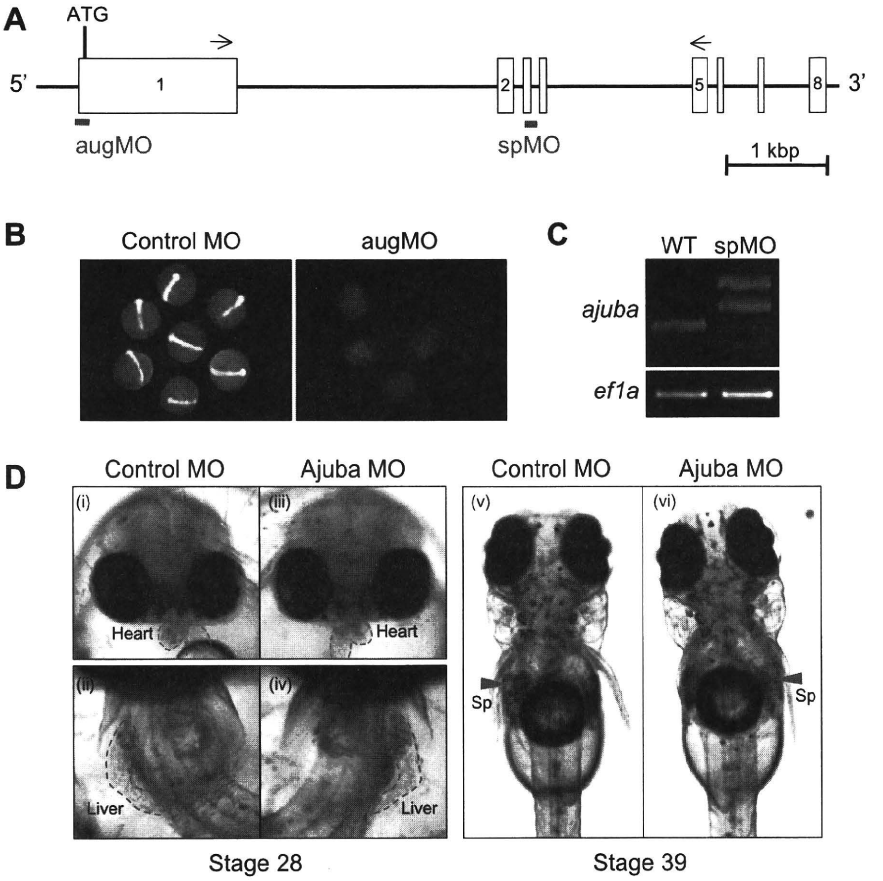

Fig. 2. Ajuba knockdown embryos show inverted positioning of the heart, liver and spleen. (A) Schematic diagram of exons 1–8 of the medaka *ajuba* genomic region. Arrows, positions of primer pairs used for the RT-PCR analysis in (C) below. augMO, translation-blocking morpholino; spMO, splice-blocking morpholino. (B) Validation of augMO. The cytoplasm of one-cell stage WT medaka embryos was co-injected with either control MO or augMO plus a GFP reporter gene that carries the Ajuba augMO target sequence at the 5′ UTR. Injection of augMO successfully blocked the translation of the GFP reporter protein. (C) Validation of spMO. WT medaka embryos were left untreated (WT) or injected with spMO, and RT-PCR was performed on total RNA. The *ajuba* RT-PCR product generated from RNA from spMO-injected embryos was increased in size compared to the control, reflecting the retention of intron sequences in the mature mRNA. Injection of spMO successfully interfered with the splicing of *ajuba* mRNA. (D) Laterality defects of organs in Ajuba morphants. In control morphants at stage 28, the heart tube jogged to the left and looped to the right (i) and the internal organs, including the liver, were located on the left side of midline (ii). In stage 28 Ajuba morphants, the heart tube jogged to the right and looped to the left (iii), and the position of the liver was reversed (iv). In stage 39 hatched larvae, the spleen was positioned on the left side in the control (v) but on the right side in the Ajuba morphant (vi). Sp, spleen.

the efficacy of the Ajuba augMO, this morpholino was co-injected into the cytoplasm of one-cell stage medaka embryos with an EGFP reporter containing the augMO target sequence. Co-injection of Ajuba augMO significantly reduced the translation of the EGFP reporter protein compared to co-injection of a control MO (Fig. 2B). To evaluate the efficacy of the splice-blocking morpholino (spMO), RT-PCR was carried out on total RNA prepared from embryos injected with 6 ng Ajuba spMO. The amplicon produced from gene-specific primers that span *ajuba* exons 1–5 resulted in abnormally large bands of two sizes (Fig. 2C). Sequence analysis revealed that these PCR products retained introns 2 and 3, causing a frame shift and introducing termination codons after exons 2 and 3, respectively. These data confirm the abilities of Ajuba augMO and spMO to disrupt expression and/or the function of the Ajuba protein.

Embryos injected with either Ajuba-spMO or Ajuba-augMO showed no alterations to body shape or size but did display defects in visceral asymmetries (Fig. 2D). In control morphants at stage 28, the ventricle of the heart looped towards the right and the atrium looped towards the left, and the liver and pancreas lay on the left side of the midline. In contrast, left–right asymmetry in the heart and liver was reversed in 30–32% of Ajuba morphants (Table S2). A similar rate of reversal was observed in the position of the spleen in Ajuba morphants at stage 39. Indeed, Ajuba morphants exhibited complete inversion of the positions of the abdominal and thoracic organs, an event reminiscent of the human disorder *situs inversus*. These results reveal that Ajuba regulates the left–right asymmetry of organs during medaka development.

3.3. Ajuba morphants display ectopic expression of left–right determinant genes

To understand the abnormal laterality phenotype of Ajuba morphants at the molecular level, we analyzed the expression patterns of genes that are normally expressed only on the left side of the embryo. In control morphants at stage 21, the nodal-related gene *southpaw* (*spaw*) was expressed in the left lateral plate mesoderm (LPM) (Fig. 3A, top left). However, Ajuba morphants showed a randomization of *spaw* expression, with 43–52% showing normal left-sided *spaw* expression but the remainder exhibiting right-sided (24–36%) or bilateral (21–24%) *spaw* expression in the LPM (Fig. 3A, top right and Table S3). The bilateral expression of *spaw* in the tailbud was unaffected in these mutants. The expression of another left-sided marker gene, *pitx2*, was also altered in Ajuba morphants. Control morphants showed normal left-sided expression of *pitx2* in the left diencephalon and the left LPM (Fig. 3A, bottom left). However, this asymmetric gene expression pattern was once again randomized in Ajuba morphants, with 27–38% of mutants exhibiting ectopic expression of *pitx2* (Fig. 3A, bottom right).

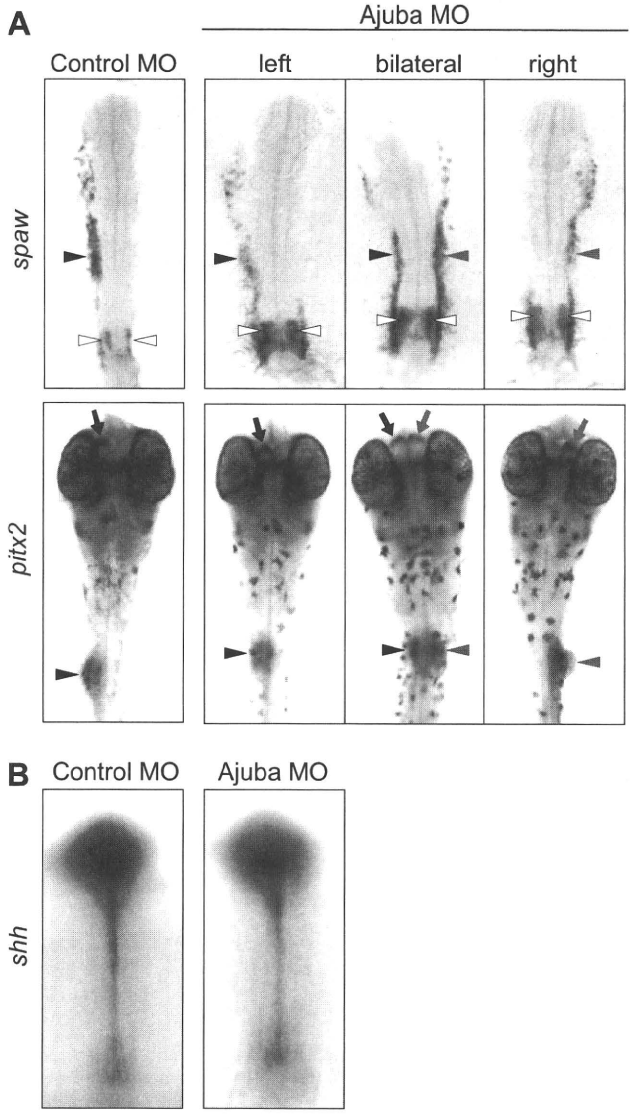

Fig. 3. Ajuba affects the expression of left-sided genes in the LPM and dorsal diencephalon. (A) Ectopic expression of *spaw* and *pitx2*. Control and *ajuba* morphants were analyzed by whole mount *in situ* hybridization to detect *spaw* expression at stage 21 (top) and *pitx2* expression at stage 26 (bottom). Dorsal views are shown. Top: *spaw* is normally expressed in the left LPM (black arrowheads). Altered bilateral or right expression of *spaw* (red arrowheads) appeared in Ajuba morphants. Normal bilateral expression of *spaw* at the KV (white arrowheads) was unaffected. Bottom: Left-sided expression of *pitx2* in control dorsal diencephalon (black arrows) was randomized (red arrows) in Ajuba morphants. (B) Normal expression of *shh* in the midline. Dorsal views of *shh* expression in control and Ajuba morphants are shown.

Because defects in embryonic midline integrity can lead to abnormal left–right development of internal organs [22], we compared the midline structure of control and Ajuba morphant embryos by examining *shh* expression via *in situ* hybridization. However, *shh* expression was not affected by the Ajuba knockdown (Fig. 3B), confirming that the midlines in these morphants were intact. These data demonstrate that Ajuba acts upstream of *spaw* and *pitx2* in the establishment of left–right asymmetry of the body plan.

3.4. Ajuba is essential for ciliogenesis of Kupffer's vesicle

One model for the establishment of the vertebrate left–right axis postulates the involvement of a ciliated organ known as the embryonic node in mice [2] or Kupffer's vesicle (KV) in zebrafish and medaka [23,24]. Motile cilia in the KV are thought to produce a leftward flow of extracellular fluid that induces the asymmetric expression of downstream genes such as *nodal* and *pitx2*. This model, combined with our observation of preferential expression of *ajuba* mRNA in cells surrounding the KV (Fig. 1C), prompted us to investigate the role of Ajuba in the development of ciliated cells in medaka. To determine if depletion of Ajuba caused defects in KV cilia, we subjected stage 21 Ajuba MO embryos to immunohistochemistry to detect acetylated tubulin, a major component of cilia. In contrast to control morphants, Ajuba augMO- or spMO-injected embryos developed shorter cilia (Fig. 4A). Quantification of ciliary lengths revealed a significant difference between control embryos and morphants (Fig. 4B), suggesting that KV cilia require Ajuba for normal development. In addition, the primary cilia of the telencephalon in Ajuba morphants were also shorter (Fig. S3).

The retina is another tissue heavily dependent on cilia, since photoreceptor cells have a highly specialized primary cilium called the outer segment (OS) that is essential for photoreception [25]. To determine whether loss of Ajuba affected retinal organization, we analyzed cross-sections of control and Ajuba morphant larvae but detected no alterations in OS or other components of photoreceptor cells in Ajuba morphants (Fig. 4C). Thus, Ajuba is required for normal ciliogenesis in some, but not all, cilium-dependent structures, most notably the KV governing left–right asymmetry.

4. Discussion

In this study, we cloned the medaka *ajuba* gene and discovered new roles for the Ajuba protein in early developmental processes. We have demonstrated that knockdown of Ajuba by antisense morpholinos disrupts the positions of the visceral organs and induces abnormal expression of genes involved in early left–right axis determination. In particular, Ajuba knockdown embryos showed shortened cilia lengths in the KV. Our results suggest a model in which Ajuba regulates left–right asymmetry by supporting the normal assembly of cilia in the KV.

Our subcellular localization studies showed that the Ajuba protein was present on basal bodies, which are ciliary initiators derived from centrosomes [4,5]. Another protein located on both the centrosome and the basal body is EB1, which is a microtubule-associated protein involved in mitosis and ciliogenesis [26]. A recent study has revealed that Ajuba is also associated with microtubules and may be important to ensure proper chromosome segregation [13]. Taken together, these observations suggest that Ajuba's function in ciliogenesis may depend on its ability to associate with microtubules. Studies are under way to address this issue.

The LIM domain has been identified in a highly diverse group of proteins [21]. We performed a phylogenetic analysis revealing that medaka express three closely related LIM proteins, Ajuba, Limd1 and Wtip, which are clustered with the three corresponding mammalian subfamily members (Fig. 1A). In contrast, *Xenopus* and *Drosophila* have no Ajuba homolog. *Xenopus* does have two homologs of Limd1 and Wtip, but *Drosophila* has only one homolog called CG11063 that belongs to the Wtip/Limd1 subfamilies. Recent studies have demonstrated that *Xenopus* Limd1 and Wtip are essential for epithelial-mesenchymal transitions in the neural crest during early development [27]. In addition, *Drosophila* CG11063 has been shown to negatively regulate the Hippo intracellular signaling pathway that controls cell survival and proliferation in the epithelial organs [28]. In our study, Ajuba-deficient medaka embryos displayed apparently normal development of the neural crest and epithelial organs (data not shown), implying that the functions of members of the Ajuba and Wtip/Limd1 subfamilies may overlap during early development. Pratt et al. have reported that Ajuba null mice are viable and reach adulthood without any obvious

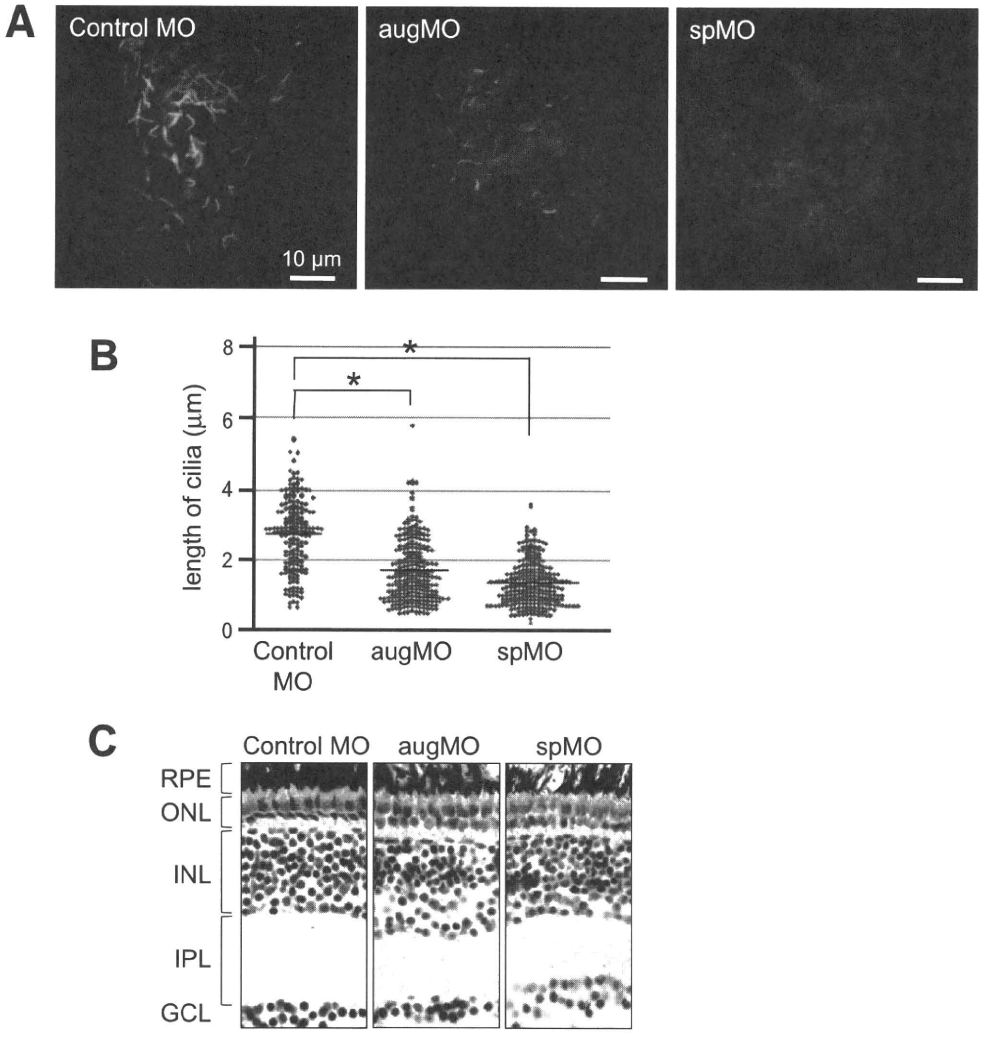

Fig. 4. Ajuba knockdown decreases ciliary length in the Kupffer's vesicle. (A) Defective cilia outgrowth but normal KV development. Stage 21 control, augMO and spMO morphant embryos were immunostained with anti-acetylated tubulin to visualize KV cilia. Whole-mount fluorescent images are shown. Cilia appear shorter in the absence of Ajuba. (B) Quantitation of decreased ciliary length. Lengths of primary cilia in the KV of the embryos in (A) were quantified using Image Browser software. Mean absolute cilium length was 2.78 μm for control embryos ($n = 197$ cilia), 1.72 μm for augMO morphants ($n = 224$ cilia), and 1.37 μm for spMO morphants ($n = 260$ cilia). $^{*}P < 0.01$. Horizontal line, mean. (C) Normal retinal structure. Transverse sections of the eyes of hatched control MO-, spMO- or augMO-injected embryos were stained with hematoxylin/eosin. There were no obvious structural defects in the retinas of Ajuba morphants. RPE, retinal pigment epithelium; ONL, outer nuclear layer (photoreceptor nuclei); INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

developmental pathologies [11]. A recent functional genomic screen using siRNA technology revealed that several dozen proteins are critical for the human ciliogenesis, but human Ajuba did not hit in the screen [29]. In Ajuba null medaka embryos, however, although the visceral organs develop normally, they are abnormally positioned in the body cavity (Fig. 2D). Precisely why Ajuba deficiency produced no significant abnormal phenotype in mice is not known. It may be that other unknown molecule(s) that do not exist in medaka serve redundant functions in the regulation of mammalian ciliogenesis. This discrepancy between the mammalian and medaka models highlights the dangers of relying on one system to define the function of a given gene, and further validates the use of our medaka knockdown system to explore the developmental roles of vertebrate gene families.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.05.017.

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ARTICLE

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Negative Regulation of *wnt11* Expression by Jnk Signaling During Zebrafish Gastrulation

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ABSTRACT

Stress-induced Sapk/Jnk signaling is involved in cell survival and apoptosis. Recent studies have increased our understanding of the physiological roles of Jnk signaling in embryonic development. However, still unclear is the precise function of Jnk signaling during gastrulation, a critical step in the establishment of the vertebrate body plan. Here we use morpholino-mediated knockdown of the zebrafish orthologs of the Jnk activators Mkk4 and Mkk7 to examine the effect of Jnk signaling abrogation on early vertebrate embryogenesis. Depletion of zebrafish Mkk4b led to abnormal convergent extension (CE) during gastrulation, whereas Mkk7 morphants exhibited defective somitogenesis. Surprisingly, Mkk4b morphants displayed marked upregulation of *wnt11*, which is the triggering ligand of CE and stimulates Jnk activation via the non-canonical Wnt pathway. Conversely, ectopic activation of Jnk signaling by overexpression of an active form of Mkk4b led to *wnt11* downregulation. Mosaic lineage tracing studies revealed that Mkk4b-Jnk signaling suppressed *wnt11* expression in a non-cell-autonomous manner. These findings provide the first evidence that *wnt11* itself is a downstream target of the Jnk cascade in the non-canonical Wnt pathway. Our work demonstrates that Jnk activation is indispensable for multiple steps during vertebrate body plan formation. Furthermore, non-canonical Wnt signaling may coordinate vertebrate CE movements by triggering Jnk activation that represses the expression of the CE-triggering ligand *wnt11*. J. Cell. Biochem. 110: 1022–1037, 2010.

KEY WORDS: JNK; MKK4; GASTRULATION; CONVERGENT EXTENSION; *WNT11*; ZEBRAFISH

Abbreviations used: CE, convergent extension; Jnk, c-Jun N-terminal kinase; Mkk, mitogen-activated protein kinase kinase; Dpp, decapentaplegic; MO, morpholino; aa, amino acid; hpf, hours post-fertilization; WT, wild type; slb, silberblick; ppt, pipetail; caMkk4b, constitutively active Mkk4b.Jungwon Seo and Yoichi Asaoka contributed equally to this work.

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Stress-activated protein kinase/c-Jun N-terminal kinase (Sapk/Jnk) is activated in response to a variety of cellular stresses. Once activated, Jnk phosphorylates downstream targets, including the c-Jun component of the activator protein-1 (AP1) transcription factor. Mkk4 and Mkk7 are two upstream Mapk kinases that interact with downstream kinases and scaffold proteins to activate Jnk. The outcome of the signaling cascades initiated by Mkk4 and Mkk7 is the phosphorylation of the Tyr and Thr residues, respectively, in the Jnk's Thr-Pro-Tyr motif [Davis, 2000; Chang and Karin, 2001]. However, there is evidence that the specific transmission of signals from these upstream kinases to Jnk may rely on different sets of, and/or interactions with, downstream kinases and scaffold proteins [Whitmarsh et al., 1998], such that Mkk4 and Mkk7 have distinct biological functions in vivo. For example, Mkk7 (but not Mkk4) is an essential and specific component of the Jnk signaling pathway activated by proinflammatory cytokines [Tournier et al., 2001]. In addition, although they both die of massive liver cell apoptosis, $mkk4^{-/-}$ mice die on embryonic day 10.5 (E10.5), whereas $mkk7^{-/-}$ mice die on E11.5 [Nishina et al., 1997, 1999; Yang et al., 1997; Ganiatsas et al., 1998; Watanabe et al., 2002; Wada et al., 2004].

Analyses of various *jnk* knockout mice have revealed much about the physiological role of Jnk signaling in embryogenesis. In mammals, the Jnk family consists of three related genes, *jnk1*, *jnk2*, and *jnk3* [Derijard et al., 1994; Kallunki et al., 1994; Mohit et al., 1995]. A role for Jnk in tissue morphogenesis was first suggested by the observation that $Jnk1^{-/-}Jnk2^{-/-}$ double mutant mice died at E11 with defective closure of the neural tube in the hindbrain [Kuan et al., 1999]. In this case, Jnk was required to control the survival and apoptosis of neuronal cells. More recently, Jnk has emerged as a critical regulator of cell migration and the morphogenetic movement of epithelial sheets. In *Drosophila*, a well-orchestrated Jnk signaling pathway is required for the sealing of embryonic epidermis in a process known as dorsal closure [Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996]. Jnk is activated in the leading edge of the dorsal epidermis at the onset of dorsal closure and drives the expression of the TGFβ homolog Decapentaplegic (Dpp), a secreted morphogen that regulates dorsal closure [Reed et al., 2001]. Little is known as yet about the function of the Jnk pathway in vertebrate morphogenesis, particularly when the body plan is first laid down.

The basic body plan of vertebrate embryos is established during gastrulation by a series of coordinated cell movements that lead to the formation of endoderm, mesoderm, and ectoderm, and overtly shape the embryonic axis [Keller, 2002]. A major driving force of vertebrate gastrulation is convergent extension (CE), a mechanism in which dorsal mesodermal cells polarize, elongate along the mediolateral axis, and intercalate toward the midline (convergence), leading to the extension of the anterior–posterior axis of the embryo [Keller, 2002; Tada et al., 2002; Seifert and Mlodzik, 2007]. CE is well conserved among vertebrate species, including in frog (*Xenopus laevis*) and zebrafish (*Danio rerio*) [Solnica-Krezel, 2005].

The study of gastrulation in mammals is difficult because these animals develop in utero, preventing direct observation of the embryos. In contrast, fertilized zebrafish eggs develop ex utero into transparent embryos that can be directly observed and are highly amenable to manipulations such as tissue transplantation and molecular perturbation. There is a high degree of conservation between zebrafish and mammalian genes, and a shared developmental path that results in fundamental similarities in many tissues and organs. In addition, there exists a wide selection of mutant zebrafish lines with developmental abnormalities, including gastrulation defects. Thus, zebrafish provide a very attractive alternative to mammals for studying the molecular and cellular bases of vertebrate morphogenesis.

Genetic analyses of gastrulation mutants in zebrafish and functional studies in *Xenopus* have revealed that CE is regulated by the non-canonical Wnt signaling pathway, which does not involve β-catenin [Seifert and Mlodzik, 2007]. Wnt11 and Wnt5 have been found to be essential ligands for normal cell movements during vertebrate CE. The zebrafish mutants *silberblick* (*slb*) and *pipetail* (*ppt*) have a mutation in *wnt11* or *wnt5*, respectively, and *slb–ppt* double mutants show severe CE defects [Heisenberg et al., 2000; Kilian et al., 2003]. Interestingly, *wnt5* mRNA can partially rescue the *slb* phenotype [Kilian et al., 2003], indicating a partial redundancy of Wnt11 and Wnt5 functions. In *Xenopus*, Wnt11 and Wnt5 bind to the Frizzled receptor, initiating non-canonical Wnt signaling that leads to activation of RhoA and Rac [Habas et al., 2001, 2003]. This RhoA and Rac activation triggers Jnk signaling critical for CE in *Xenopus* [Habas et al., 2003; Kim and Han, 2005]. Notably, depletion of Jnk in *Xenopus* causes defective gastrulation in these animals [Yamanaka et al., 2002]. All these findings suggest that Jnk is an essential component of the non-canonical Wnt pathway involved in vertebrate CE. However, the precise molecular mechanism by which Jnk signaling regulates CE has remained obscure. In an effort to elucidate the downstream targets of Jnk signaling in the non-canonical Wnt pathway that is associated with early embryogenesis, we have analyzed the functions of Mkk4 and Mkk7 orthologs in zebrafish (Mkk4a, Mkk4b, and Mkk7). Using morpholino-mediated knockdown, we demonstrate that Mkk4b is essential for CE movements. Furthermore, we provide the first evidence that *wnt11* itself is a downstream target of the Jnk cascade in the non-canonical Wnt pathway associated with early embryogenesis.

MATERIALS AND METHODS

ZEBRAFISH STRAINS

The AB and TL wild-type (WT) strains were maintained essentially as described in "The Zebrafish Book" [Westerfield, 1994]. Embryos were produced by natural matings and staged by standard morphological criteria or by hours post-fertilization (hpf), as described [Kimmel et al., 1995; Asaoka et al., 2002].

CLONING OF ZEBRAFISH MKK4 AND MKK7 GENES

Zebrafish sequences highly homologous to mouse *mkk4* and *mkk7* cDNAs were identified by database searching. Full-length zebrafish cDNAs were obtained by 5′- and 3′-RACE PCR according to the manufacturer's protocols (Invitrogen). RACE-PCR fragments were purified and subcloned into pGEM-T easy (Promega).

SEMI-QUANTITATIVE RT-PCR ANALYSIS

Total RNA was isolated from embryos at various developmental stages using TRIzol reagent according to the manufacturer's