

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

Jungwon Seo,^{1,2} Yoichi Asaoka,^{1*} Yoko Nagai,^{1,2} Jun Hirayama,³ Tokiwa Yamasaki,^{1,2} Misako Namee,¹ Shinya Ohata,^{1,2} Nao Shimizu,^{1,2} Takahiro Negishi,¹ Daiju Kitagawa,¹ Hisato Kondoh,^{4,5} Makoto Furutani-Seiki,^{4,6} Josef M. Penninger,⁷ Toshiaki Katada,² and Hiroshi Nishina^{1*}

¹Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan

²Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan

³Medical Top Track Program, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan

⁴SORST, Kondoh Differentiation Signaling Project, Japan Science and Technology Corporation, Kyoto 606-8305, Japan

⁵Graduate School of Frontier Biosciences, Osaka University, Osaka 565-0871, Japan

⁶Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

⁷Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohr-gasse 3, Vienna A-1030, Austria

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. © 2010 Wiley-Liss, Inc.

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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*Correspondence to: Dr. Yoichi Asaoka, PhD and Dr. Hiroshi Nishina, PhD, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan.

E-mail: y-asaoka.dbio@mri.tmd.ac.jp, nishina.dbio@mri.tmd.ac.jp

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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 [Tourmier 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 TGF β 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

protocol (Invitrogen). First-strand cDNA was synthesized from 1 μ g total RNA using SuperscriptIII reverse transcriptase (Invitrogen) and a random or oligo-dT primer. Semi-quantitative PCR was done essentially as described [Okuda et al., 2006]. Primers used for RT-PCR analysis of mRNA expression in zebrafish extracts were as follows: for *mkk4a*, 5'-CGTTC AACAG TAGAC GAGCG-3' and 5'-AATCA CTTCG TCTAA AGAGG-3'; for *mkk4b*, 5'-CGCTC CACGG TGGAT GAGAA-3' and 5'-AATGA CGTCA TCTGA CGCAC-3'; for *mkk7*, 5'-TGGCC ATGTC ATCGC AGTCA-3' and 5'-GGAAA CTGTG CTGTG GCTAG-3'; for β -actin, 5'-CAGCT TCACC ACCAC AGC-3' and 5'-GTGGA TACCG CAAGA TTCC-3'; for *jnk1a-1*, 5'-AGCGT ATGAC CACGT CCTCG-3' and 5'-GGGCC AGACC GAAAT CCAAG-3'; for *jnk1a-2*, 5'-GGATG CTTAC ACATC GACTT CAC-3' and 5'-CATCC ATCAG CTCCA TTAGT AGG-3'; for *jnk2*, 5'-ATCTG GACCA TGAGA GGATG TC-3' and 5'-CTGGG GTTTG TTCAT CACAT AG-3'; for *e-cadherin*, 5'-ACAAA CTTAG GGCTC ATGCG-3' and 5'-ACAGA TGACA TGAC GAGGA-3'; for *stat3*, 5'-TGAAT GGAAA CAGCC AGGCA-3' and 5'-TTTGA TGACA AGGGG TCGGT-3'; for *liv1*, 5'-CGGTT GCCAA TATGA TTGGC-3' and 5'-GGTGG ATTCC TGGTT CATCT-3'; for *wnt5*, 5'-CCGGA GATGT ACATC ATTGG-3' and 5'-TTCTC ACGTT CACGA GCGTC-3'; for *wnt11*, 5'-GTAAA CTCTT GGACG GGCTC-3' and 5'-CGAAG GTTAT CTCCA CATCC-3'.

ZEBRAFISH EMBRYO PREPARATION

Embryos were de-yolked with de-yolk buffer (1/2 Ginzburg Fish Ringer) without calcium [Link et al., 2006]. Subsequently embryos were lysed in buffer containing 100 mM NaCl, 40 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.05% 2-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 4 μ g/ml aprotinin, 100 μ M Na₃VO₄, and 50 mM NaF, and sonicated for 1 min. Lysates were centrifuged at 20,000g to pellet cellular debris. Protein concentration was measured by BCATM Protein Assay Kit (Pierce).

ES CELL LINES

Mkk4^{-/-} and *mkk7*^{-/-} murine ES cell lines were generated as previously described [Nishina et al., 1997; Fleming et al., 2000; Kishimoto et al., 2003].

ANTIBODIES

Anti-Jnk1 polyclonal Ab (FL), which recognizes both the 46 and 55 kDa splice variants of Jnk1, was from Santa Cruz Biotechnology, Inc. Anti-phospho-Jnk (9251) and anti-FLAG (M2) Abs were from Cell Signaling Technology and Sigma-Aldrich Co., respectively.

CONSTRUCTION OF PLASMIDS AND TRANSFECTION

cDNAs encoding FLAG-tagged zebrafish *Mkk4a-1*, *Mkk4b*, and *Mkk7* were cloned into the mammalian expression vector pCE-IRES2-EGFP [Ura et al., 2007]. ES cells were plated at 1 \times 10⁶ cells/35-mm dish and transfected 1 day later with expression construct (4 μ g) plus Lipofectamine 2000 reagent (Invitrogen). Transfected cells were stimulated with UV light (1 kJ/m²) and subjected to standard Western blot analysis as described below.

WESTERN BLOT ANALYSIS

Proteins were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane that was then incubated for 1 h

in blocking solution (2% or 5% skim milk in Tris-buffered saline [TBS]). The blocked membrane was incubated for 1 h in blocking solution containing anti-phospho-Jnk, anti-Jnk1, or anti-FLAG Ab. The membrane was then washed in TBS-Tween 20 (0.05%), incubated with anti-mouse/rabbit horseradish peroxidase-conjugated Ab (Jackson ImmunoResearch Laboratory) for 30 min, and washed three times in TBS-Tween 20. Proteins were visualized using Immobilon HRP (Millipore) or the SuperSignal West Femto Kit (Pierce) and a ChemiDoc XRS system (Bio-Rad), as described [Kishimoto et al., 2003].

ANTI-SENSE MORPHOLINO (MO) AND MRNA INJECTIONS

All MOs were designed to bind to exon-intron junctions and were synthesized by Gene Tools (Philomath, OR). Sequences of splice-blocking MOs were as follows: *mkk4a* MO, 5'-GATGA AACAG ACGAA CCTCT CTGAA-3'; *mkk4b* MO, 5'-TGTGT GTGTC TGACC TCTCT GAAGA-3'; *mkk7* MO, 5'-AGAGG AACTC ACCAG AGAAA TGCCA-3'; *jnk1a-1* MO, 5'-AGACA AATAA CTTAC ACATC CTGGA-3'; *jnk1a-2* MO, 5'-ATTTC AGTGT CTTGA CTTAC ATTTT-3'; *jnk2* MO, 5'-AAAAA CAGCA TTACC ATTCT CCTTG-3'. Sequences of translation-blocking MOs were: *mkk4a-1* atgMO, 5'-GCGTC GCCAT TTGGG TTTGA CTCTT-3'; *mkk4b* atgMO, 5'-AGAGT CACTG CTGGG AGCCG CCATT-3'; *mkk7* atgMO, 5'-AGAGT CTCTG CTCCA GCGAC GACAT-3'; *mkk4a-s* atgMO, 5'-GCCAT CTTGT TGACC GAGCC ATACG-3'. The standard control MO was: 5'-CCTCT TACCT CAGTT ACAAT TTATA-3'.

For knockdown, MO solution was injected into the yolks of one- to four-cell stage zebrafish embryos immediately beneath the cell body. For *wnt5* or *wnt11* overexpression, a construct designed to produce mRNA encoding full-length zebrafish *wnt5* or *wnt11* was created by cloning the relevant fragment (amplified by high-fidelity PCR) into the pCS2+ plasmid. A constitutively active form of *Mkk4b* (ca*Mkk4b*) was constructed by replacing Ser275 and Thr279 with aspartic acid and glutamic acid, respectively, and subcloning into the pCS2+ plasmid. Sense strand capped mRNA was synthesized using SP6 RNA polymerase and the mMACHINE mMACHINE system (Ambion). RNA injections were performed as described [Shinya et al., 2000].

WHOLE-MOUNT IN SITU HYBRIDIZATION

Digoxigenin-labeled RNA probes were synthesized using the DIG RNA Labeling Kit (Roche Diagnostics). Primers used for mRNA expression analysis in whole zebrafish embryos were as follows: for *dlx3*, 5'-TCCGA CTCT AAGGA CTCTC-3' and 5'-TTCAC CTGTG TCTGT GTGAG-3'; for *ntl*, 5'-AGACG AATGT TTCCC GTGCT-3' and 5'-CTTCT CTCTT TGGCA TCGAG-3'; for *hgg1*, 5'-ATGAG GAGTT CAGAC AGGCA-3' and 5'-ATTAC CGCTG GGAAT GTCCA-3'; for *par2.1*, 5'-CCGCG AGTAT TAAAC CTGGA-3' and 5'-AGGTG CTCC GTAAA CTCTC-3'; for *gsc*, 5'-GTCAC TATGA AGGAC ACTCG TGC-3' and 5'-TTTGT TCCTG TTTC AGGCG AC-3'; for *wnt5*, 5'-GTAGC AGACG TGAGC ACTGG-3' and 5'-CGCAT TCCGA AAGTT CTAA GAG-3'. Mixtures of three riboprobe pairs were used to detect *wnt11*, as follows: (1) 5'-GTAAA CTCTT GGACG GGCTC-3' and 5'-CTAAA GTCCT GTGGG CTGA-3'; (2) 5'-CCGGA ATTCA TGACA GAATA CAGGA ACT-3' and 5'-CGAAG GTTAT CTCCA CATCC-3'; (3) 5'-GGTGC TTATG GACTC TCTAG-3' and 5'-GAGTC GACTC

ACTTC GAGAC GTATC TCT-3'. Riboprobes for *myoD* and *krox20* were used as described [Shinya et al., 2000]. Whole mount in situ hybridization procedures were performed essentially as described [Thisse et al., 1993].

QUANTITATIVE REAL-TIME RT-PCR

Quantitative real-time RT-PCR analyses were performed using the Chromo4 real-time detection system (Bio-Rad). The PCR primers used were as follows: for *wnt11*, 5'-CACAA CAATG CTGTT GGCAG ACAGG TG-3' and 5'-GGAGA TGGTG CTGAT GTCTT GAAGA CC-3'; for β -actin, 5'-GCAGA TGTGG ATCAG CAAGC AGG-3' and 5'-CTGAG TCAAT GCGCC ATACA GAG-3'. For a 20 μ l PCR reaction, cDNA template was mixed with 10 μ l iQ SYBR Green Supermix (Bio-Rad) plus the appropriate primers to a final concentration of 200 nM each. The reaction was first incubated at 95°C for 3.5 min, followed by 41 cycles of 95°C for 12 s, 60°C for 13 s, and 72°C for 18 s.

RESULTS

CLONING AND CHARACTERIZATION OF ZEBRAFISH MKK4A, MKK4B, AND MKK7 GENES

To unravel the role of Jnk signaling in early zebrafish development, we first determined whether zebrafish *mkk4* and *mkk7* could function as direct activators of Jnk. We performed BLAST searches with mouse *mkk4* and *mkk7* to enable predictions of zebrafish *mkk4* and *mkk7* cDNA sequences. The obtained EST sequences were subjected to 5'- and 3'-RACE methodology to acquire the full-length cDNA sequences. The zebrafish has two *mkk4* genes, named *mkk4a* and *mkk4b* (GenBank accession no. AB438979), but only one *mkk7* ortholog (GenBank accession no. AB438980). The predicted amino acid (aa) sequences of the proteins encoded by these zebrafish genes are 81–84% identical to those of the mouse, and the Mkk phosphorylation sites are conserved (Fig. S1A). Two splice variants of zebrafish *mkk4a* were identified: *mkk4a-s* and *mkk4a-l* (GenBank accession nos. AB030901 and AB438978). Mkk4a-s (281 aa) is an N-terminal truncated form of Mkk4a-l (404 aa). A phylogenetic analysis of vertebrate *mkk4* genes revealed that zebrafish *mkk4a* was clustered with mammalian *mkk4* genes, whereas zebrafish *mkk4b* was clustered with those of other teleosts (Fig. S1B). These phylogenetic relationships suggest that the duplication of the *mkk4* gene occurred in the common ancestors of teleosts and tetrapods.

We next used semi-quantitative RT-PCR to examine the expression dynamics of *mkk4a*, *mkk4b*, and *mkk7* during early zebrafish development. All three genes were continuously expressed from the one-cell stage to the blastula (4.7 hpf) (Fig. 1A). Levels of both *mkk4a* and *mkk7* were markedly decreased by the shield stage and became relatively low at the tailbud stage. In contrast, *mkk4b* expression was essentially constant from the one-cell stage through to the shield stage and relatively high during later gastrula stages.

To monitor Jnk activity during zebrafish embryogenesis, we prepared protein extracts from zebrafish embryos collected at each of the 30% epiboly, shield, 75% epiboly, and tailbud stages, and examined the relative amounts of the active, phosphorylated form of Jnk by Western blot analysis. Jnk activity was detected from 30%

epiboly and relatively high during later gastrula stages (Fig. 1B), suggesting the possibility that Jnk plays a role in the gastrulation stage.

In mammals, Mkk4 and Mkk7 have distinct biochemical properties and preferentially phosphorylate the Tyr and Thr residues, respectively, within the Thr-Pro-Tyr motif of Jnk [Lawler et al., 1998; Kishimoto et al., 2003]. To qualitatively determine whether these distinctions applied to the zebrafish orthologs of Mkk4 and Mkk7, and to gauge the functional relatedness of the mammalian and zebrafish enzymes, we analyzed whether zebrafish Mkks could compensate for the lack of mouse Mkk4 and Mkk7 in *mkk4*^{-/-} and *mkk7*^{-/-} murine ES cells, respectively. We transfected zebrafish *mkk4a-l*, *mkk4b*, and *mkk7* expression vectors separately into *mkk4*^{-/-} or *mkk7*^{-/-} ES cells, which lack the capacity to activate Jnk in response to UV irradiation [Nishitai et al., 2004]. We then subjected the transfected cells to UV irradiation and assessed Jnk activation. We found that zebrafish Mkk4a-l or Mkk4b, but not Mkk7, rescued UV-induced Jnk activation in *mkk4*^{-/-} ES cells (Fig. 1C, lanes 2–4). Conversely, zebrafish Mkk7, but not Mkk4a-l or Mkk4b, rescued UV-induced Jnk activation in *mkk7*^{-/-} ES cells (Fig. 1C, lanes 6–8). These results suggest that zebrafish Mkk4 and Mkk7 are analogous in function to their murine counterparts and play distinct biochemical roles during stress-induced Jnk activation.

ZEBRAFISH MKK4B IS INDISPENSABLE FOR NORMAL CE REGULATION

To elucidate the physiological roles of Mkk4a, Mkk4b, and Mkk7 during early zebrafish development, we performed anti-sense morpholino (MO)-mediated knockdown of each gene's mRNA and analyzed its residual expression by RT-PCR. Microinjection of *mkk4b* MO effectively prevented correct splicing of target pre-mRNA from the shield to tailbud stages (Fig. 2A). As a result, *mkk4b* morphants exhibited severe defects in anterior–posterior extension after gastrulation. At 11 hpf, *mkk4b* morphants displayed an MO dose-dependent shortening of body length (Fig. 2B, row 1) that did not recover at later developmental stages (Fig. 2B, rows 2 and 4). In addition, *mkk4b* morphants displayed broader notochords and somites during early segmentation than did control MO-injected embryos (Fig. 2B, row 3), indicating that CE is defective in the absence of Mkk4b. Quantification of the anterior–posterior axis extension and mediolateral convergence at 16 hpf revealed that *mkk4b* knockdown significantly increased the angle between the anterior and posterior ends of the embryo, as well as its mediolateral distance, in a MO dose-dependent manner (Fig. 2C).

With respect to marker expression patterns, *mkk4b* morphants showed a more posteriorly and broadly positioned prechordal plate (*hgg1*), a wider neural plate (*dlx3* and *pax2.1*), and a shorter and broader notochord (*ntl*) at the tailbud stage than did controls (Fig. 2D). Although their body axis was shorter and their somites wider, *mkk4b* morphants had the same numbers and shapes of rhombomeres (marked by *krox20* expression) and somites (*myoD*) as control embryos at the eight-somite stage (Fig. 2E), implying that Mkk4b has no obvious role in specifying cell fate.

To further confirm that it was loss of Mkk4b that was responsible for the observed CE defects, we co-injected *mkk4b*

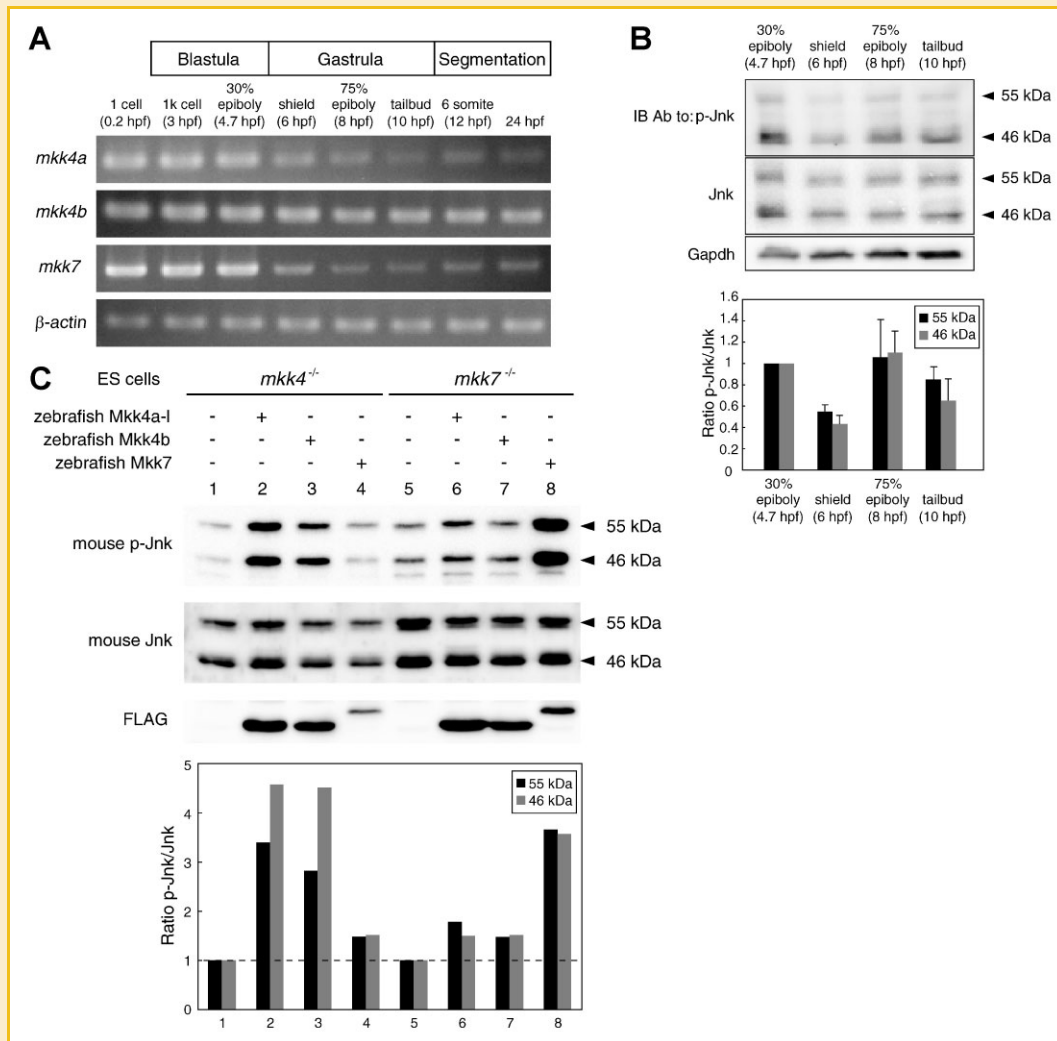


Fig. 1. Expression and biochemical properties of zebrafish *mkk4a*, *mkk4b*, and *mkk7* gene products during early development. A: Temporal expression patterns of zebrafish *mkks*. Semi-quantitative RT-PCR reveals relatively high expression of *mkk4b* up to 24 hpf in WT embryos, whereas *mkk4a* and *mkk7* levels abruptly decrease around the shield stage. Lanes 1–8 correspond to template cDNA derived from 0.2 to 24 hpf embryos ($n = 30, 30, 50, 20, 20, 20, 30, 50$ embryos, respectively). β -Actin, loading control. B: Analysis of Jnk activity during zebrafish embryogenesis. Approximately 50 μ g of protein was loaded in SDS-PAGE in each lane. Activated Jnk and total Jnk were detected with anti-phospho-Jnk (p-Jnk) and anti-total-Jnk antibodies, respectively. Image shown is representative of three separate experiments with similar results (top). Graphic results are expressed as a ratio of p-Jnk to total Jnk and the value of 30% epiboly stage is assigned an arbitrary value of 1 (bottom). Data shown are the mean \pm SEM of three independent experiments. C: Effects of zebrafish Mkk4 or Mkk7 expression on stress-induced Jnk activation in *mkk4*^{-/-} or *mkk7*^{-/-} mouse ES cells. *Mkk4*^{-/-} or *mkk7*^{-/-} mouse ES cells were transfected with zebrafish *mkk4a-l*, *mkk4b*, or *mkk7* expression vectors, cultured for 24 h, and stimulated with UV (1 kJ/m²). Expression levels of zebrafish Mkks (FLAG) and endogenous levels of p-Jnk and total Jnk (46 and 55 kDa isoforms) in cell lysates were detected by Western blotting (top). The histogram shows quantitative representations of the relative activated Jnk, which was expressed as a ratio of p-Jnk to total Jnk (bottom). The values of lanes 1 and 5 are assigned an arbitrary value of 1.

MO with in vitro-transcribed *mkk4b* mRNA and assayed for the rescue of the CE defects. Inspection of live co-injected embryos showed that the synthetic *mkk4b* mRNA was able to prevent *mkk4b* MO-induced defects (Fig. S2). Thus, the observed phenotypes are the result of specific knockdown of Mkk4b by the *mkk4b* MO.

To rule out the possibility that the observed phenotypes in *mkk4b* knockdown embryos were secondary to a patterning alteration, we evaluated the distribution of *gooseoid* (*gsc*), a dorsal mesoderm “organizer” marker at the shield stage. No alterations to the *gsc* expression pattern were observed in *mkk4b* morphants (Fig. 2F), indicating that dorsoventral specification is not disturbed by *mkk4b* MO treatment.

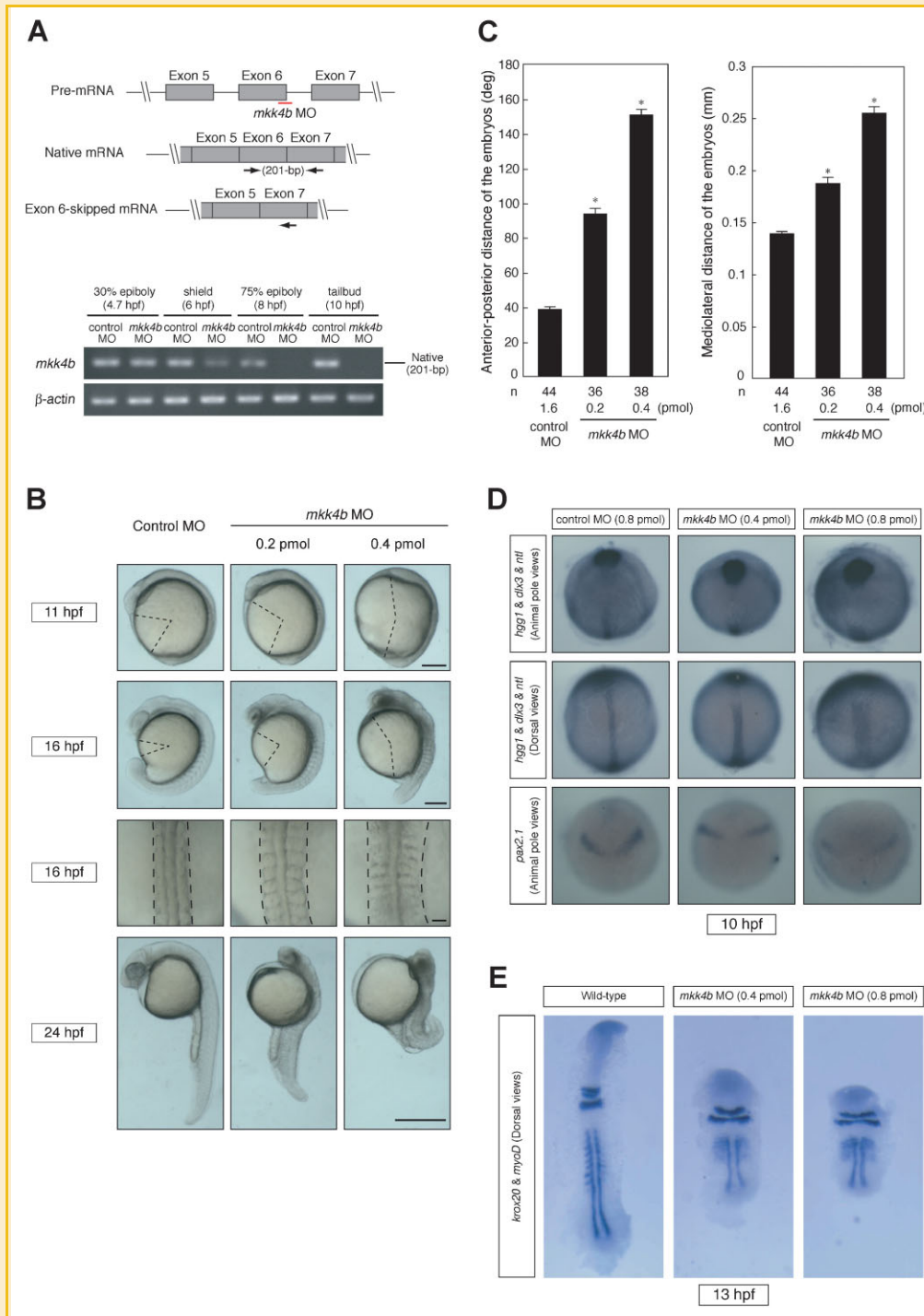
The data in Figure 2D suggested that *mkk4b* morphants developed slightly slower than controls. To determine if Mkk4b influences epiboly, we monitored the development of live embryos over a time course of 6–10 hpf. As was true for control embryos, gastrulation began at 6 hpf in *mkk4b* morphants and 75% epiboly was reached at 8 hpf (Fig. S3). However, whereas control embryos had completed their epiboly movements by 10 hpf, *mkk4b* morphants exhibited a mild delay in epiboly that extended it to 11 hpf (Figs. S3 and 2B). Thus, Mkk4b activity is not required for the initiation or progression of epiboly but does influence its late phase.

When parallel experiments were used to examine the importance of Mkk4a in early zebrafish development, a very different result was

obtained. Microinjection of 0.8 pmol of *mkk4a* MO reduced WT *mkk4a* mRNA beginning at the shield stage (Fig. S4A). Unlike *mkk4b* morphants, however, *mkk4a* morphants had no gross morphological abnormalities (Fig. S4B), and the expression patterns of *hgg1*, *dlx3*, *ntl*, and *pax2.1* were completely normal (Fig. S4C).

In morphants injected with 0.8 pmol of *mkk7* MO, expression of the WT *mkk7* transcript declined by the shield stage and was

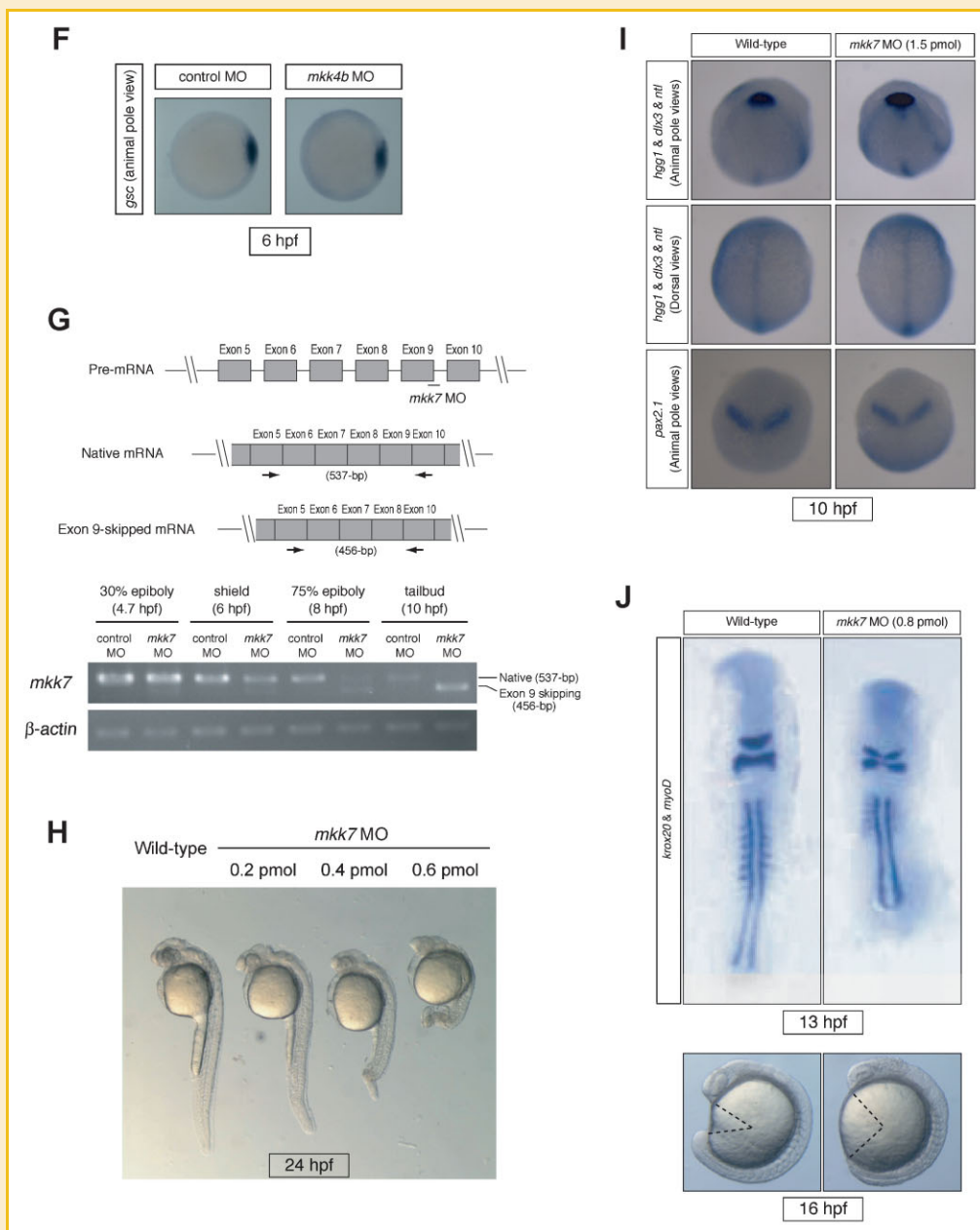
undetectable at the tailbud stage (Fig. 2G). Instead, a shorter amplification product was detected. Like *mkk4b* morphants, *mkk7* morphants exhibited an MO dose-dependent shortening of body length at 24 hpf (Fig. 2H). However, the expression patterns of *hgg1*, *dlx3*, *ntl*, and *pax2.1* were normal in *mkk7* morphants, even at a high dose of MO (1.5 pmol) (Fig. 2I). These data suggest that *mkk7* is dispensable for proper gastrulation. Nevertheless, during



segmentation, the *mkk7* morphants displayed somites of abnormal patterning and morphology (Fig. 2J). Thus, Mkk7 function is essential for normal somite morphogenesis.

Because the *mkk4a*, *mkk4b*, and *mkk7* genes are maternally expressed, it was possible that their expression products might persist throughout gastrulation. To rule out this possibility, we used translation-blocking MOs (atgMOs) that would specifically target maternal and zygotic *mkk* transcripts and prevent their translation. We injected embryos with a specific translation-blocking MO (*mkk4a-l* atgMO, *mkk4b* atgMO, or *mkk7* atgMO) and examined their development using gross morphological analysis and in situ hybridization. By 23 hpf, *mkk4b* atgMO- or *mkk7* atgMO-injected

embryos showed reduced body length compared to control-injected embryos, whereas few or no morphological differences were apparent in *mkk4a* atgMO-injected embryos (Fig. S5A). *Mkk4b* atgMO-injected embryos also showed altered expression of several marker genes (*hgg1*, *dlx3*, *ntl*, and *pax2.1*; Fig. S5B), highlighting the morphological abnormalities associated with defective CE movements at 10 hpf. This phenotype was strikingly similar to that of embryos subjected to *mkk4b* splice-blocking MO knockdown (Fig. 2D). Taken together, these results demonstrate that Mkk4b but not Mkk4a is essential for co-ordinated CE. In the following analyses, we focused on the specific role of Mkk4b–Jnk signaling in proper CE movements during gastrulation.



ZEBRAFISH JNK2 IS NECESSARY FOR CORRECT CE MOVEMENTS

It has previously been reported that non-canonical Wnt signaling regulates CE movements in *Xenopus* through the activation of Jnk [Yamanaka et al., 2002]. We therefore asked whether overexpression of *wnt11* or *wnt5* could activate Jnk during zebrafish gastrulation. Extracts from zebrafish embryos overexpressing *wnt11* or *wnt5* mRNA were prepared at 75% epiboly (8 hpf) and subjected to Western blotting with anti-phospho-Jnk antibody. This analysis confirmed that *wnt11* or *wnt5* overexpression significantly increased levels of the active, phosphorylated form of Jnk (Fig. 3A). Furthermore, analysis of extracts from embryos injected with 0.4 pmol *mkk4a* MO, *mkk4b* MO, or *mkk7* MO showed that Jnk phosphorylation was significantly decreased in *mkk4b* morphants but still detected in *mkk4a* and *mkk7* morphants (Fig. 3B). These data indicate that Mkk4b (exclusively) has a profound effect on CE, and that this effect is mediated through activation of the Jnk pathway. Thus, Mkk4b–Jnk signaling plays a critical role in proper CE movements during gastrulation.

To examine Mkk4b–Jnk signaling in zebrafish embryos, it was first necessary to identify zebrafish Jnk enzymes. To isolate zebrafish *jnk* genes, we used cDNA sequences for mammalian Jnks to perform BLAST searches of the zebrafish genomic database and identified four zebrafish *jnk* genes. A phylogenetic analysis based on the aa sequences of vertebrate Jnks revealed that the four zebrafish Jnk homologs closely resembled members of the three mammalian Jnk subfamilies, Jnk1, Jnk2, and Jnk3. We therefore designated the zebrafish proteins as Jnk1a-1, Jnk1a-2, Jnk2, and Jnk3 (Fig. S6). During gastrulation, mRNAs for Jnk1a-1, Jnk1a-2, and Jnk2 (but not Jnk3) were transcribed (data not shown).

To ascertain the functional importance of these zebrafish Jnk(s) for normal CE movements, we performed MO-mediated knockdown of the *jnk1a-1*, *jnk1a-2*, and *jnk2* genes and analyzed residual expression by RT-PCR. Co-injection of *jnk1a-1* MO and *jnk1a-2* MO efficiently prevented the correct splicing of *jnk1* pre-mRNAs during

gastrulation (Fig. 3C). However, unlike the *Xenopus* Jnk1 morphant [Yamanaka et al., 2002], *jnk1a-1* and *jnk1a-2* morphants showed no gross morphological abnormalities (Fig. 3E, center), and the expression patterns of *hgg1*, *dlx3*, *ntl*, and *pax2.1* were completely normal (Fig. 3F, center). On the other hand, injection of *jnk2* MO not only prevented correct splicing of *jnk2* pre-mRNA (Fig. 3D) but also induced severe defects in anterior–posterior extension and mediolateral convergence, as assessed by analysis of morphology and molecular markers (Fig. 3E,F, right). These results indicate that Jnk2, but no form of Jnk1, is required for normal CE movements during zebrafish gastrulation.

TRANSCRIPTION OF ZEBRAFISH *WNT11* IS REPRESSED BY *MKK4B*–*JNK* SIGNALING

The molecular mechanism by which Jnk regulates CE movements is poorly understood. To identify relevant transcriptional targets downstream of the Mkk4b–Jnk signaling cascade associated with zebrafish CE, we used RT-PCR to survey mRNA levels of candidate genes in control and Mkk4b morphants at 75% epiboly (8 hpf). Because normal gastrulation requires modulation of cadherin-mediated cell–cell adhesion, we first investigated *e-cadherin* expression but found that its mRNA levels were unchanged in our morphants (Fig. 4A, row 1). Similarly, although *stat3* and *liv1* are reportedly essential for zebrafish gastrulation [Yamashita et al., 2004], the expression of these mRNAs was also normal in our *mkk4b* morphants (Fig. 4A, rows 2 and 3). We next screened components of the non-canonical Wnt pathway and found that, although *wnt5* expression was normal (Fig. 4A, row 4), *wnt11* expression was highly upregulated in Mkk4b-depleted embryos at 75% epiboly (8 hpf) (Fig. 4A, row 5), as well as at the 30% epiboly, shield, and tailbud stages (Fig. 4B, left). In contrast, upregulation of *wnt11* expression was not detected in Mkk4a- or Mkk7-depleted embryos (Fig. 4A). When we analyzed the expression of *wnt11* transcripts using quantitative real-time RT-PCR, we confirmed that levels

Fig. 2. Mkk4b–Jnk signaling is required for proper CE movements. A: Validation of *mkk4b* MO efficacy. The *mkk4b* MO targets the exon 6/intron 6 junction of *mkk4b* pre-mRNA as shown in the diagram (top). WT zebrafish embryos were injected with 0.8 pmol of *mkk4b* MO or control MO, and total RNA was extracted from the 30% epiboly to tailbud stages and subjected to RT-PCR analysis (bottom). Arrows in the diagram represent the primer pairs used for the RT-PCR analysis. The *mkk4b* MO efficiently prevents correct splicing of *mkk4b* pre-mRNA beginning at the shield stage (6 hpf), conceivably leading to a functional impairment of Mkk4b. B: Gross appearance of *mkk4b* MO-injected embryos. Images of a live control MO-injected embryo and live *mkk4b* morphants injected with the indicated doses of *mkk4b* MO were acquired at 11, 16, and 24 hpf (rows 1, 2, and 4, respectively). These embryos are viewed laterally, with anterior to the top. *Mkk4b* morphants exhibit a shortening of body length and brain degeneration in a MO dose-dependent manner. Row 3 shows that the notochord and somites are wider in the *mkk4b* morphant than in the control at 16 hpf (dorsal view). Scale bars = 200 μ m (row 1); 200 μ m (row 2); 50 μ m (row 3); 500 μ m (row 4). C: Quantification of anterior–posterior axis extension and mediolateral convergence at 16 hpf. Left panel shows the average angle between the anterior and posterior ends of embryos ($n = 44, 36, 38$) that were injected as indicated. Right panel shows the average mediolateral width of embryos ($n = 44, 36, 38$) that were injected as indicated. Data shown are the mean \pm SEM. * $P < 0.01$ versus control. D: Impaired CE in *mkk4b* morphants. Control MO-injected embryos and *mkk4b* morphants were analyzed by whole-mount in situ hybridization for the expression of tissue-specific genes at the tailbud stage (10 hpf). The position of the prechordal plate (*hgg1*) is more posterior and broader in *mkk4b* morphants compared to the control. Expression of *dlx3* (neuroectoderm) reveals a broader neural plate, and the notochord (*ntl*) is shorter and broader. The midbrain/hindbrain boundary (*pax2.1*) has expanded laterally. E: Normal rhombomeres in *mkk4b* morphants. WT embryos and *mkk4b* morphants had the same number and shape of rhombomeres (*krox20*) and somites (*myoD*) at the eight-somite stage (13 hpf), although the body axis was shorter and somites were wider in the *mkk4b* morphants. F: Expression of the dorsoventral patterning marker gene *gsc* is normal in *mkk4b* morphants. Control MO (0.8 pmol) or *mkk4b* MO (0.8 pmol) was injected into 1- to 4-cell embryos, which were analyzed by whole-mount in situ hybridization at the shield stage. Shown are animal pole views with dorsal to the right. For B and D–F, results shown are one experiment representative of at least three trials. G: Validation of *mkk7* MO efficacy as for A. Top: The *mkk7* MO targets the exon 9/intron 9 junction of *mkk7* pre-mRNA as shown in the diagram. Arrows represent primer pairs used in RT-PCR analysis. Bottom: The *mkk7* MO (0.8 pmol) causes a marked reduction in *mkk7* expression beginning at the shield stage (6 hpf). H: Gross appearance of *mkk7* MO-injected embryos. Images of a live untreated WT embryo and *mkk7* morphants injected with the indicated doses of *mkk7* MO were acquired at 24 hpf. All embryos are viewed laterally, with anterior to the top. *Mkk7* morphants exhibit weak, MO dose-dependent defects in the morphogenesis of the somites and a slightly shortened body length. I: Normal CE in *mkk7* morphants. WT embryos and *mkk7* morphants were analyzed for tissue-specific gene expression as for D. Even at a high dose of *mkk7* MO (1.5 pmol), the expression patterns of all tissue-specific genes examined were normal. J: Impaired somitogenesis in *mkk7* morphants. Top: The morphologies of rhombomeres (*krox20*) and somites (*myoD*) were examined at the eight-somite stage (13 hpf) in WT embryos and *mkk7* morphants. The body axis was shorter and the somites were wider in the latter (dorsal view). Bottom: Images of a live WT embryo (left) and live *mkk7* morphant (right) were acquired at 16 hpf (lateral view).

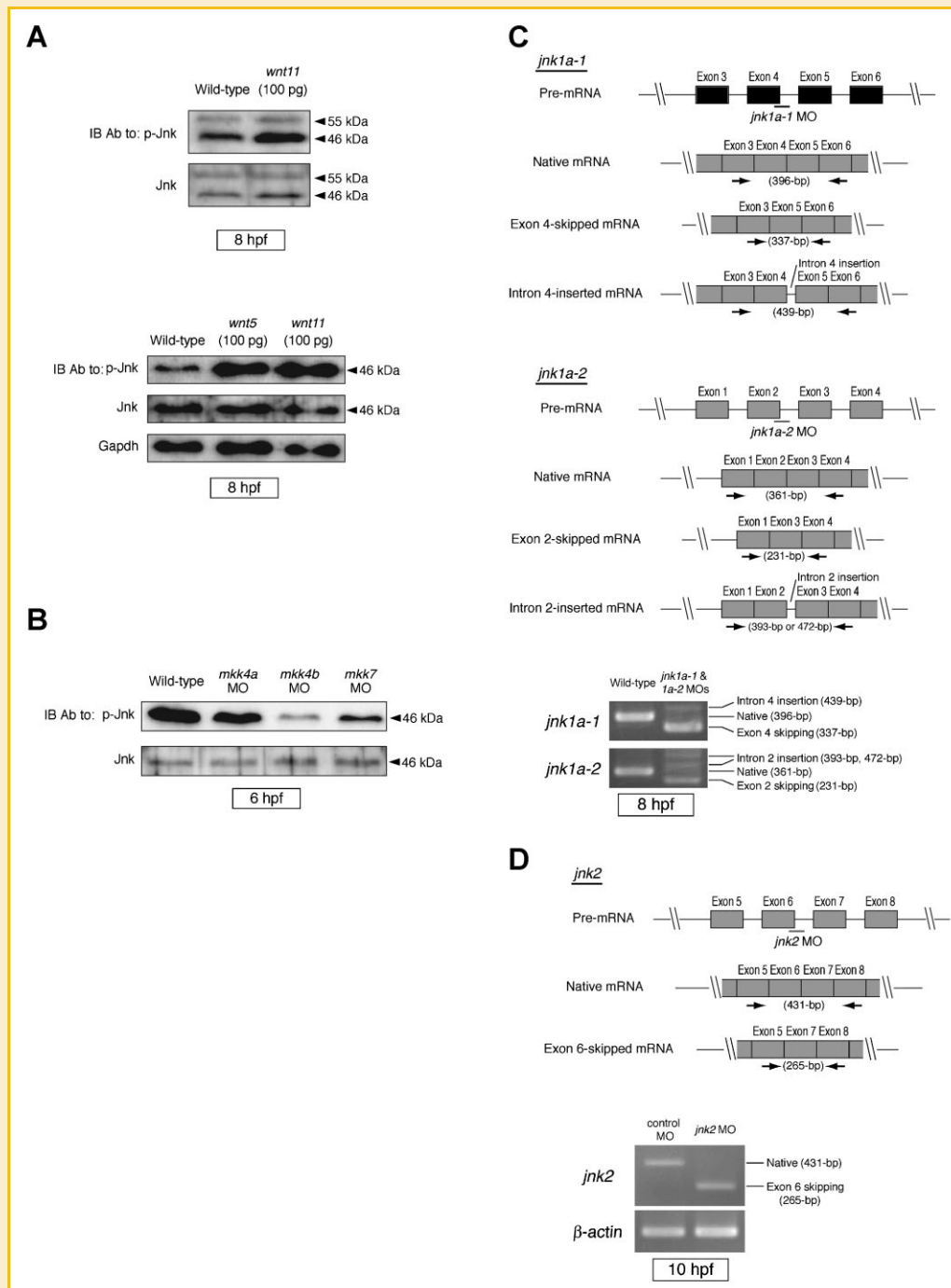
of *wnt11* mRNA were increased in *mkk4b* morphants (Fig. 4C). On the other hand, Mkk4b knockdown did not affect *wnt5* expression at any stage (Fig. 4B, right). These data indicate that Wnt11 itself is one of the molecular components downstream of the Jnk cascade in the non-canonical Wnt pathway associated with early embryogenesis.

We next examined whether activation of the Mkk4b–Jnk signaling pathway was sufficient to repress *wnt11* expression. Overexpression of a constitutively active form of Mkk4b (caMkk4b)

markedly reduced the level of *wnt11* expression at 50% epiboly (Fig. 4D), reinforcing the notion that *wnt11* is a transcriptional target downstream of the Mkk4b–Jnk signaling cascade.

OVEREXPRESSION OF *WNT11* INDUCES ABNORMAL CE MOVEMENTS

To ascertain whether the increased *wnt11* expression in *mkk4b* morphants induced CE defects, we injected WT embryos with *wnt11* mRNA. Embryos overexpressing *wnt11* mRNA showed a shortening



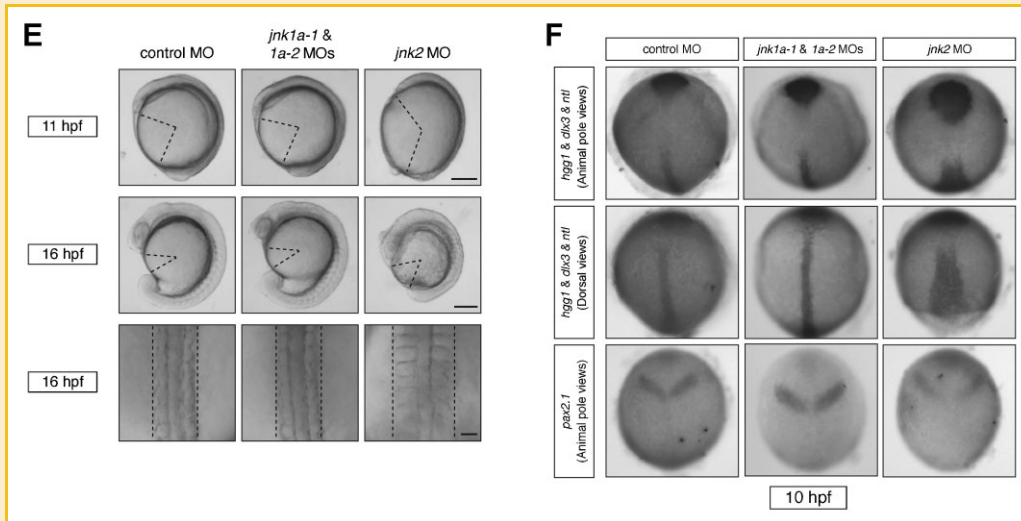


Fig. 3. Jnk2 is required for proper CE movements in zebrafish. **A:** Jnk is activated by *wnt11* or *wnt5* overexpression. Pooled extracts of 25 embryos overexpressing *wnt11* were analyzed by Western blotting (top). Levels of phospho-Jnk (p-Jnk) were increased compared to the control but total Jnk (55 and 46 kDa isoforms) was unchanged. Pooled extracts of 25 embryos overexpressing *wnt5* were analyzed by Western blotting (bottom). Gapdh, internal control. **B:** Markedly reduced Jnk phosphorylation in *mkk4b* morphants. Twenty-five embryos injected with 0.4 pmol of *mkk4a* MO, *mkk4b* MO, or *mkk7* MO were analyzed at the shield stage by Western blotting to detect Jnk and p-Jnk. **C:** Validation of *jnk1a* MOs. Schematic illustrations of the target sites of the *jnk1a-1* MO and *jnk1a-2* MO are shown in the top and center panels, respectively. Arrows represent the primer pairs used in the RT-PCR evaluation of MO efficacy. The bottom panel shows an RT-PCR analysis of the effects of these MOs on *jnk1a-1* and *jnk1a-2* expression. Total RNA was extracted at 8 hpf from untreated WT embryos, or WT embryos co-injected with *jnk1a-1* MO and *jnk1a-2* MO (0.8 pmol each). *Jnk1a-1* MO and *jnk1a-2* MO prevent correct splicing of *jnk1a-1* and *jnk1a-2* pre-mRNAs, respectively, resulting in a reading-frame shift and production of mutant proteins. **D:** Validation of *jnk2* MO. Top panel depicts the location of the target site of *jnk2* MO and the primer pairs used for RT-PCR. The bottom panel shows an RT-PCR analysis of the effect of *jnk2* MO. Total RNA was extracted from WT embryos injected with control MO (0.8 pmol) or *jnk2* MO (0.7 pmol). *Jnk2* MO induces defective splicing. **E:** Gross appearance of *jnk* morphants. Images of live embryos injected with control MO (0.8 pmol), *jnk1a-1* MO plus *jnk1a-2* MO (0.8 pmol each), or *jnk2* MO (0.7 pmol) were acquired at 11 and 16 hpf. These embryos are viewed laterally, with anterior to the top. *Jnk1* MOs cause no gross morphological defects, whereas *jnk2* morphants exhibit a shortening of body length. At 16 hpf, the notochord and somites are wider in the *jnk2* morphant than in the control (dorsal view). Scale bars = 200 μ m (row 1); 200 μ m (row 2); 50 μ m (row 3). **F:** Impaired CE in *jnk2* morphants. *Jnk* morphants and controls were analyzed by whole-mount in situ hybridization for the expression of tissue-specific genes at the tailbud stage (10 hpf). *Jnk1a* morphants showed normal marker expression. In *jnk2* morphants, the position of the prechordal plate (*hgg1*) is more posterior and broader than in the control. Expression of *dlx3* (neuroectoderm) reveals a broader neural plate, and the notochord (*ntl*) is shorter and broader. The midbrain/hindbrain boundary (*pax2.1*) has expanded laterally.

of body length at 11, 16, and 24 hpf and exhibited broader notochords and somites at 16 hpf (Fig. 5A), reminiscent of the *mkk4b* morphant phenotype (Fig. 2B). With respect to marker expression patterns, the expression of the organizer marker *gsc* was not changed in *wnt11*-overexpressing embryos compared to control embryos injected with *egfp* mRNA (Fig. 5B, top). At the tailbud stage, *wnt11*-overexpressing embryos showed a more posteriorly positioned prechordal plate (*hgg1*), a wider neural plate (*dlx3*), a shorter and broader notochord (*ntl*), and a laterally expanded midbrain/hindbrain boundary (*pax2.1*) than did controls (Fig. 5B, bottom). These observations imply that the level of *wnt11* mRNA present critically influences CE.

MKK4B MORPHANTS SHOW INCREASED WNT11 EXPRESSION IN THE ORGANIZER AND MARGIN REGIONS

We next used whole-mount in situ hybridization to localize the increased *wnt11* expression in Mkk4b-depleted embryos. During gastrulation in control MO-injected embryos, *wnt11* expression became prominent in the lateral and ventral germ ring (margin region) but was downregulated within the region of shield formation (i.e., the organizer) (Fig. 6A; 6 hpf). *Wnt11* expression near the margin persisted throughout gastrulation, although the staining

intensity gradually decreased in the ventral region. *Wnt11* expression remained high in the dorsal region, which converges with the tailbud (Fig. 6A; 8 and 10 hpf). In contrast, during gastrulation in *mkk4b* morphants, *wnt11* expression was significantly increased in both the organizer and margin regions (Fig. 6A; 6 hpf). By late gastrulation, *wnt11* expression was strongly upregulated in all domains (Fig. 6A; 8 and 10 hpf). On the other hand, the expression pattern of *wnt5* in *mkk4b* morphants matched that of controls (Fig. 6B).

IMPAIRED MKK4B FUNCTION PROMOTES WNT11 TRANSACTIVATION IN A NON-CELL-AUTONOMOUS MANNER

To determine whether *wnt11* was a direct transcriptional target of Mkk4b-Jnk signaling, we generated small clones of *mkk4b*-deficient zebrafish cells within whole embryos by co-injecting *mkk4b* MO together with *egfp* mRNA into one blastomere of a 32- or 64-cell embryo (Fig. 7A). We subsequently examined the location of the EGFP-labeled descendant cells at the shield stage (Fig. 7B, top row) and analyzed *wnt11* expression pattern by whole-mount in situ hybridization (Fig. 7B, bottom row). *Wnt11* expression was significantly elevated even in the embryonic region that did not overlap with the lineage label marking the progeny of *mkk4b* MO-

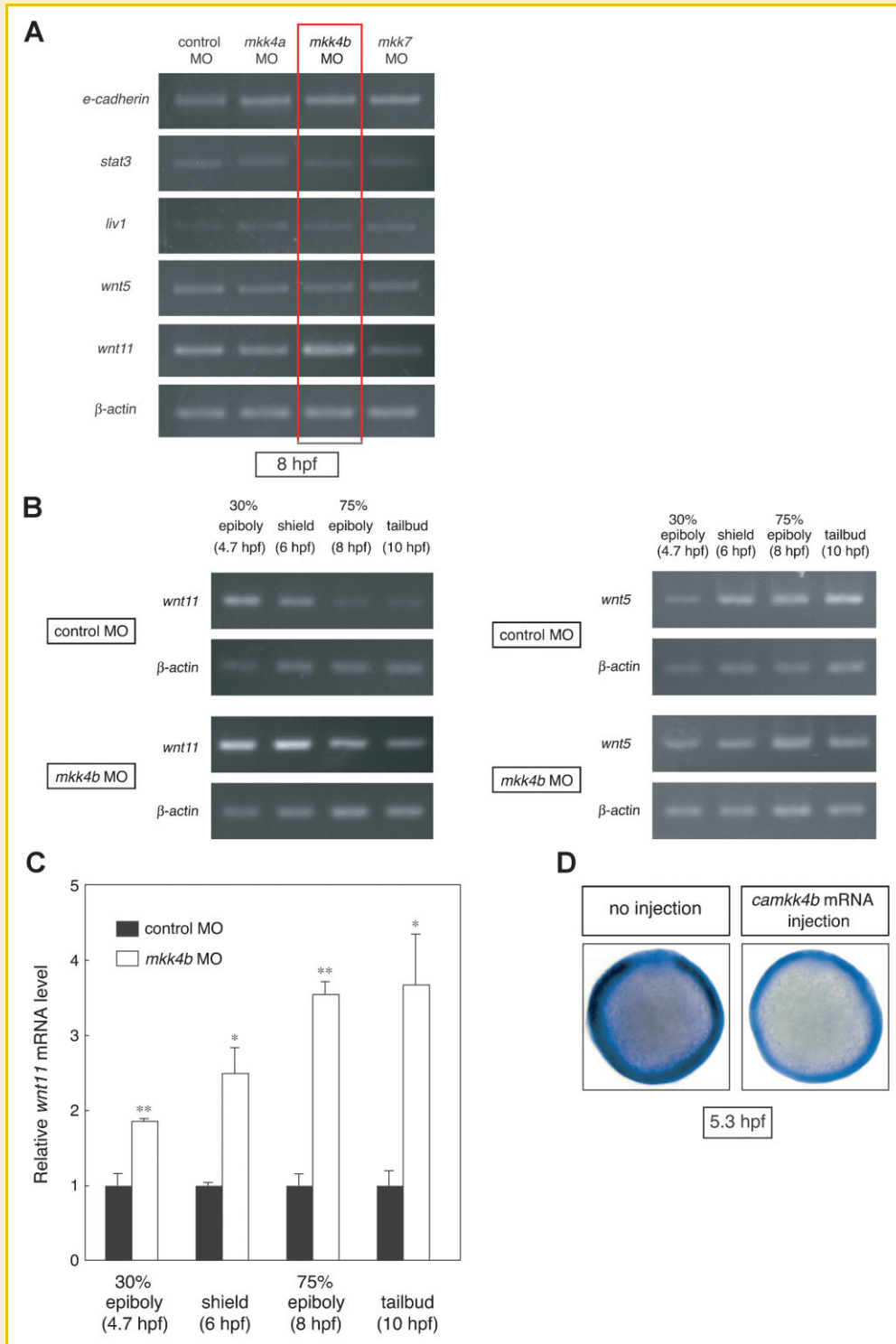


Fig. 4. Identification of *wnt11* as a downstream component of Mkk4b–Jnk signaling in the non-canonical Wnt pathway. A: Specific upregulation of *wnt11* in *mkk4b* morphants. Total RNA was isolated from control, *mkk4a*, *mkk4b*, and *mkk7* morphants at the 75% epiboly stage (8 hpf) and subjected to RT-PCR to detect expression of the indicated mRNAs. β -Actin, internal control. B: Upregulation of *wnt11* but not *wnt5* in *mkk4b* morphants throughout early embryogenesis. Total RNA was isolated from control and *mkk4b* morphants at the indicated stages and subjected to RT-PCR to detect expression of *wnt11* (left) and *wnt5* (right) mRNAs. C: Quantitative RT-PCR analysis of *wnt11* mRNA expression in *mkk4b* morphants. For all quantitative PCR experiments, *wnt11* cDNA amplification was standardized to the amplification of β -actin cDNA. *Wnt11* expression in *mkk4b* morphants was normalized to that in controls (assigned an arbitrary value of 1). Data shown are the mean \pm SEM of three independent experiments (* $P < 0.05$; ** $P < 0.01$ vs. control). D: Mkk4b–Jnk signaling represses *wnt11* expression. WT embryos were left untreated or injected with *camkk4b* mRNA (40 μ g) and subjected to whole-mount in situ hybridization to detect *wnt11* at the 50% epiboly stage (5.3 hpf; animal pole views). Decreased *wnt11* expression was observed in *camkk4b*-overexpressing embryos (right) compared with uninjected embryos (left).

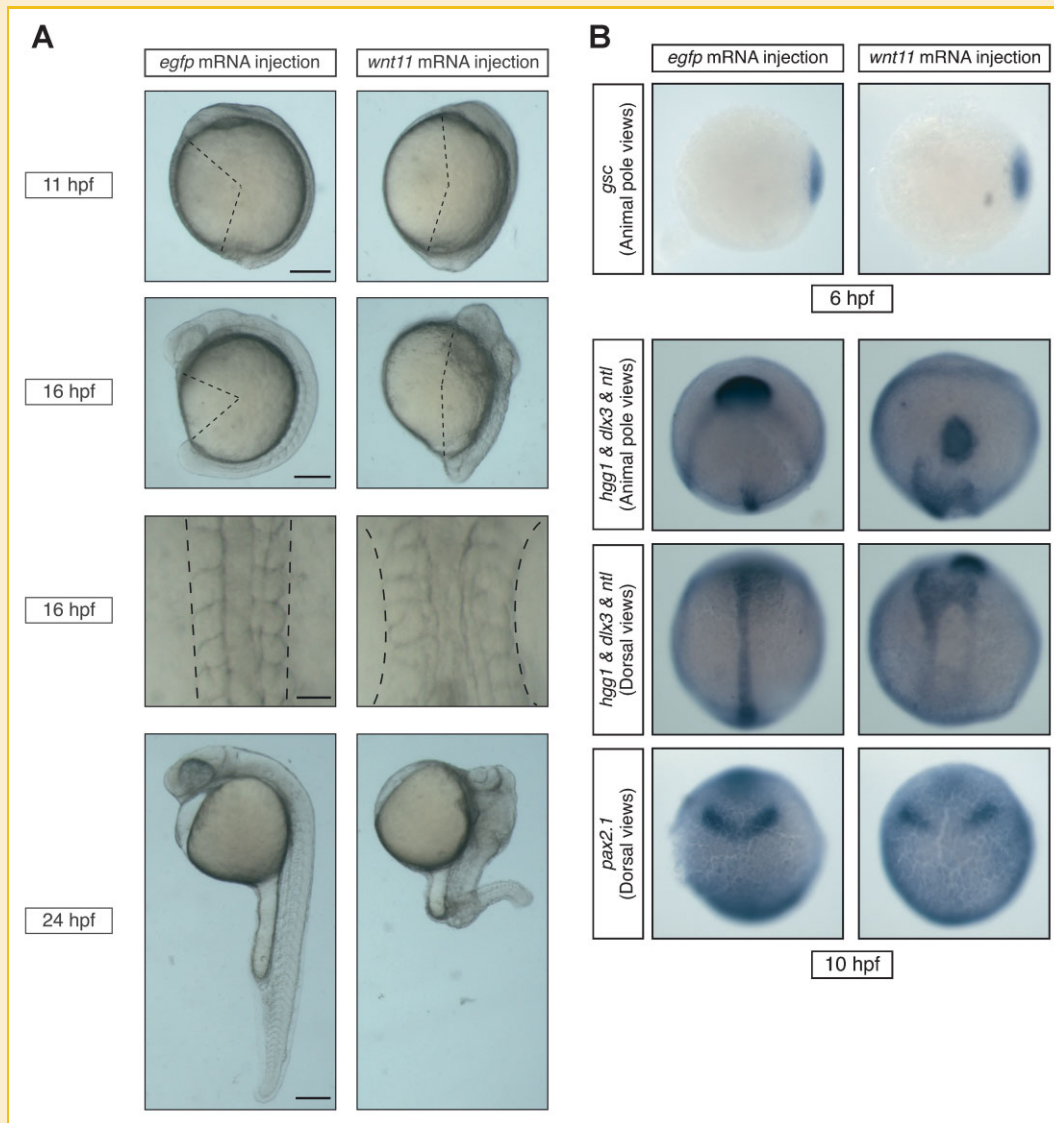


Fig. 5. Overexpression of *wnt11* induces CE abnormalities. **A:** Altered gross appearance. WT embryos were injected with either *egfp* mRNA (100 pg; control) or *wnt11* mRNA (75 pg), and the gross phenotype of the embryos was examined at 11, 16, and 24 hpf (rows 1, 2, and 4, respectively). These embryos are viewed laterally, with the anterior to the top. A shortening of body length can be seen as early as 11 hpf in the *wnt11*-overexpressing embryos. Row 3 shows that the notochord and somites are wider in the *wnt11*-overexpressing embryos than in the controls at 16 hpf (dorsal view). Scale bars = 200 μ m (row 1); 200 μ m (row 2); 50 μ m (row 3); 200 μ m (row 4). **B:** Impaired CE in *wnt11*-overexpressing embryos. WT embryos were injected with *egfp* or *wnt11* mRNA (100 pg), and analyzed by whole-mount in situ hybridization for the expression of tissue-specific genes at the shield stage (6 hpf) and the tailbud stage (10 hpf). The expression pattern of the organizer marker *gsc* is similar in the *wnt11*-overexpressing embryos and controls. However, the position of the prechordal plate (*hgg1*) is more posterior in *wnt11*-overexpressing embryos compared to the control. Expression of *dlx3* (neuroectoderm) reveals a broader neural plate, and the notochord (*ntl*) is shorter and broader. The midbrain/hindbrain boundary (*pax2.1*) has expanded laterally.

injected cells (Fig. 7B). Thus, activation of the Mkk4b–Jnk signaling pathway in zebrafish embryos represses *wnt11* expression in a non-cell-autonomous fashion.

Taken together, our findings suggest that the suppression of *wnt11* transcription by Jnk activation is important for the precise regulation of vertebrate CE, and establish a model in which non-canonical Wnt signaling leading to Jnk activation represses expression of the CE-triggering ligand Wnt11 (Fig. S7).

DISCUSSION

In this study, we examined the role of Jnk signaling during the early embryogenesis of zebrafish by carrying out MO-mediated knock-down of the orthologs of the *mkk4* and *mkk7* genes. We found that *mkk4a* MO-injected zebrafish embryos had no phenotype (Fig. S4B,C), whereas *mkk4b* MO-injected embryos exhibited axial tissues that were abnormally short and wide due to defective CE

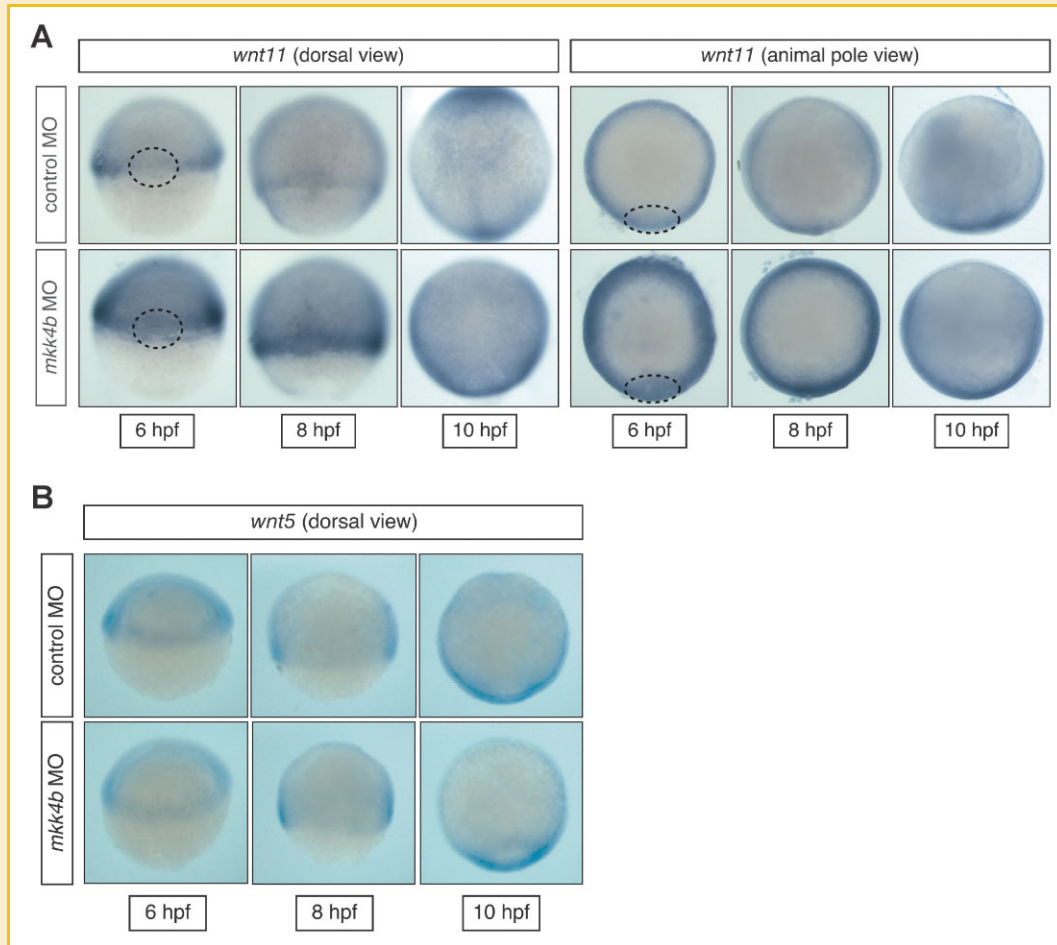


Fig. 6. Elevated expression of *wnt11* in the organizer and margin regions of *Mkk4b*-depleted embryos. A: Localization of increased *wnt11* expression in *mkk4b* morphants. Whole-mount in situ hybridization was used to monitor *wnt11* expression in developing control and *mkk4b* morphants from the shield stage (6 hpf) to tailbud stage (10 hpf). Two views are shown as indicated, with dashed lines outlining the boundaries of the organizer region. B: Localization of *wnt5* expression in *mkk4b* morphants. Whole-mount in situ hybridization was used to monitor *wnt5* expression in developing control and *mkk4b* morphants from the shield stage (6 hpf) to tailbud stage (10 hpf). *Wnt5* showed the same expression pattern in control and *mkk4b* morphants.

(Fig. 2B–E). These observations suggest that *Mkk4b* is functionally redundant with *Mkk4a* and can compensate for its loss during gastrulation, but that *Mkk4a* cannot compensate for a lack of *Mkk4b*. *Mkk7* morphants had no phenotype during gastrulation but showed abnormal somite morphologies during segmentation (Fig. 2H–J). *Mkk7* is thus critical for a slightly later stage of development.

Recently, Rui et al. [2007] demonstrated that zebrafish *mkk4a-s* plays an important role in dorsoventral patterning in zebrafish blastulas. These authors reported that *mkk4a-s* knockdown with a translation-blocking MO prior to gastrulation reduced the expression of dorsal markers but expanded the expression of ventral markers. In our study, we performed *mkk4a-l* knockdown with translation-blocking MO but could not detect any alteration to the expression of the dorsal marker *gsc* (Fig. S8). This discrepancy implies that there is functional diversity between the two splicing isoforms of *mkk4a*. The results of our study, together with those of Rui et al., reinforce the idea that Jnk signaling plays multiple roles in

major developmental processes, including the specification of the dorsoventral axis, cell movements required during gastrulation, and somite morphogenesis.

Our previous studies of disruption of the *mkk4* and *mkk7* genes in murine ES cells revealed that *Mkk4* and *Mkk7* preferentially phosphorylate the Tyr and Thr residues, respectively, within the Thr-Pro-Tyr motif of Jnk [Kishimoto et al., 2003]. In this study, we expressed zebrafish *Mkk4* and *Mkk7* in mouse ES cells and obtained results indicating that these Mapks also differ in their biochemical properties (Fig. 1C). These data strongly suggest that the use of Jnk activation as a molecular switch has been evolutionarily conserved from teleosts to mammals. In zebrafish, loss of *Mkk4b* leads to severe defects in CE movements during gastrulation, whereas our *mkk7* morphants had no phenotype during gastrulation (Fig. 2D,I). These observations indicate that *mkk4* has a more prominent role than *mkk7* in early embryogenesis. Consistent with this hypothesis, *mkk4*^{-/-} mouse embryos die earlier than *mkk7*^{-/-} embryos [Nishina et al., 1999; Wada et al., 2004]. This conserved functional

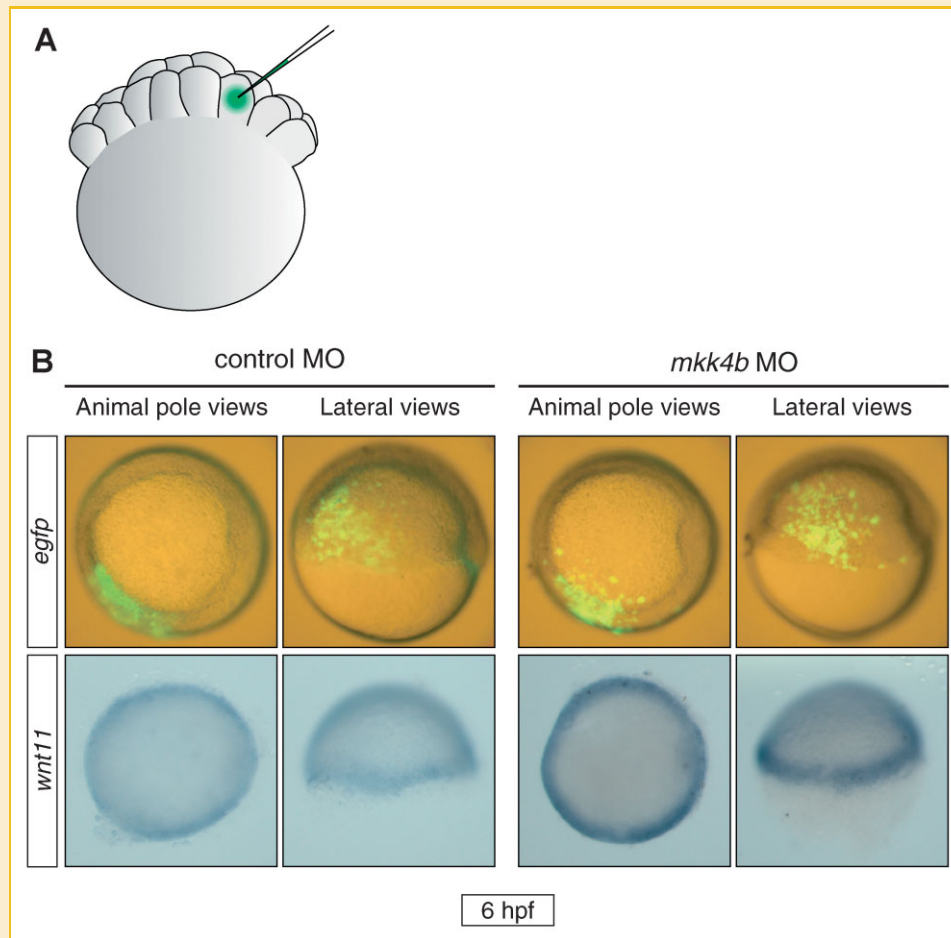


Fig. 7. Abrogation of Mkk4b function induces elevated expression of *wnt11* in a non-cell-autonomous manner. A: Schematic representation of MO microinjection into one cell of a 32- or 64-cell zebrafish embryo. B: Control MO (0.4 pmol) or *mkk4b* MO (0.4 pmol) was injected into one cell of a 32- or 64-cell embryo to generate randomly distributed *mkk4b*-knockdown cell clones. *Egfp* mRNA was co-injected as a cell lineage tracer. Top, EGFP-labeled cells were observed at the shield stage by fluorescence microscopy using a halogen lamp and blue light. Bottom, *wnt11* expression was monitored in developing control and *mkk4b* morphants at the shield stage by whole-mount in situ hybridization. Two views are shown, with dorsal to the right.

diversity of Mapks may be related to the need to activate Jnk at different times and locations during early development. Indeed, we found that Mkk4b depletion reduced Jnk phosphorylation more dramatically than did Mkk7 depletion, at least at the shield stage (Fig. 3B). Alternatively, the functional properties of Mkk4b–Jnk and Mkk7–Jnk signaling modules may be governed in part by scaffold proteins such as the Jnk-interacting proteins (JIPs) that confer specificity to kinase actions [Whitmarsh, 2006].

Jnk1^{−/−}*jnk2*^{−/−} mice die at about E11 with defective neural tube morphogenesis [Kuan et al., 1999]. Based on our results in zebrafish, we speculate that the failure to close the neural tube in *jnk1*^{−/−}*jnk2*^{−/−} mice may be due to defective CE in the neural plate. In support of this idea, disruption of non-canonical Wnt pathway genes (such as *dishevelled*) in *Xenopus* inhibits CE in the neural plate and results in abnormal, widely spaced neural folds that do not close [Copp et al., 2003]. Invertebrate development may also rely on Jnk-mediated events that share molecular similarities with vertebrate CE. In *Drosophila*, dJnk (Basket) is activated by dJnkk (Hemipterous), a homolog of vertebrate Mkk7 [Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996]. *Basket* and *hemipterous* are

important for morphogenesis, and loss of function of either gene inhibits dorsal closure [Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996]. These findings emphasize the notion that Jnk activation is utilized in parallel morphogenetic events among widely divergent species.

Signals initiated by non-canonical Wnt ligands such as Wnt11 and Wnt5 are important for: vertebrate morphogenetic processes that require directed cell movements; cell–cell adhesion; and the establishment of cell polarity. Zebrafish with mutations in *wnt5* (*ppt*) or *wnt11* (*slb*) exhibit defects that reduce CE without affecting cell fate [Heisenberg et al., 2000; Kilian et al., 2003]. The *slb* mutant shows more anterior CE defects, including a shortened and broadened body axis at the end of gastrulation and a slight fusion of the eyes (cyclopia) at later developmental stages. In contrast, the Wnt5 protein functions in posterior regions. The *ppt* mutant exhibits a shortened body axis and compressed tail, whereas the position of the eyes is only mildly affected. These observations indicate that Wnt11 and Wnt5 possess distinct and non-redundant functions in regulating zebrafish gastrulation, which may be accounted for by differences in spatiotemporal expression. Although both *wnt5* and

wnt11 are expressed in the germ ring at the shield stage, *wnt11* is specifically expressed in anterior tissues at the end of gastrulation, whereas *wnt5* is specifically expressed in posterior mesendodermal tissues (Fig. 6) [Heisenberg et al., 2000; Kilian et al., 2003]. Our study demonstrates that Mkk4b–Jnk signaling precisely regulates *wnt11* transcription. This Mkk4b–Jnk-mediated control may contribute to the mechanisms segregating *wnt11* and *wnt5* expression domains during late gastrulation.

In *Drosophila*, a role for Dpp in dorsal closure during early morphogenesis has been clearly established [Hou et al., 1997; Riesgo-Escovar and Hafen, 1997]. During dorsal closure, Jnk activates the expression of *dpp* in leading-edge epithelial cells, and Dpp subsequently acts as a secreted signal that controls the elongation of lateral epidermis in a paracrine fashion [Reed et al., 2001]. Our analyses demonstrate that Mkk4b–Jnk signaling regulates *wnt11* expression during zebrafish gastrulation in a non-cell-autonomous manner (Fig. 7). Our results raise the possibility that, like *Drosophila* dorsal closure, normal vertebrate CE movements depend on Jnk signaling that regulates the expression of a secreted signaling molecule capable of promoting concerted movements of neighboring cells (Fig. S7).

Our proposed model of CE movement regulation by *wnt11* expression can be summarized as follows: In normal zebrafish embryos, activated Mkk4b transmits a signal leading to Jnk activation. This activated Jnk may induce secreted factor X, which exerts moderate suppression of *wnt11* transcription in neighboring organizer and margin cells, and results in proper co-ordination of CE. In *mkk4b* morphants, however, JNK activation is blocked such that the expression of factor X is downregulated. As a result, *wnt11* is expressed at an abnormally high level that may impair CE (Fig. 7). We undertook preliminary studies to examine whether the *mkk4b* morphant phenotype could be suppressed by a low dose of *wnt11* MO, but found that rescue did not occur (data not shown). This inability to rescue the CE defect may be due to a failure to strictly control the expression pattern and amount of *wnt11* expression, since Wnt11 levels and localization must be tightly regulated for normal embryonic development [Heisenberg et al., 2000].

A previous study demonstrated that Bmp activity regulates CE movements during zebrafish gastrulation [Myers et al., 2002]. This report prompted us to investigate whether zebrafish Bmp2 and Bmp4 (homologs of *Drosophila* Dpp) function downstream of the Mkk4b–Jnk signaling cascade. However, expression levels of *bmp2* and *bmp4* were not downregulated in Mkk4b-depleted embryos (data not shown). Future identification of secreted factor(s) responsible for the transmission of Jnk signals to neighboring cells may provide insight into the complex mechanisms controlling vertebrate CE.

Our proposed model postulates that *wnt11* expression is under precise spatiotemporal control during zebrafish development. Previous studies have shown that Wnt11 expression in zebrafish starts in the dorsal part of the blastoderm margin at the oblong stage, and that the entire margin becomes Wnt11⁺ by the late blastula stage [Makita et al., 1998]. Our present work shows that, at the onset of gastrulation, *wnt11* is highly expressed around the circumference of the germ ring, with slightly reduced expression in the region of the organizer (Fig. 6A). It has been established that *wnt11* is

expressed predominantly in epiblast cells of the germ ring (ectodermal germ layer), whereas ingressing hypoblast cells (mesendodermal germ layer) show no detectable *wnt11* expression [Makita et al., 1998; Ulrich et al., 2003]. This profile of early *wnt11* expression in zebrafish is virtually identical to that seen in *Xenopus* [Ku and Melton, 1993]. Taken together, these results suggest an evolutionary conservation of *wnt11* function during gastrulation, and a prominent role for the control of *wnt11* expression by Jnk signaling.

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