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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

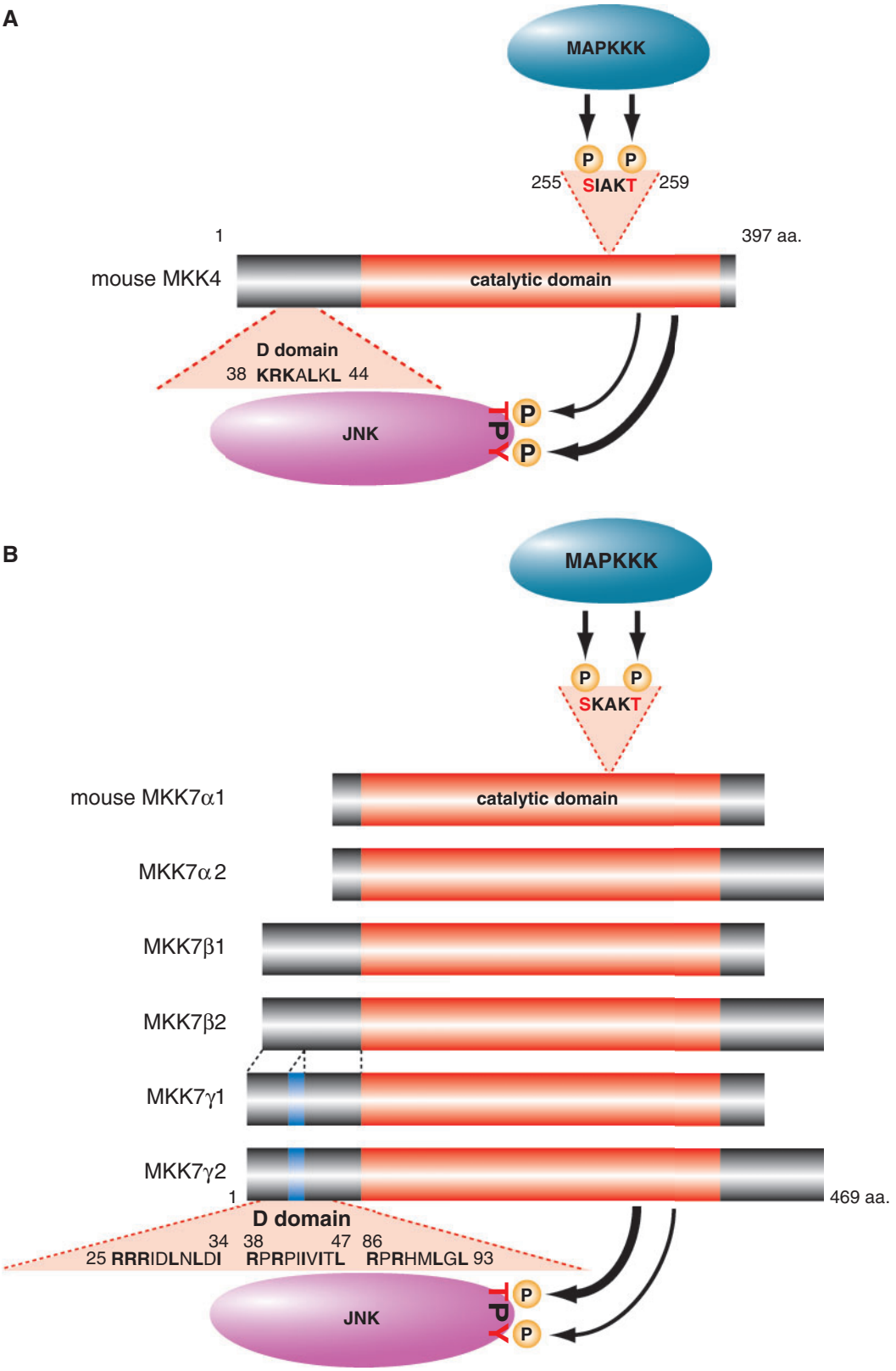

Fig. 1 Function and structure of murine MKK4 and MKK7 isoforms. A specific MAPKKK phosphorylates (A) MKK4 and (B) MKK7 at Ser (S) and Thr (T) residues within the Ser-X-Ala-Lys-Thr (SXAKT) motif of the catalytic domain. Activated dual specificity kinases, MKK4 and MKK7, in turn activate JNK by preferentially 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 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

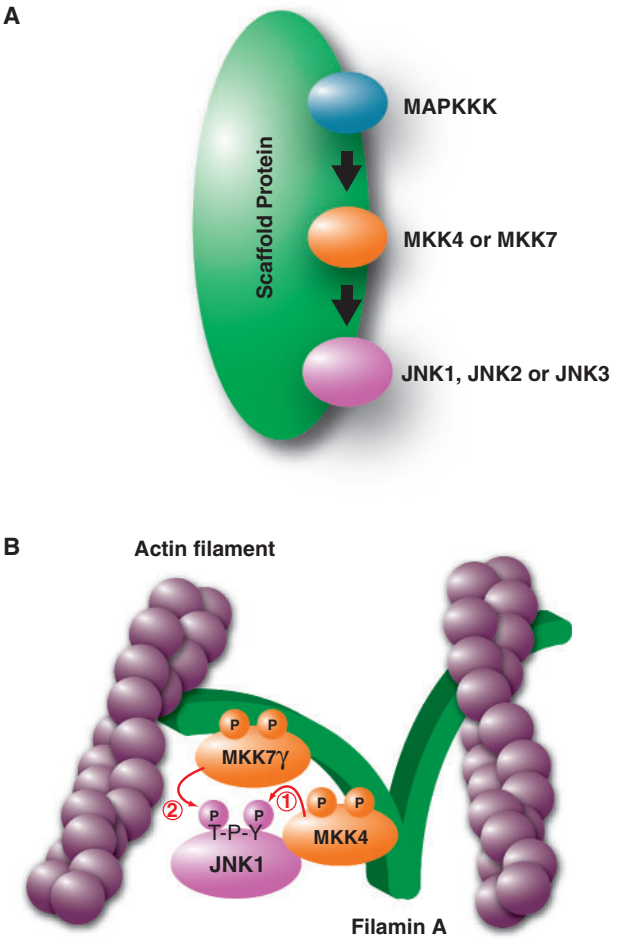

Fig. 2 Scaffold proteins mediating the structural and functional organization of the three-tier JNK signalling module. (A) Model of how a typical scaffold protein supports the assembly of a three-tier JNK signalling module consisting of a MAPKKK, a MAPKK (MKK4 or MKK7) and a JNK. Such scaffold proteins 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.

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

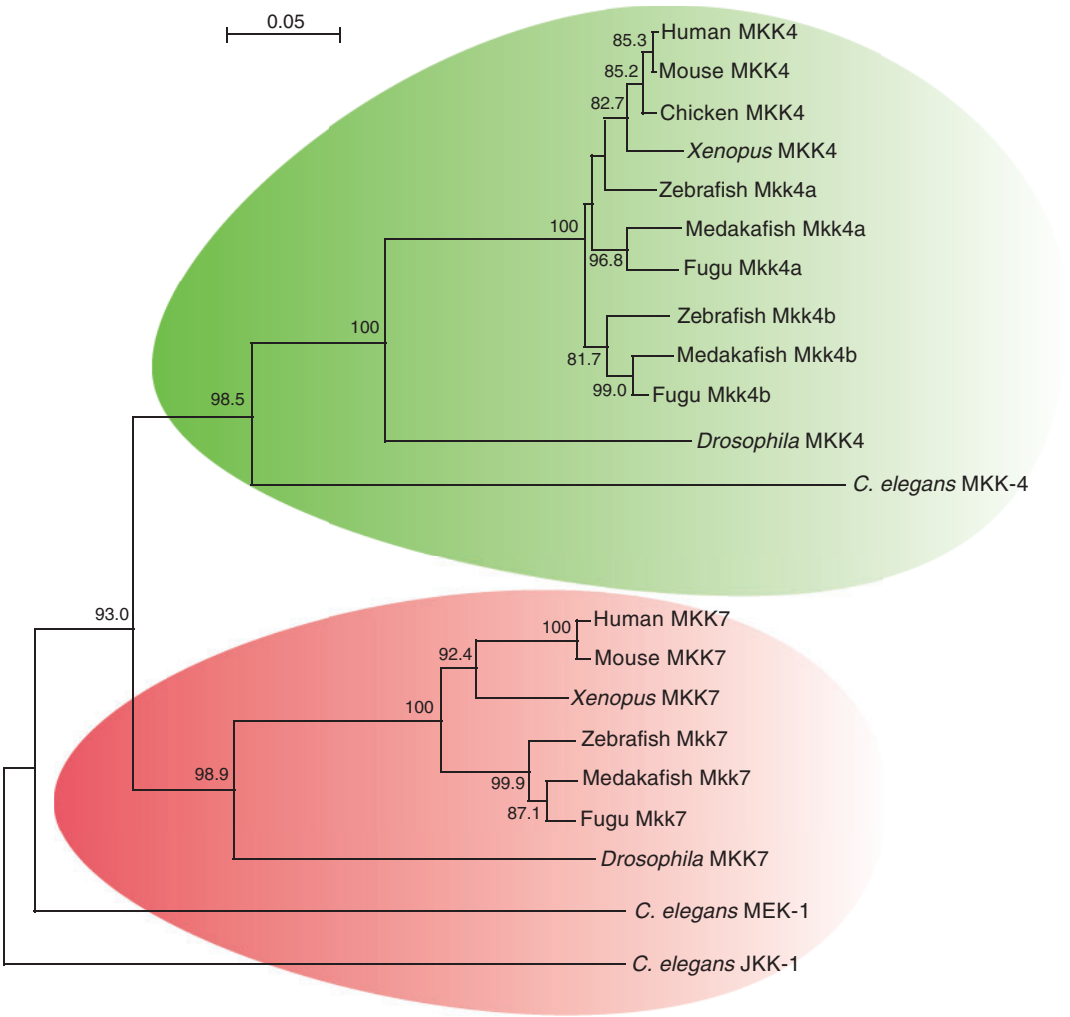

Fig. 3 Molecular phylogenetic tree relating the MKK4 and MKK7 proteins of nine species. The tree was constructed using the neighbour-joining method on the basis of the amino acid identity. The estimated bootstrap probabilities (percent) of local topologies are shown on each node. The length of the scale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

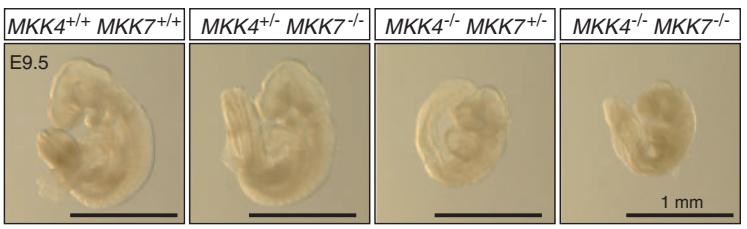

Fig. 4 Embryonic lethality in progeny of $Mkk4^{+/-}$ $Mkk7^{+/-}$ intercrosses. $Mkk4^{+/-}$ $Mkk7^{+/-}$ mice were intercrossed and the genotypes and viability of the progeny embryos were determined at the indicated time points of gestation. Dead embryos (numbers in parentheses) were defined as those 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*

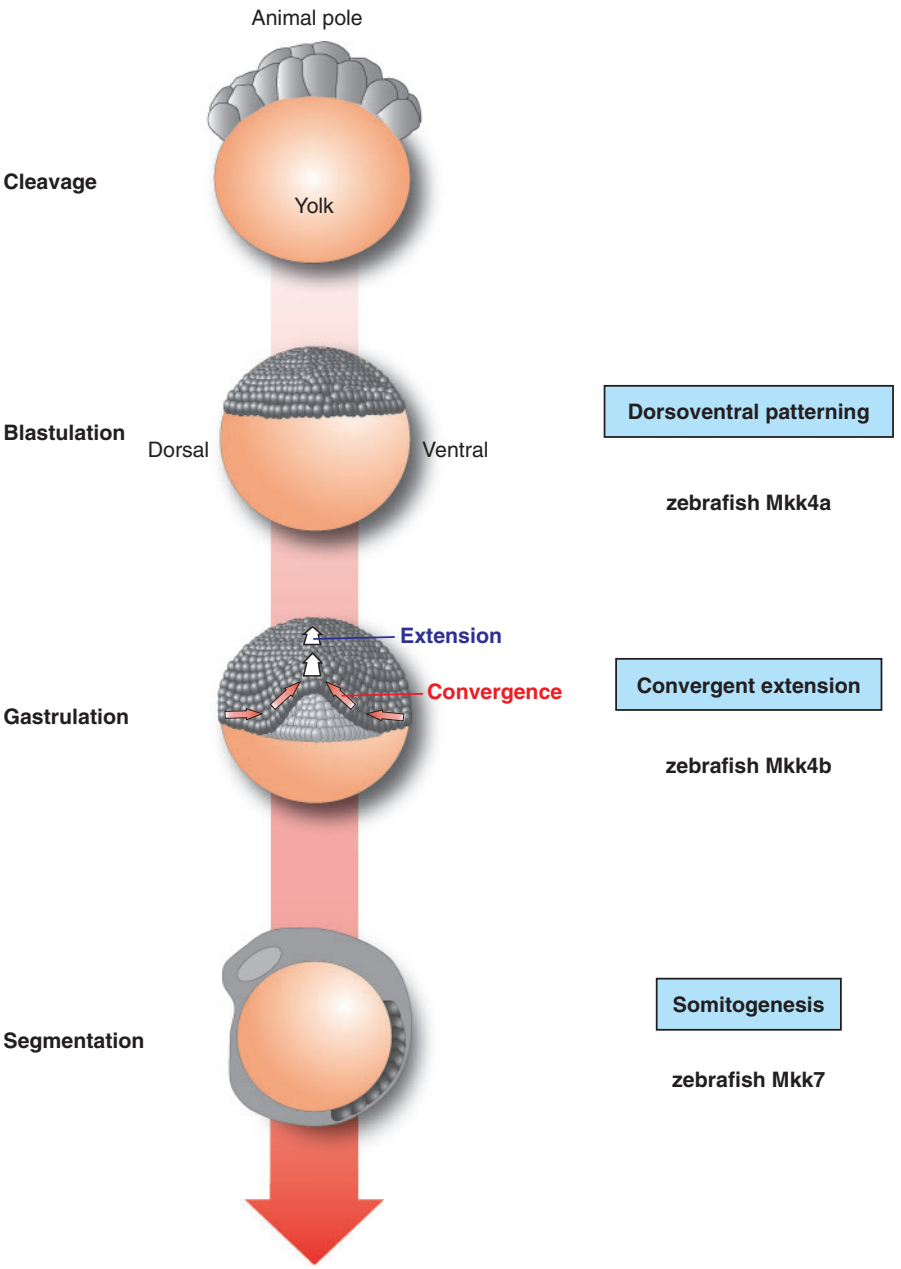

Fig. 5 Multiple roles of zebrafish Mkk4 and Mkk7 during body plan formation. The early embryonic development of the zebrafish occurs in four stages: cleavage, blastulation, gastrulation and segmentation. As indicated, Mkk4a is crucial for dorsoventral patterning, Mkk4b for convergent extension and Mkk7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