Doctoral thesis

The Planar Cell Polarity Gene *Prickle* in Vertebrates Regulates Gastrulation Cell Movements.

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CONTENTS

ABBREVIATIONS

PCP	Planar cell polarity
Fz	Frizzled
Dsh	Dishevelled
Xpk	*Xenopus prickle*
Zpk	*Zebrafish prickle*
XPIK	*Xenopus prickle interacting kinase*
DN	dominant negative
JNK	Jun N-terminal kinase
Wnt	wingless, Int
GTP	guanosine triphosphate
EST	expression sequence tag
XDB	*Xenopus* data base
Ci-pk	*Ciona prickle*
Xbra	*Xenopus brachyury*
mo	Morpholino Oligonucleotide
MyoD	myogenic determination gene
N-CAM	neural cell adhesion molecule
GFP	Green fluorescent protein
UTR	untranslated region
mRNA	messenger ribonucleic acid
wt (WT)	wild type
P/L	PET, LIM domain
cDNA	complementary deoxyribonucleic acid
HA	hemmagglutinin
GST	glutathione S-transferase
St	stage
ODC	ornithine decarboxylase
Gal4	Galactosidase 4

DBD	DNA binding domain
IP	immunoprecipitation
cdc42	Cell-division-cycle gene 42
Ste20	sterile 20 protein
JIK	JNK/SAPK-inhibitory kinase
KFC	Kinase From Chicken
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
TAO1	one thousand and one amino acid
catal	catalytic domain
Kn	kinase negative

ABSTRACT

Gastrulation is one of the most important processes during morphogenesis of early embryo, involving dynamic and coordinated cell movements that cause drastic changes in embryo shape. Although gastrulation proceeds by various types of cell movements, in *Xenopus* mesodermal cells, two cell movements known as convergence and extension in which polarized axial mesodermal cells intercalate in radial and mediolateral directions, thus elongating the dorsal marginal zone along the anterior-posterior axis, have been mainly studied (Keller and Danilchik, 1988; Keller, 1992; Shih and Keller, 1992; Wilson and Keller, 1991). Recently, it was reported that a non-canonical Wnt signalling pathway, which is known to regulate planar cell polarity (PCP) in *Drosophila* (Adler, 1992; Eaton, 1997; Mlodzik, 1999), participates in the regulation of convergent extension movements in *Xenopus* as well as in the zebrafish embryo (Djiane et al., 2000; Heisenberg et al., 2000). The Wnt5a/Wnt11 identified as ligands of PCP signalling are mediated by members of the seven-transmembrane receptor Frizzled (Fz) and the signal transducer Dishevelled (Dsh), through the Dsh domains that are selectively required for the PCP signal (Moon et al., 1993; Sokol, 1996). It has also been shown that the relocalization of Dsh to the cell membrane is required for convergent extension movements in *Xenopus* gastrulae (Wallingford et al., 2000). Although it appears that signalling via these components leads to the activation of JNK (Boutros et al., 1998) and rearrangement of microfilaments, the precise interplay among these intercellular components is largely unknown.

In chapter 1, I show that *Xenopus prickle* (*Xpk*), a *Xenopus* homologue of a *Drosophila* PCP gene (Gubb et al., 1999; Mlodzik, 2000), is an essential component for gastrulation cell movement. *Xpk* encodes a protein that includes conserved PET and triple LIM domains in its N-terminal half. In gastrula, *Xpk* transcripts are restricted to the marginal zone with a steep gradient from dorsal to ventral side, suggesting that *Xpk* plays a role during gastrulation. Both gain-of-function and loss-of-function of *Xpk* severely perturbed gastrulation and caused *spina bifida* embryos without affecting

mesodermal differentiation. Loss-of-function of zebrafish *prickle* (*Zpk*) also disrupted the dorsal convergence, caused a phenotype similar to PCP mutants *knypeck*and *trilobite* (Marlow et al., 1998), which have recently been found to encode a glypican and *strabismus* (Jessen et al., 2002), respectively. The structure-activity relationship of XPK was examined by overexpression of some deletion constructs. As a result, triple LIM domain-deleted XPK mutants could act as the dominant-negative way to wild-type XPK, whereas XPK mutants containing the domain were still able to transmit signals similar to that of wild type. Although XPK alone showed no effect on the JNK activation as output of PCP signaling, XPK could enhanced the JNK activation mediated by Dsh. Importantly, I also demonstrated that XPK physically binds to *Xenopus* Dsh as well as to JNK. This suggests that XPK plays a pivotal role in connecting Dsh function to JNK activation.

In chapter 2, I discuss about the identification of *Xenopus* Ste20-like kinase (Dan et al., 2001) as a XPK interacting protein. It has been identified by the yeast two hybrid screening with XPK as a bait, and named *Xenopus Prickle* Interacting Kinase; XPIK. XPIK is expressed in dorsal side of early gastrula embryo overlapping with the expression domain of XPK during gastrulation, suggesting that XPIK play a role in Wnt/PCP signaling like XPK. In fact, I have found that XPIK could activate JNK via its kinase domain. Both gain-of-function and loss-of-function of XPIK interfered gastrulation movements, phenocopying XPK-overexpressed or -disrupted embryos. In the overexpression analysis of some deletion mutants of XPIK, degree of gastrulation defects caused by each mutant was well-correlated with its activity level of JNK activation. I also designed a dominant negatively acting version of XPIK (D165A) (Hutchison et al., 1998), XPIK-KN which is able to inhibit the JNK activation mediated by wild type XPIK and found that not only it could restore the gastrulation defective phenotype of dorsally overexpressed XPIK but also it alone perturbed gastrulation suggesting that XPIK-KN inhibited the endogenous XPIK activity of JNK activation. XPIK-KN also inhibited the JNK activation mediated by Dsh. Taken these observations together, I propose that XPIK is an essential components of the Wnt/PCP signaling linking Dsh function to JNK activation, thereby regulating

gastrulation cell movements.

In conclusion, the analysis of Prickle function in this study confirmed that the mechanism of cell polarity establishment by PCP signaling in *Drosophila* is commonly utilized beyond animal species and adopted to the correct gastrulation cell movements in vertebrates. The action mechanism of Prickle was identified as a modulator protein of Dsh-JNK pathway. In addition, XPK Interacting Kinase (XPIK) was found to be required for activation of JNK mediated by Dsh. Accordingly, our results strongly suggest that non-canonical Wnt (PCP) pathway regulates gastrulation cell movements in vertebrate through activation of JNK mediated or modulated by Dsh, XPK and XPIK. To further understand the pathway, how Dsh, XPK and XPIK regulate each other remains to be explored.

INTRODUCTION

Embryonic development involves cell determination and differentiation, and also morphogenesis; the construction of the embryo by the coordinated mechanical activities of cell populations such as cell movements. While the mechanisms of cell type specification are now quite well understood in many cases, relatively little is known about the control of cell behavior in development. The dynamic cell movements of gastrulation and neurulation, which establish the vertebrate body plan, constitute particularly dramatic events of morphogenesis, involving cell migration, intercalation and shape change. Although these tissue movements have been described in some detail, particularly in *Xenopus*, many questions remain to be solved. Specially, the signaling events that regulate morphogenesis remain largely mysterious. How are regional patterns of cell behavior established? What are the cues that coordinate the timing and orientation of cell motility? How do extracellular signals provoke changes in cytoskeleton, adhesion and protrusive activity?

An important insight into the regulation of morphogenesis in the vertebrate embryo came with the discovery that an interfering version of the signal transduction molecule Dishevelled (Dsh) can profoundly disrupt dorsal tissue movements in *Xenopus*, apparently without affecting cell type specification or differentiation (Sokol, 1996). In particular, blocking Dsh function impaired convergent extension, the narrowing and elongation of the dorsal mesoderm and posterior neural plate, resulting in a drastically shortened trunk. Convergent extension, driven by active cell intercalation, not only shapes the embryo by elongating the dorsal axial structures, but also plays important roles in gastrulation, at least in amphibians, contributing to involution of the mesoderm and closure of the blastopore (Keller et al., 2000). Although Dsh was initially recognized to have a central role in the canonical Wnt/beta-catenin signaling cascade, in *Drosophila* and later in the axis determination of vertebrate embryos, it also participates in a second, largely separate genetic network known as the "planar cell polarity" pathway (Boutros and Mlodzik, 1999). Subsequent work in *Xenopus* and in the zebrafish, making use of mutants specific to one pathway or the other strongly suggests that Dsh regulates

convergent extension as a part of the vertebrate planar cell polarity pathway (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000).

Planar cell polarity (PCP) in *Drosophila*, also known as tissue polarity, describes the coordinated orientation of cells or structures within the plane of an epithelium (this is distinct from the better understood apical-basal polarity; (Adler, 1992; Mlodzik, 1999; Shulman et al., 1998). The two most studied examples are the wing, in which the hairs produced by individual epithelial cells pointed toward the distal tip, and the compound eye, whose component ommatidia acquire a precisely coordinated orientation. Genetic analysis have identified a number of genes affect specifically wing or eye, some are required in both tissues as well as in other polarized developmental fields, implying that a common system for coordinating tissue polarity operates in very different contexts. In addition to Dsh, this core group of genes includes the transmembrane receptor Frizzled (Vinson and Adler, 1987), the atypical cadherin Flamingo (Usui et al., 1999), also known as Starry night (Chae et al., 1999), the small GTPase RhoA (Strutt et al., 1997), the ankyrin repeat protein Diego (Feiguin et al., 2001), the putative integral membrane protein Strabismus/Van Gogh (Taylor et al., 1998; Wolff and Rubin, 1998) and the LIM domain containing protein Prickle/Spiny legs (Gubb et al., 1999). Although epistasis experiments have established that Frizzled functions upstream of Dsh/RhoA (Krasnow et al., 1995; Strutt et al., 1997) to JNK activation, it has been difficult to further define a pathway.

In *Xenopus*, inhibition or misexpression of either Frizzled-8 or Frizzled-7 gives a phenotype very similar to that of dominant-negative Dsh (Deardorff et al., 1998; Djiane et al., 2000; Medina et al., 2000; Sumanas and Ekker, 2001). That negative or positive manipulation of gene function often have indistinguishable effects seems to be a general feature of this pathway. A Wnt ligand is apparently involved as well: the *Xenopus* Dsh phenotype is mimicked by injection of dominant-negative Wnt11 and closely resembles the zebrafish Wnt-11 mutant *silberblick* (Heisenberg et al., 2000; Tada and Smith, 2000). Moreover, Wnt-5A can also disrupt dorsal morphogenetic movements (Torres et al., 1996). Thus, the signaling system suggested by these observations is often considered as an alternative or 'non-canonical' Wnt pathway, although its relationship to the

Wnt/β-catenin pathway and to a recently proposed Wnt signaling mechanism involving calcium (Kuhl et al., 2001) remains obscure. Curiously, no strong evidence yet implicates a Wnt ligand in *Drosophila* planar cell polarity.

In chapter 1, I present that a *Xenopus* homolog of the *Drosophila* PCP gene *Prickle* is involved in the regulation of convergent extension. *Xpk* is expressed in marginal zone with a steep gradient from dorsal to ventral side where convergent extension occurs, and dorsal overexpression or reduction of expression with a morpholino antisense-oligo causes gastrulation defects similar to those produced by Dsh, Fz, Wnt11 and Wnt5A (Djiane et al., 2000; Moon et al., 1993; Smith et al., 2000; Wallingford et al., 2000). The effect is specific to morphogenesis: the specification and differentiation of mesodermal tissues is essentially normal. Although XPK alone had no effect on the JNK activation as output of PCP signaling, XPK enhanced the JNK activation triggered by Dsh. In chapter 2, I describe about *Xenopus Prickle* Interacting Kinase, XPIK that has been identified by the yeast two-hybrid screening using XPK PET/LIM domain as a bait. XPIK could activate JNK via its kinase domain. Thus, non-canonical Wnt (PCP) pathway is likely to regulate gastrulation cell movements in vertebrate through activation of JNK mediated by Dsh and is modulated by XPK and XPIK.

CHAPTER1

Prickle-related gene in vertebrates is essential for gastrulation cell movements.

The effects of pathway-specific mutant forms of Dsh in *Xenopus* and zebrafish suggest that a vertebrate orthologue of the *Drosophila* PCP pathway genes may participates in the regulation of convergent extension (Wallingford et al., 2000; Wallingford and Harland, 2001). To explore this hypothesis and to identify other molecules with roles in the same process, I focused on Prickle known as one of core group PCP genes in *Drosophila* and expressed in ascidian notchord where convergent extension occurs (Hotta et al., 2000; Keys et al., 2002). Soon after, homologous genes have been identified in *Xenopus laevis* (Wallingford et al., 2002).

The sequence and structure of Xenopus prickle protein

The gene was found in our own *Xenopus* EST database (XDB; http://xenopus.nibb.ac.jp/) as a homolog of ascidian and *Drosophila prickle* that was reported to be a critical gene for establishing PCP (Gubb et al., 1999) (Mlodzik, 2000). *Xpk* encodes an 835 amino acid protein with a single PET domain conserved among *Drosophila prickle*, *espinas*, and vertebrate testin and three repetitive LIM domains in its N-terminal half (Figure 1A). The primary structure of XPK is highly homologous to *prickle*-related proteins in other organisms from the fly to the human, particularly in PET and triple LIM domains (Figure 1B,C). Phylogenetic tree based on the amino acid sequences of PET and triple LIM domains shows that *prickle* and *espinas* fall into the same family but are distantly related to testin (Figure 1C). Together, these observations suggested that XPK is an evolutionary conserved protein that may play an essential role during development.

The temporal and spacial expression pattern of Xpk and its regulation

Northern blot analysis demonstrated that two *prickle* transcripts of approximately 5 kb are maternally encoded and expressed throughout the development at least to the tadpole stage with slight changes in its level being peaked around the onset of gastrulation (Figure 2B). Whole-mount *in situ* hybridization of developing *Xenopus* embryos revealed that *Xpk* is expressed as a maternal mRNA localized in the animal hemisphere of early blastula embryo

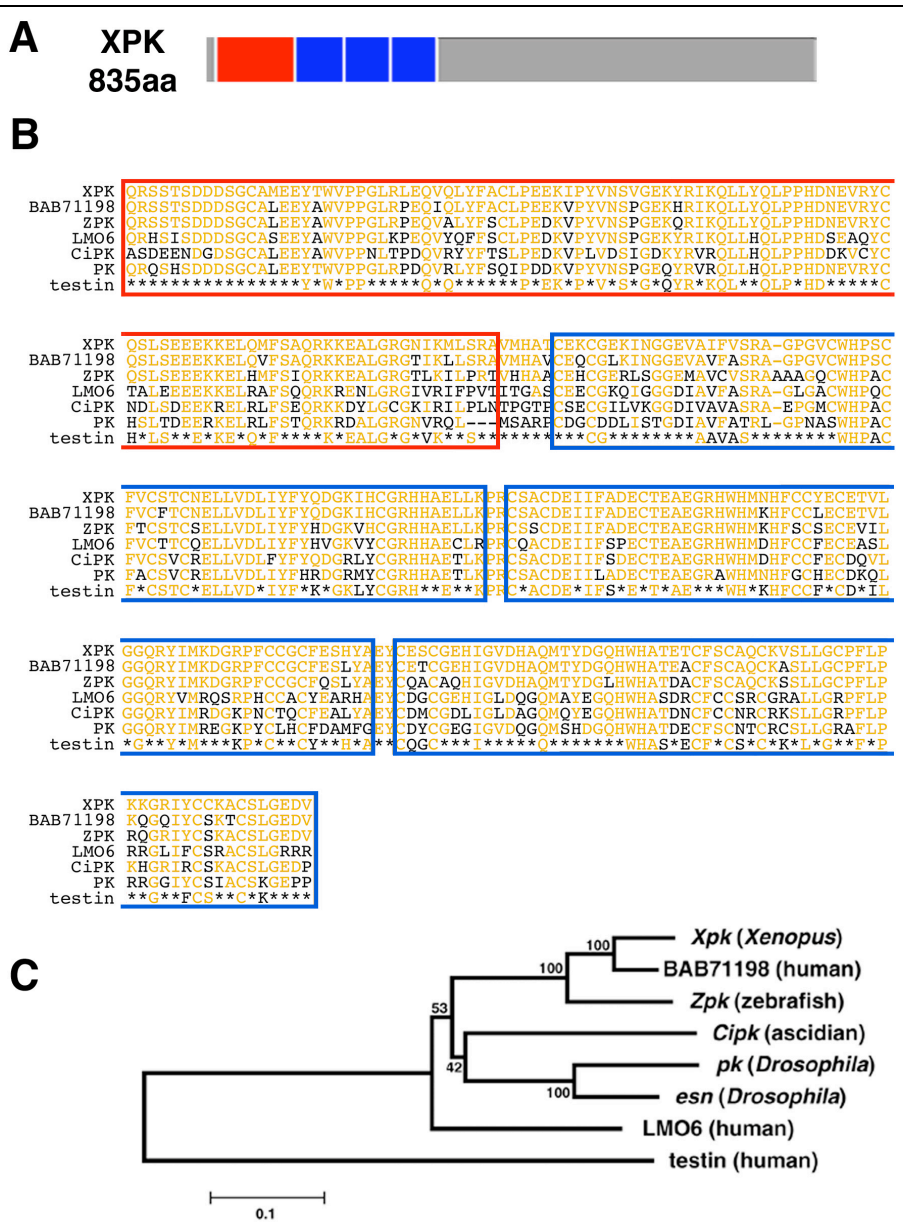


Figure 1 **The structure of *Xpk*, which encodes a protein homologous to *Drosophila prickle* (*pk*).**

A, *Xpk* encodes an 835-amino acid protein with a single PET domain and three tandem LIM domains (triple LIM), indicated in red and blue box, respectively.

B, Alignment of PET/LIM domain amino acid sequence of Prickle related genes. Yellow letters were indicated identical residues as XPK's and itself. Since the sequence of testin is not more similar than others, only conserved residues were shown.

C, A phylogenetic tree based on P/L amino-acid sequences reveals that the *pk* family genes are more closely related to one another than to testin, which also contains PET and LIM domains. The bar indicates an evolutionary distance of 0.1 amino acid substitution per position. The numbers at the node represent the bootstrap value (%) for the grouping.

(Figure 2A a, b). As gastrulation starts, the transcripts become restricted to the marginal zone with a steep gradient from dorsal to ventral side (c, d). As gastrulation further proceeds, the expression domain becomes localized in the involuting edge around blastopore and accumulates towards posterior end of the midline (future tail bud) (Figure 2A e, f) and then extends along anterior-posterior (AP) axis (Figure 2A g, h). Expression pattern of *Xpk* during gastrulation is similar to those of *Xba* and eFGF (Isaacs et al., 1994; Smith et al., 1991), but not identical in that *Xpk* is eventually fated to somites (Figure 2A i, j) but not to anterior notochord. By sectioning the stained embryo, the intensive staining was found in the midline ectoderm (Figure 2A j inset) but not in anterior notochord (arrow head). In stage 28 tadpole, intensive *Xpk* expression was also observed in pronephros (Figure 2A k). These expression profiles of *Xpk* gene spatially tracing the convergent extension movement suggested that XPK plays a role during gastrulation.

Ciona intestinalis prickle (*Ci-pk*) was previously identified to be a gene expressed specifically in notochord and induced by *Ci-bra*, an acidian homolog of *brachyury* (*T*) gene (Hotta et al., 2000). In fact, *Xpk* has also been shown to be induced in animal cap cells by the overexpression of *Xenopus brachyury* (*Xbra*) in a dose-dependent manner (Figure 2C), suggesting that regulation of *prickle* genes by *brachyury* is conserved. In addition, it is noteworthy that expression pattern of *Xenopus Wnt11* (Ku and Melton, 1993; Tada and Smith, 2000) known as a Wnt/PCP signalling ligand, also a target of *Xbra* is extremely similar to that of *Xpk* during somite stages.

Xpk specific antisense morpholino oligo. perturbs gastrulation movement, but did not inhibit the mesoderm induction.

To understand *in vivo* role of *Xpk*, we first attempted to block endogenous translation of XPK protein by a morpholino antisense-oligonucleotide mo-Xpk designed to hybridise with the nucleotide sequence including the ATG for the initiation methionine of *Xpk*. Interestingly, the mo-Xpk inhibited gastrulation of injected embryo and led to *spina bifida* (Figure 3A d left panel, 103 out of 120 [85.8%] that received an injection of 10 pmol of mo-Xpk). Apparently, involution of mesodermal cells was impaired severer than

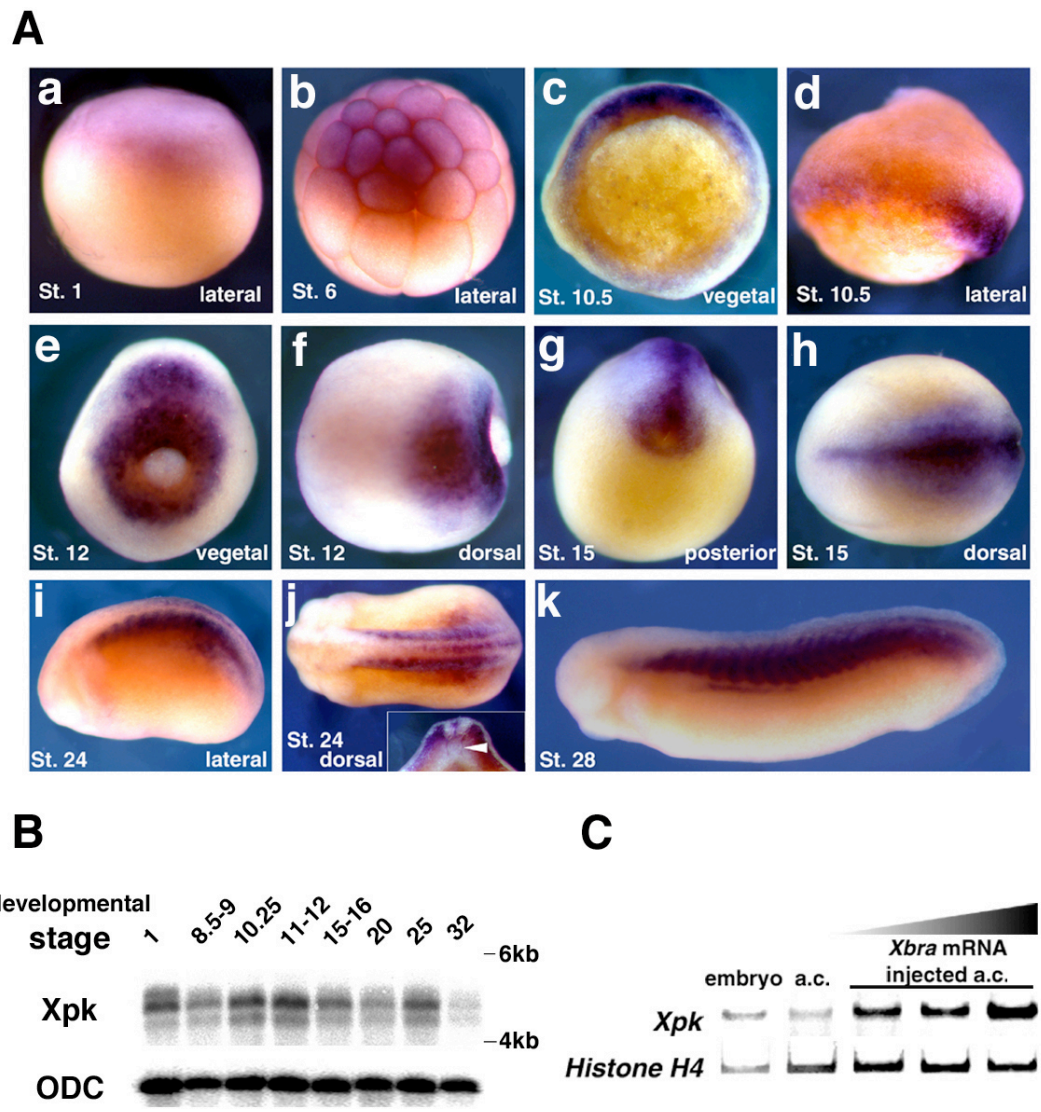


Figure 2 **The spatial and temporal expression pattern of *Xpk*.**

A, Whole-mount in situ hybridization of *Xpk* in staged embryos; Post-fertilized egg (a, lateral view), blastula stage 6 (b, lateral view), early gastrula stage 10.5 (c and d, vegetal and lateral view, respectively), late gastrula stage 13 (e and f, vegetal and dorsal view, respectively), early neurula stage 15 (g and h, posterior and dorsal view, respectively), late neurula stage 24 (i and j, lateral and dorsal view, respectively), tailbud embryo stage 28 (k, lateral view). In c, e, g, the dorsal side is at the top. In d, the dorsal side is at the right. In j inset, sagittal view, the white arrowhead points to the notochord.

B, Northern blot analysis of *Xpk*. The numbers at the top of each lane indicate developmental stages. Two *Xpk* transcripts of about 5 kb are detected in eggs to stage 32 embryos. ODC is the RNA loading quantity control.

C, *Xpk* expression is induced in animal cap cells in a dose-dependent manner by the overexpression of *Xbra*. In animal cap assay.

control embryo that uninjected (Figure 3A a). In these embryos, the expression of the mesoderm marker *Xbra*, *goosecoid* (Cho et al., 1991) and *MyoD* (Hopwood et al., 1989) and a neural marker N-CAM was not affected as revealed by whole-mount in situ hybridization (data not shown), suggesting that this defect was not due to the perturbation of mesodermal and neural differentiation. We also tested the effect of mo-Xpk on the elongation of the animal cap injected with activin mRNA elongates mimicking gastrulation cell movements (Thomsen et al., 1990). The mo-Xpk efficiently blocked the activin-induced elongation of animal caps (Figure 3A d right panel). We also confirmed that the expression of *Xbra*, *Xwnt11*, and *Xpk* itself, which are all induced by activin, were not affected by mo-Xpk (Figure 3C). Similar inhibitory effect was observed for the autonomous elongation of dorsal marginal zone (data not shown). These effects are specific to mo-Xpk, because a mo-oligo with unrelated mo or 4 mis-sense mutations (4 mis mo-Xpk) had no or little effects on gastrulation and activin-induced animal cap elongation, respectively at the same dose (Figure 3A b, c). We confirmed that mo-Xpk could prevent the translation of *Xpk*-GFP mRNA with the native sequence in 5'UTR of *Xpk*, but not of GFP mRNA (Figure 3B). However, we were unable to rescue the mo-Xpk phenotype with the *Xpk* mRNA lacking the mo target site, which will be discussed below.

mo-Zpk phenotype is very similar to it of dorsal convergence mutants.

To investigate the functional generality of the *prickle*-related gene in other organisms, we subsequently searched for zebrafish counterpart of *Xpk*. We identified its orthologous gene in the database on the web and named it *Zpk*. To confirm the role of *Zpk* in gastrulation, we employed disruption of *Zpk* with morpholino oligo nucleotides. As we expected, mo-Zpk dramatically inhibited gastrulation and caused stunted tail and proximalization of eyes (Figure 4A, B). The result suggests that vertebrate *prickle* related gene products have a conserved role in gastrulation cell movements. Very interestingly, the phenotype of 10-somite stage embryo showing the lateral expansion of somites (Figure 4C, D) and eye proximalization (Figure 4B) displayed a close resemblance with that of the zebrafish mutant *knypeck* and *trilobite* which were initially identified in a large-scale mutagenesis as gastrulation defective

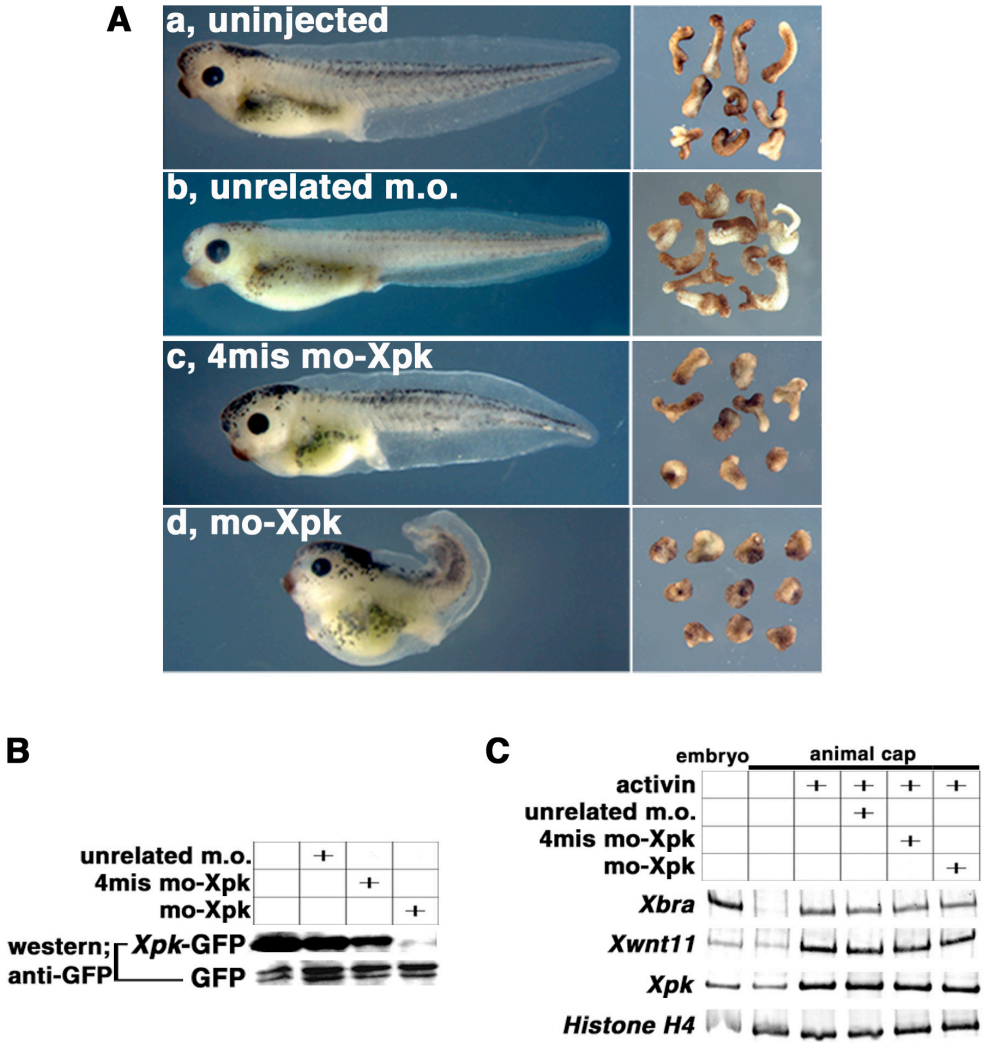


Figure 3 ***Xpk* antisense morpholino oligo. (mo-Xpk) inhibits convergent extension movements.**

A, mo-Xpk injection perturbs the correct gastrulation movements and activin-induced elongation of animal cap explants. **a**; uninjected sibling embryo at the tadpole stage (left panel) and 0.5pg activin mRNA-injected animal cap explants (right panel). **b**; unrelated m.o. has no effect when injected dorsally (left panel) or in the animal cap elongation assay (right panel). **c**; 4mis mo-Xpk has a little effect when injected dorsally (left panel) or in the animal cap elongation assay (right panel). **d**; mo-Xpk 10-pmol dorsal-injected embryo (left panel) and animal cap co-injected with activin and mo-Xpk (left panel).

B, mo-Xpk prevented the translation of overexpressed Xpk-GFP, but unrelated m.o. or 4mis mo-Xpk had no or little effects, respectively. Translation of co-injected GFP mRNA as negative control were not prevented.

C, In the animal cap elongation assay, mo-Xpk does not interfere with mesoderm induction (*Xbra* expression) or expression of *Xwnt11* or of *Xpk* itself. *Histone H4* is the cDNA loading quantity control.

mutants (Henry et al., 2000; Marlow et al., 1998). However, responsible genes for zebrafish mutants *knypek* and *trilobite* have recently been found to encode a glypican (Ohkawara et al., 2003) and is a fish homolog of a *Drosophila* PCP gene *strabismus*/*Van Gogh* (Darken et al., 2002; Jessen et al., 2002; Park and Moon, 2002), respectively, tempting to speculate that there might be another gastrulation defect mutants in which *Zpk* gene is disrupted. It was also found that the defect of gastrulation movement in *Zpk*-disrupted embryo was not due to the loss of mesodermal gene expression such as *MyoD* as their expression patterns are essentially indistinguishable from wild type embryo except that the expression domains were laterally broader compared to those in wild type embryo (Figure 4C, D). These results suggest that the gastrulation defect is attributed to the impaired cell movement of convergence and extension by the depletion of *Zpk*. Taken together, these strongly suggest that vertebrate *prickle, knypeck* and *trilobite* function in the same or an overlapping cascade of the non-canonical Wnt signalling pathway. These effects were also shown the case of mo-Zpk II (Figure 4D, G) that is designed at different target site in 5'UTR from mo-Zpk, but 4 mis mo-Zpk had no effect (Figure 4D, F) suggesting that two morpholino blocked *Zpk* specific role.

Overexpression of XPK and its deleted mutants also inhibit gastrulation movements without affecting mesodermal differentiation.

It is known that wild type Xwnt11 as well as its dominant negative form (DNXwnt11) causes gastrulation defects when overexpressed in *Xenopus* embryo (Smith et al., 2000; Tada and Smith, 2000). To test whether XPK is involved in the Xwnt11 signalling, we overexpressed wild type and XPK mutants, □PET, □LIM, □PET/LIM (□P/L), and PET/LIM (P/L) with deletions of putative functional domains (Figure 5A) in animal cap and examined their effects in activin-induced elongation. In analogy with mo-Xpk experiments, elongation of activin-induced animal cap was also significantly inhibited by injecting wild type and other mutant *Xpk* mRNAs (Figure 5B). They all inhibited animal cap elongation without affecting the expression level of mesodermal marker genes such as *Xbra* and *Xwnt11* (Figure 5C).

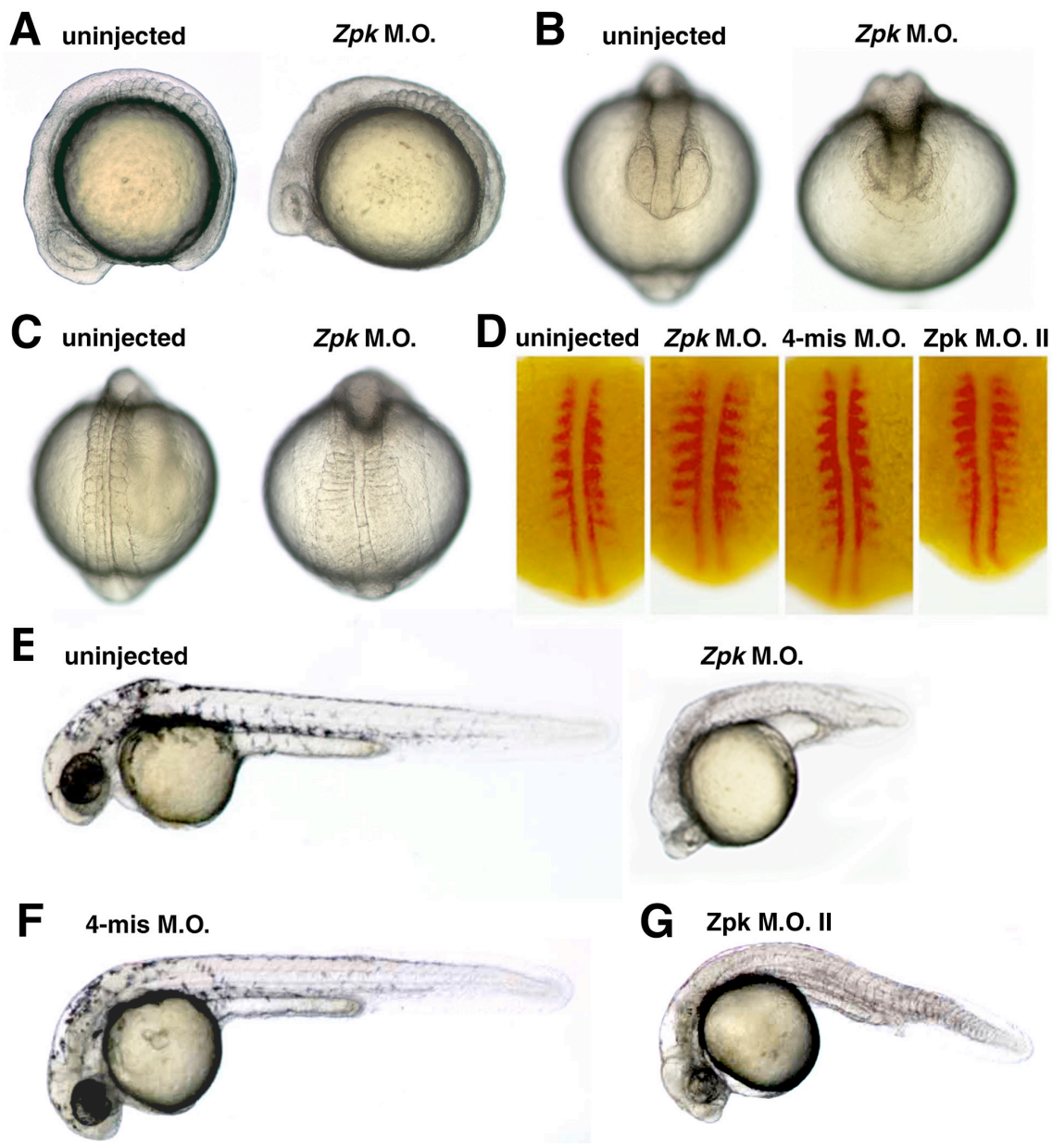


Figure 4 ***Zpk* knockdown using mo-Zpk inhibits dorsal convergence.**

A- C, Phenotypes are observed at the10 somites stage, 17 hpf. **A**, lateral view; **B,** anterior view; **C**, dorsal view, each left panel and right panel are uninjected and mo-Zpk 0.5 pmol injected embryo, respectively.

D, Whole mount *in situ* hybridization of *MyoD* expressed in somite (red). The knock-down of *Zpk* embryo is laterally expanded somite, indicated in fig. **C** too, but not uninjected.or 4-mis mo-Zpk injected embryos.

E, In prim-20 stage (33 hpf), m.o. phenotype is very similar to *knypec* and *trilobite*, those are identified as PCP signal components mutants with dorsal convergence defects.

F, 4-mis mo-Zpk is not affected. **G,** mo-Zpk II has similar effects with mo-Zpk.

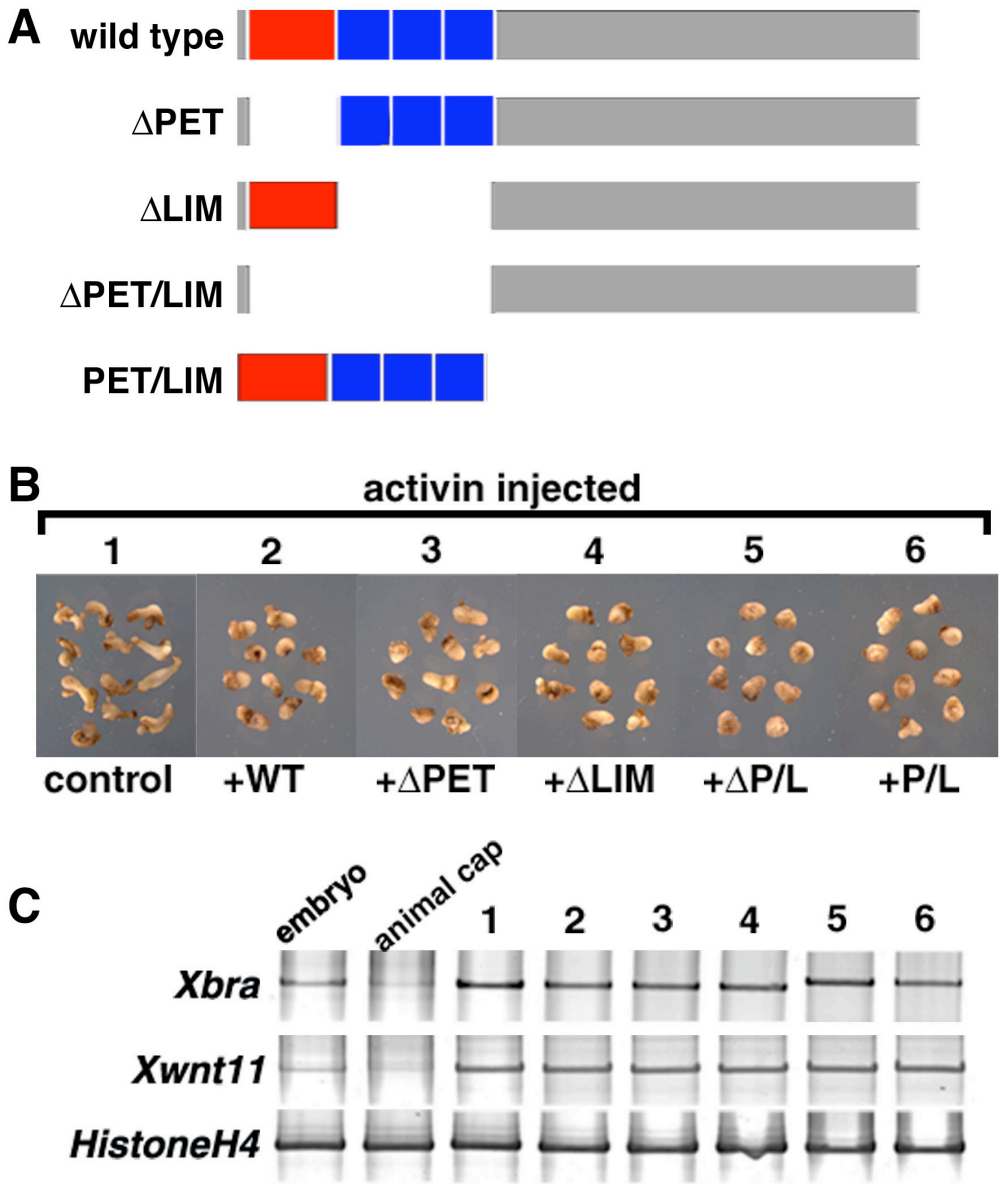


Figure 5 **Wild type XPK and all of deleted XPKs overexpression also inhibits gastrulation cell movements.**

A, Structures of wild-type XPK and deletion constructs; ΔPET, ΔLIM, ΔP/L, and P/L are illustrated.

B, All of these constructs inhibit activin-induced elongation of animal cap explants. (wild-type and ΔPET 500 pg, ΔLIM 1 ng, ΔP/L and P/L 250 pg)

C, None of the XPK constructs affect activin-induced *Xbra* and *Xwnt11* expression in animal cap explants at stage10.5. The indicated numbers correspond to B 1-6.

Triple LIM domain deleted XPKs could act as the dominant-negative way to wild-type XPK.

To examine the structure-activity relationship of XPK and investigate its functional properties, we next tested activity of above mentioned XPK mutant proteins (Figure 5A), focusing on gastrulation phenotype. We overexpressed them in either ventral or dorsal side of embryo by injecting its mRNA into two blastomeres of 4-cell embryo and affected embryos were classified into 3 grades from 1 to 3 (Figure 6A) and embryos counted according to the score. Ventral overexpression of XPK and its mutants also blocked ventral gastrulation movements although resulting phenotype was relatively subtle (data not shown). As we expected, dorsal overexpression of wild type *Xpk* mRNA significantly perturbed gastrulation movement (259 out of 312, 83.0% of embryos injected with 1 ng of RNA were scored between grade 2,3) and in the most severe case, it caused *spina bifida* embryos phenocopying the mo-Xpk injected embryos.

It should be noted that ΔP/L and P/L gave the most severe phenotype even at lower doses. These results suggest every construct retains some functional domains for signalling. To ascertain whether each XPK protein signaled in positive or negative direction, we then co-injected each mutant mRNA with wild type mRNA and observed phenotype (Figure 6B). Given wild type XPK signals in positive direction, ΔLIM and ΔP/L (Figure 6B a, b, respectively and C) seemed to signal in a negative way as their co-injection with wild type XPK mRNA reverted the phenotype (average score; 1.6 to 1.2, 2.2 to 1.7, respectively). In contrast, phenotype of ΔPET and P/L (Figure 6B c, d, respectively) was not rescued but rather enhanced by co-injection of wild type XPK mRNA, suggesting that they act in the same direction as wild type XPK. Interestingly, ΔPET or P/L could be rescued with ΔP/L (Figure 6B e, f, respectively), suggesting that the C-terminal domain negatively interacts with LIM domain, possibly interfering the LIM domain from interacting with other partners. In summary, it seems that XPKs lacking LIM domain act antagonistically to wild type XPK and that XPKs lacking domains other than LIM domain still be able to transmit signals similar to wild type.

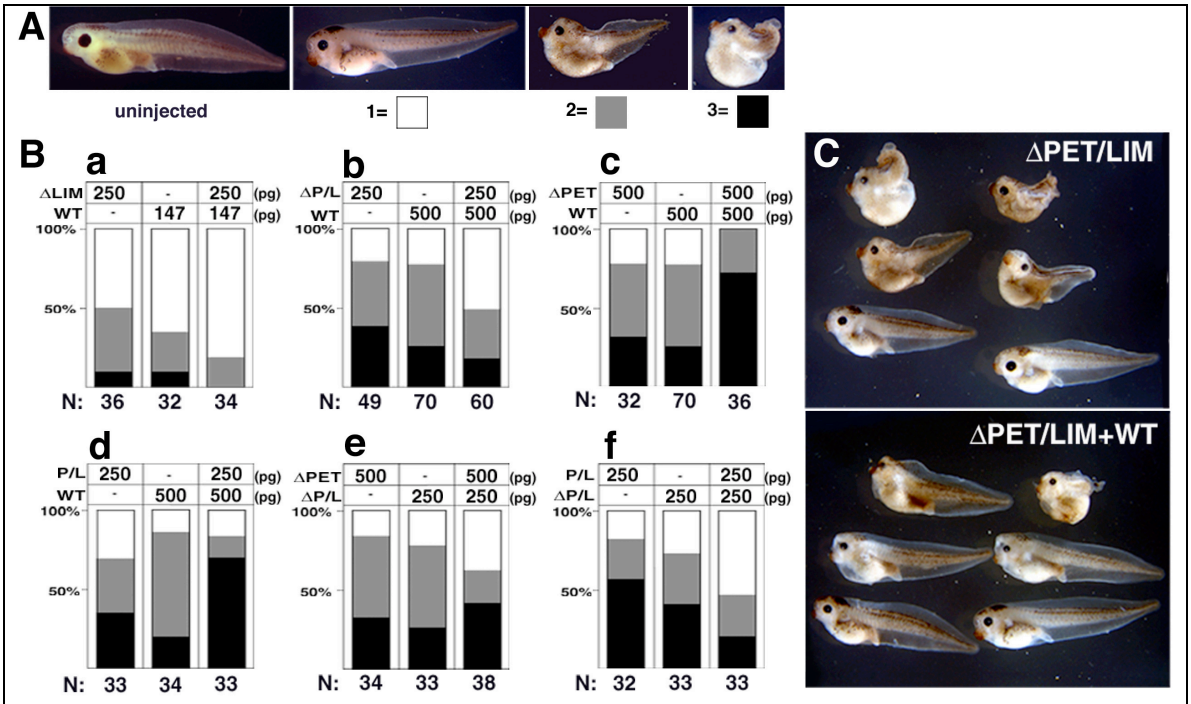


Figure 6 **Analysis of XPK structure-function relationship based from dorsal co-injection of each deleted Xpks and wild type.**

A, Index of gastrulation defective phenotypes. All constructs perturb normal gastrulation movements when injected into the dorsal embryo. Phenotypes of abnormal gastrulation were classified into 3 grades; 1, normal or weak phenotype showing a small head but nearly normal-sized trunk and tail; 2, intermediate phenotype showing a short and curved body axis; 3, severe phenotype showing an open blastopore and *spina bifida*-like.

B, In this bar graph, the phenotypes caused by injecting these constructs into the dorsal embryo were scored as 1, 2, or 3 and are indicated with white, gray, and black color, respectively. In **a, b,** effects of LIM domain-deleted *Xpks* injection were rescued by the co-injection of wild-type *Xpk* mRNA. In **c, d,** effects of LIM domain-containing *Xpks* were enhanced the wild type effect in gastrulation. In **e, f,** effects of LIM domain-containing *Xpks* were rescued by the co-injection of ΔPET/LIM.

C, An example of phenotype rescue, the result of injecting ΔP/L at 250 pg is shown in the upper panel and the phenotype resulting from injecting ΔP/L with wild-type *Xpk* at 500 pg is shown in the lower panel. The ratio of abnormal embryos was reduced by the co-expression of wild-type *Xpk*.

It is intriguing to find that both gain-of-function and loss-of-function of XPK resulted in a similar gastrulation-defective phenotype. This is often observed for PCP signals in vertebrates, and is well explained by the idea that the PCP signal, which is regulated by Wnt11/*silberblick*, must be fine tuned to some appropriate level that is essential for proper cell movements during gastrulation (Smith et al., 2000; Tada and Smith, 2000). It is also possible that localization of signalling components such as Xdsh and Fz in the pathway is strictly regulated sensing the proper level of external signals. This may also explain why rescuing the mo-XPK or loss-of function of upstream component with wt XPK is difficult. Nevertheless, these cellular responses to *Xpk* signal manipulations imply that *Xpk* may act in the PCP signalling pathways regulated by Wnt11/*silberblick*.

Xpk did not interfere membrane localization of Dsh depending on Fz signaling.

To gain an insight into the cellular mechanism by which XPK regulates gastrulation cell movements, we examined localization of Xdsh in relation to the XPK activity and subcellular localization of signalling components. In *Drosophila*, overexpression of Prickle has been shown to inhibit membrane-localizaton of Dsh to block PCP signal at least in cultured cells (Tree et al., 2002). To examine the possibility in *Xenopus*, wild type XPK, P/L or other mutant XPKs was expressed in animal cap cells by mRNA injection and myc-tagged Xdsh was visualized with an anti-myc antibodies. N-terminally flag-tagged wt XPK was found to be localized ubiquitously and distributed to nucleus, cytoplasm and membrane (left). Interestingly, P/L was sharply localized in nucleus (Figure 7A, right) although the physiological significance is unclear. Xdsh protein is also ubiquitously distributed in the cells when exogenous Fz7 is absent regardless of the presence of wild type, P/L, or other mutant XPKs. Xdsh becomes associated with membrane when Fz7 that activates non-canonical Wnt signal is co-injected (Figure 7B left) regardless of the presence of wild type *Xpk*, P/L (Figure7B, bottom two panels) nor other XPK mutants including and □P/L (data not shown). In addition, presence of neither Fz7 nor Xdsh affected the localization of XPK and P/L (Figure 7B, top panels). These results suggest that at least in *Xenopus*, XPK is not

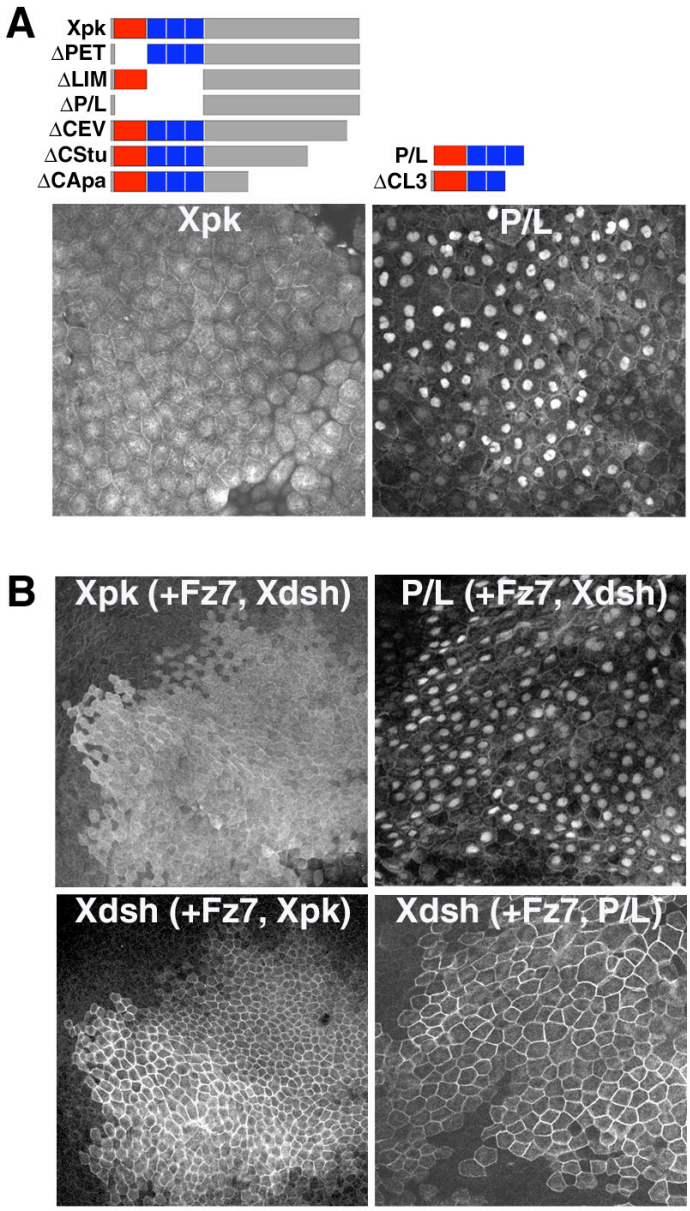


Figure 7 **Intracellular localization of the XPK constructs; Neither XPK nor its deletion mutants regulate Xdsh membrane localization.**

A, Intracellular localization of XPK constructs in animal cap cells. The FLAG-tagged constructs illustrated in the upper panel were analyzed by confocal microscopy. wt XPK was expressed ubiquitously but its expression was slightly greater at the cell membrane (bottom left). Deletion constructs ΔPET, ΔLIM, ΔP/L, and the C-terminal deletion mutants ΔCEV, ΔCstu, and ΔCApa were localized similarly to wt XPK, but P/L and ΔCL3 were found to be localized solely to the nucleus (bottom right).

B, The localization of the XPK constructs was not altered by the membrane localization of myc-Xdsh in response to the Fz7 signal, and *vice versa*. The XPK constructs and Xdsh did not interfere with each other's localization.

localization of Xdsh.

XPK modulates JNK activation mediated by Dsh, and physically interacts with Dsh as well as JNK.

To further investigate whether XPK function is correlated to the non-canonical Wnt pathway regulating PCP, we examined the possibility that XPK activates JNK, as JNK has been reported to be acting in non-canonical Wnt pathway downstream of Dsh (Boutros et al., 1998; Lisovsky et al., 2002; Yamanaka et al., 2002). To evaluate JNK activation, phsphorylation of a target of JNK c-Jun in HEK293T cells transfected with *Xdsh*, *Xpk*, or both cDNAs. XPK alone failed to activate JNK, while Xdsh activated JNK in a dose dependent manner (Figure 8B). However, co-transfection of Xdsh with Xpk but not with ΔP/L cDNAs dramatically increased the level of c-Jun phosphorylation, at the level which Xdsh alone could not activate JNK efficiently (Figure 8C). This result suggests that XPK cooperates with Xdsh to activate JNK through its PET/LIM domain. At a high level of Xdsh, it alone can activate JNK to a certain level and interestingly, wt XPK or ΔP/L but not P/L suppressed the JNK activation by Xdsh (Figure 8D), suggesting the part of XPK excluding PET/LIM domain may act negatively to Xdsh mediated JNK activation at high levels of Xdsh. This is consistent with the observation that ΔP/L and *Xpk* including LIM domain counteract each other in dorsal overexpression (Figure 6B). As a general rule, it seems that Wnt/PCP pathway and Wnt/β-catenin canonical pathway are regulated antagonistically by each other (Kuhl et al., 2001), but Xpk was not affect to Wnt/β-catenin signalling (Figure 8A).

These observations prompted us to test whether XPK physically interacts with Xdsh. To test this possibility, GST fusion protein of either XPK or Xdsh was transiently expressed with HA-tagged Xdsh or flag-tagged XPK, respectively in 293T cells and precipitated with glutathione-Separose beads, and the resulting precipitate was probed with anti-HA or anti-flag antibodies by western blotting. HA-Xdsh or flag-XPK was efficiently precipitated with the GST fusion protein of the other (Figure 9A), indicating that XPK and Xdsh physically interact each other. The interaction between XPK and Xdsh is conserved among species as *Drosophila* Prickle has been reported to bind Dsh through DEP domain of Dsh

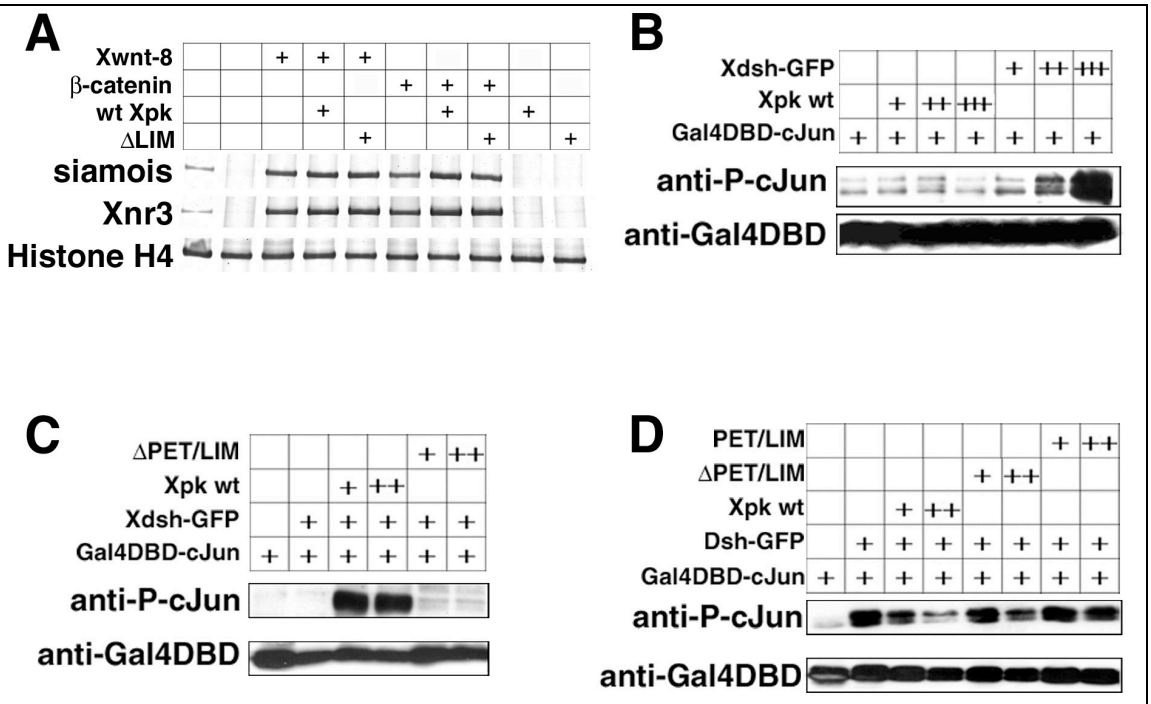


Figure 8 **XPK modulates the activation of JNK mediated by Dsh without interferes to canonical Wnt/β-catenin pathway.**

A, *Xpk* dose not interfere with the expression of siamois or Xnr3 targetted by canonical Wnt ligand, Xwnt-8, and β-catenin in animal cap assy.

B, JNK activation could not detected in *Xpk* transfected cells, but Xdsh alone could activate to JNK in dose dependent manner.

C,D, Two-phase activity of XPK, depending on the Xdsh dose. **C,** At a low Xdsh dose, wild-type XPK enhances Xdsh-mediated JNK activation. **D,** At a high Xdsh dose, wild-type XPK or ΔP/L suppresses the activation of JNK, which is mediated by Xdsh. HEK293T cells were transfected with the indicated expression plasmids. The activation of JNK was monitored using an anti-phosphorylated c-Jun antibody. Western blotting using an anti-Gal4DBD antibody was the control for the transfection and loading quantity.

(Tree et al., 2002). Our yeast two hybrid assay also demonstrated that PET/LIM domain of XPK is sufficient to bind Xdsh (data not shown). As we speculated that XPK might play as a scaffold for JNK activation, next we tested whether XPK binds to JNK. Again, 293T cells were transfected with flag-tagged XPK or its derivatives. Neither the N-terminal region containing PET/LIM domain nor the C-terminal region of XPK was found to be sufficient to bind JNK, and only wt XPK could bind JNK significantly (Figure 9B). Nevertheless, this further

suggests that XPK forms ternary complex with Dsh and JNK, binding Xdsh and JNK via PET/LIM and the C-terminal domain of XPK, respectively.

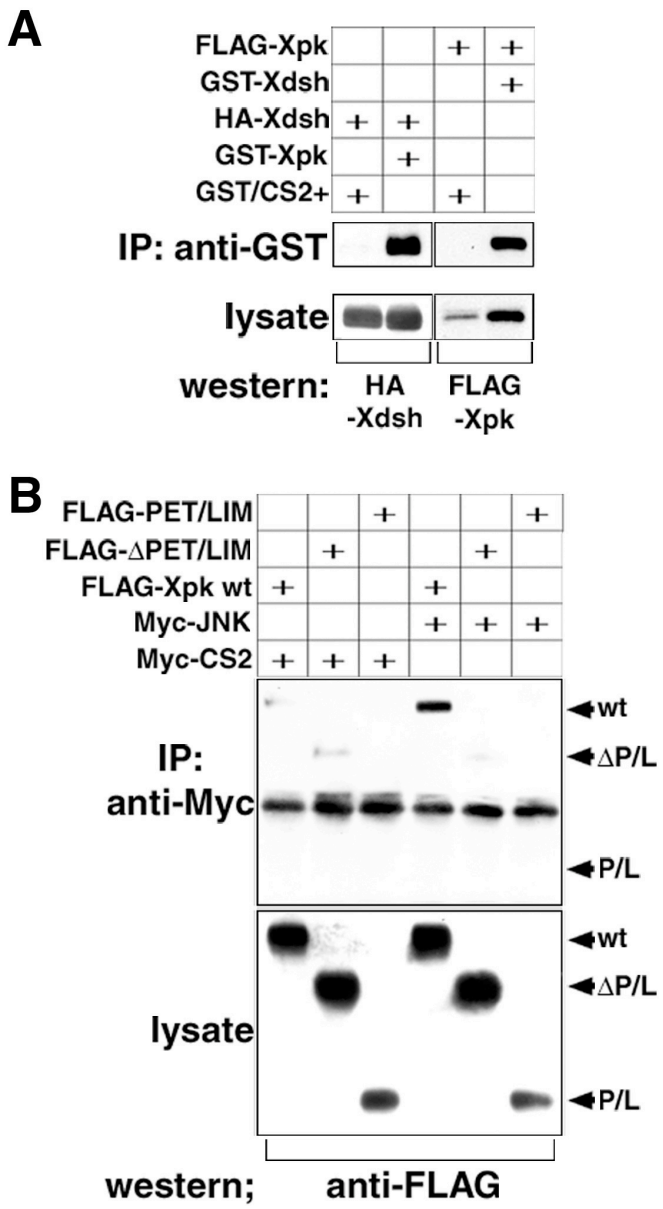


Figure 9 **XPK interacts physically with Dishevelled as well as JNK.**
A, HA-Xdsh or flag-XPK was performed the pull-down assay with GST fusion protein of the other, showing in left or right, respectively.
B, In immunoprecipitation, wild-type, but not deleted XPKs interacts physically with JNK1.

In *Drosophila* wing, it has been proposed that Prickle generates asymmetric Frizzled and Dishevelled localization through the suppression of Fz and Dsh localization at the proximal cell cortex (Tree et al., 2002). In this study, we showed that XPK is a key component connecting Dsh to JNK activation during *Xenopus* gastrulation. It has been predicted from *Drosophila* genetics that JNK is one of the downstream targets of PCP pathway (Boutros et al., 1998). Our results reinforce the idea that non-canonical Wnt (PCP) pathway regulates gastrulation cell movements in vertebrate through activation of JNK. To further understand the pathway, we are currently undertaking to identify XPK-interacting components that regulate JNK activation.

CHAPTER2

PIK; Prickle-Interacting-Kinase is required for JNK activation mediated by Wnt/PCP signaling during *Xenopus* gastrulation.

In chapter 1, I demonstrated that XPK plays an essential role in gastrulation movements through the regulation of JNK activation mediated by Wnt/PCP component Dsh. I also showed that XPK physically interacts with Dsh as well as with JNK and proposed that XPK connects Dsh to JNK in this signalling pathway. Although XPK is thought to be a necessary component for Dsh to indirectly activate JNK/MAPKinase pathway, XPK itself does not seem to have catalytic activities to activate JNK/MAP kinase from its primary structure. Thus, it was predicted that XPK is functionally associated with molecule(s) that is regulated by Dsh/XPK and such factors may be responsible for initiating the MAPK pathway, resulting in JNK activation.

Isolation of Xenopus Prickle Interacting Kinase; XPIK and its structure.

To identify a possible adapter protein of XPK, we used PET/LIM domain of XPK as bait for yeast two hybrid screening (Figure 1AB). As a result of the screening of a *Xenopus* cDNA library, a cDNA encoding the C-terminal half of XPIK was identified as one of positive clones. Full length XPIK was obtained by 5'-RACE method, and it was found to encode an 896 amino acids protein containing a typical kinase domain in its N-terminal half. As shown in Fig. 2, XPIK is grouped into the TAO family which includes TAO1 (Hutchison et al., 1998)and JIK (Tassi et al., 1999), and keeps aloof from other Ste20 group kinases in phylogenetical distance and its structure (Dan et al., 2001; Figure 1C, 2).. Although some of Ste20 group kinases including *Drosophila* Misshapen have a conserved interacting domain to small GTPase, including RhoA, cdc42 and Rac which play a role in gastrulation (Su et al., 1998), XPIK dose not seem to possess it. XPIK is homologous gene of human Ste20-like kinase (JIK) or chick KFC (Yustein et al., 2000), and highly conserved in kinase domain and C-terminal half identified as a putative XPK interacting domain by two-hybrid assay. Based on these structural similarities, XPIK is most likely to be a MAP4K.

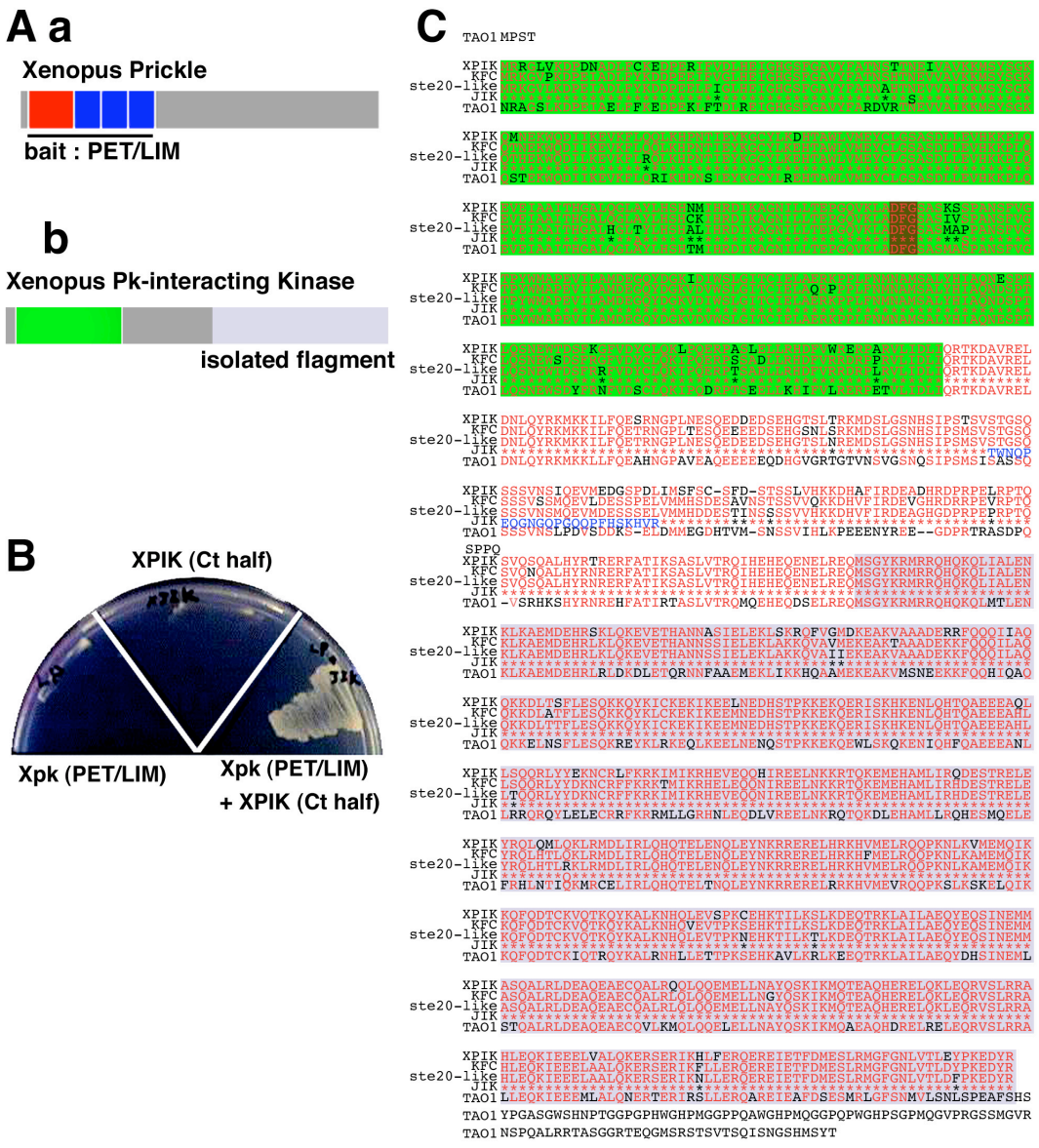
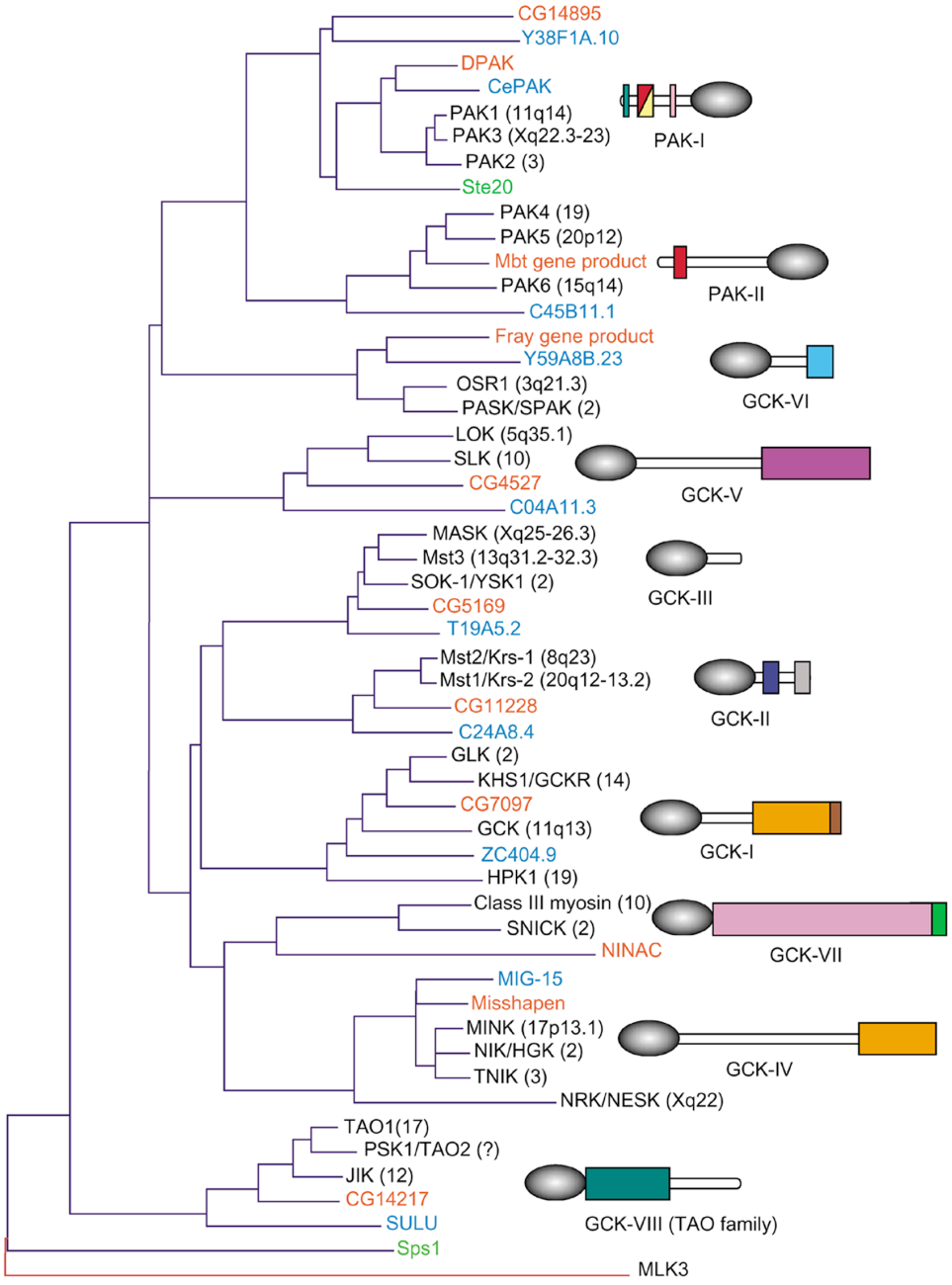


Figure 1 **Cloning of XPIK and its structure; XPIK is homologous gene of human Ste20-like kinase taxonomically classified into TAO1 group.**

A, Illustration of XPK(**a**) and XPIK(**b**) structures. We obtained XPIK using XPK PET/LIM domain as bait for yeast two-hybrid screening. In **a**, PET and LIM domains were indicated to red and blue, respectively. In **b**, XPIK kinase domain and the flagment obtained by this screening were indicated to green and amethyst, respectively.

B, From results of yeast two-hybrid assay, XPIK bind to PET/LIM domain of XPK.

C, Alignment of TAO family genes. Red letters indicated to amino asid residue conserved in TAO family. The sequence of kinase domain and PET/LIM binding flagment were boxed in green and amethyst, respectively. DFG motif was boxed in dark brown. Ste20-like and JIK are estimated in splicing variant.



quote from TRENDS in Cell Biology Vol.11 No.5 May 2001, Dan et. al.

Figure 2 Phylogenetic relations among mammalian Ste20 group kinases and their schematic structures in each subfamily.

The expression pattern of XPIK during gastrulation.

By an RT-PCR analysis of staged embryos, it was found that XPIK transcripts are maternally encoded and expressed during gastrulation cell movements (Figure 3B). Whole-mount *in situ* hybridization of developing *Xenopus* embryos revealed that XPIK is expressed as a maternal mRNA localized in dorsal –animal area of early blastula embryo (Figure 3A). As gastrulation starts, the transcripts become restricted to the dorsal side including marginal zone and pre-neural ectoderm (Figure 3A). This expression pattern is very similar to Wnt5a that is one of non-canonical Wnt ligands (Moon et al., 1993), and overlaps with the *Xpk* expression domain, suggesting that XPIK play a role in gastrulation movements with XPK in PCP signaling pathway.

XPIK play a role in JNK activation by its kinase activity.

Since KFC, JIK and TAO1 are capable of activating or inhibiting JNK as previously reported (Hutchison et al., 1998; Tassi et al., 1999; Yustein et al., 2000), we examined whether XPIK could activate JNK in embryos. Activation of JNK was evaluated as phosphorylation of c-Jun which was exogenously introduced into early *Xenopus* embryo as a substrate by RNA injection. As a result, overexpression of wild-type XPIK activated JNK in a dose dependent manner (Figure 4B). To identify the functional domain of XPIK acting for JNK activation, XPIK deletion mutants; XPIK-catal that is only the putative kinase domain and XPIK-C that was deleted entire kinase domain (Figure 4A) were tested for the activity of JNK activation. Interestingly, XPIK-catal had higher activity than wild-type XPIK in JNK activation, but XPIK-C had no activity, suggesting that XPIK activate JNK via its kinase domain and this activity, which in turn suggests its kinase activity is negatively regulated by C-terminal region (Figure 4C). To further investigate in vivo function of XPIK kinase domain, we tested JNK activation assay with another mutant form XPIK-KN that was altered D165 to A (Figure 4A), as such mutation was previously reported with TAO1 to diminish its kinase activity (Hutchison et al., 1998). In consequence, XPIK-KN also had no effects on JNK activation as predicted, demonstrating again that XPIK play a role in JNK activation by its kinase activity (Figure 4D). When

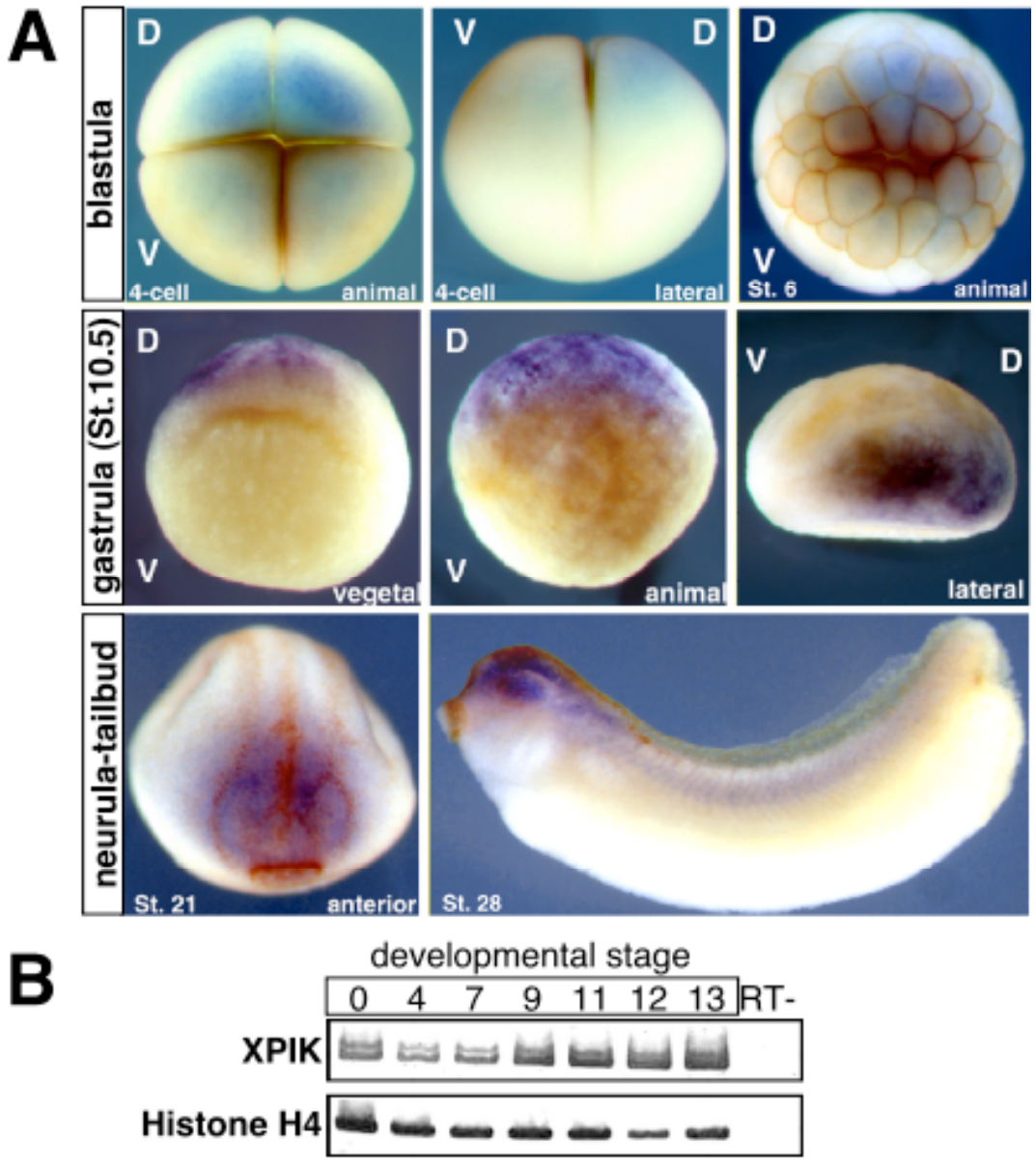


Figure 3 **Spasial and temporal expression pattern of XPIK**

A, Whole mount *in situ* hybridization of XPIK. XPIK were maternally expressed in dorso-animal area. **a**; left and middle panels are 4 cell stage embryo in animal and lateral view, respectively. light panel are animal view of st. 6 embryo. As gastrulation starts, the expression domain was dorsal side including mesodermal and neural areas. **b**; In st. 10.5 embryo, left, middle and light panels were vegetal, animal and lateral views, respectively. From neurula to tail-bud stage embryos, XPIK were expressed in eyes, b rains and neural tube, but not notochord (**c**).

B, Northern blot analysis of XPIK in early developmental stages. XPIK were highly expressed in gastrula.

XPIK-KN or XPIK-C were co-expressed with wild-type XPIK, XPIK-KN but not XPIK-C inhibited JNK activation mediated by wild-type, suggesting that XPIK-KN acts as a dominantly negative form for JNK activation (Figure 4D).

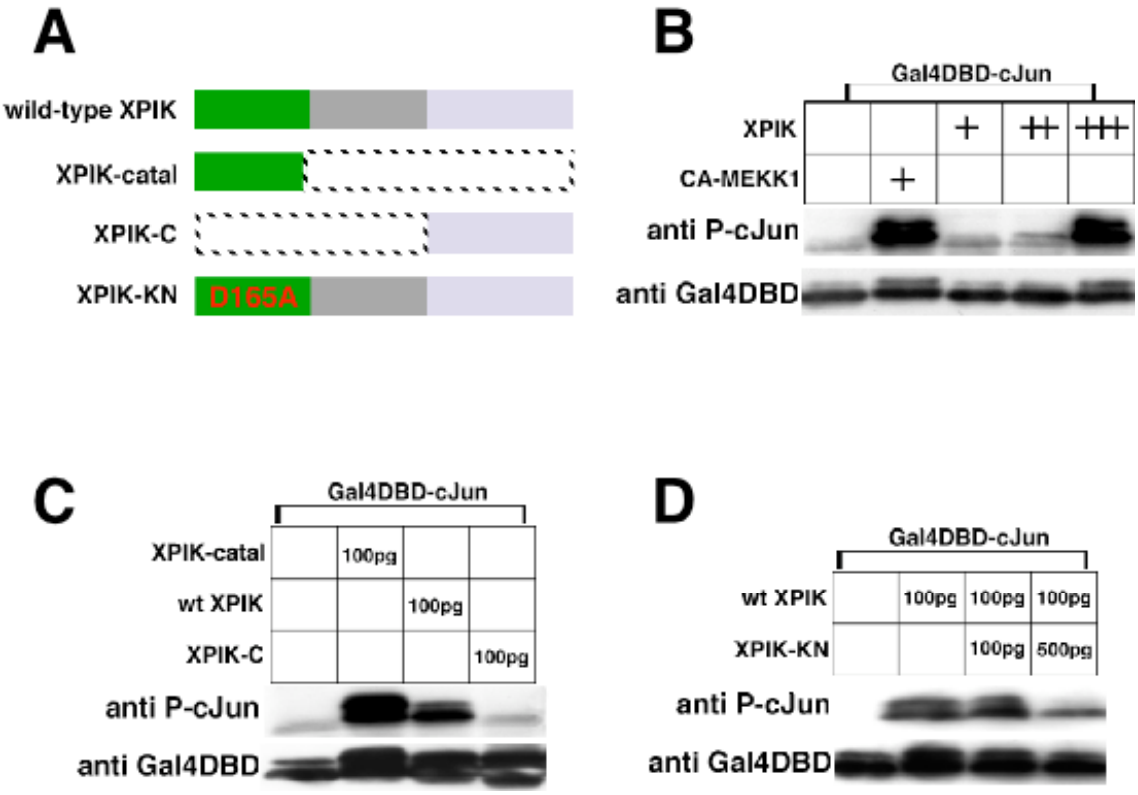


Figure 4 **XPIK acts for JNK activation via its kinase domain, and XPIK-KN acts as dominant negative manner against wild-type.**

A, Illastration of each XPIK constructs.

B, Wild-type XPIK activates JNK detected as c-Jun phosphorylation in dose dependent manner. MEKK1 known as MAP4K to JNK activation is positive control. Western blot for anti Gal4DBD is quantity controls of transfection and loding.

C, XPIK-catal has higher activity of JNK activation than wild-type XPIK in same dose. XPIK-C has no effects of JNK activation.

D, XPIK-KN inhibited JNK activation mediated by wild-type XPIK, acts as dominant negative role against wild-type XPIK.

Perturbation of gastrulation movements by XPIK overexpression is correlated with levels of JNK activation.

Overexpression of wild-type XPIK in dorsal marginal zone of 4 cell *Xenopus* embryos perturbed gastrulation movements as has been shown by XPK overexpression (Figure 5B). One striking feature was that injected embryo displayed a typical phenotype known as gastrulation defects, including shortened trunk and *spina bifida* whose neural tube was remained open. These affected embryos were classified into three grades of severity as described in Chapter 1 (Figure 5A). First, I examined whether XPIK mutants with various JNK activation/inhibition activities can disrupt the gastrulation movements and asked how phenotype is correlated with JNK activation status. Overexpression of each mutant in dorsal marginal zone demonstrated that XPIK-catal or XPIK-KN, but not XPIK-C, had effects on gastrulation (Figure 5B). Just as its ability of JNK activation, XPIK-catal caused more severe defects of gastrulation than wild-type XPIK at the same dose. In contrast, XPIK-C had no effects on gastrulation movements as did for JNK activation. These results suggested that the degree of this gastrulation defects depended on levels of XPIK expression and JNK activation; higher levels of XPIK expression concomitant with higher JNK activation are always associated with more severe phenotypes in gastrulation movements. XPIK-KN that inhibits JNK activation mediated by wild-type XPIK in a dominantly negative manner perturbed normal gastrulation and its phenotypes were rescued by XPIK-catal. It suggested that the gastrulation defect by XPIK-KN dorsal-expression is due to the reduction of endogenous XPIK activity. These results were concluded that JNK activation over the endogenous level or depletion of active JNK in dorsal side of embryos causes the gastrulation defects. Taken these observations together, XPIK is thought to play an essential role in gastrulation through the regulation of JNK activation. In addition, we tested the possibility that TAO1 has a similar effect on gastrulation or JNK activation by overexpression in dorsal marginal zone. However, TAO1 had no effect in both experiments, suggesting that these effects of XPIK were specific function of XPIK (data not shown).

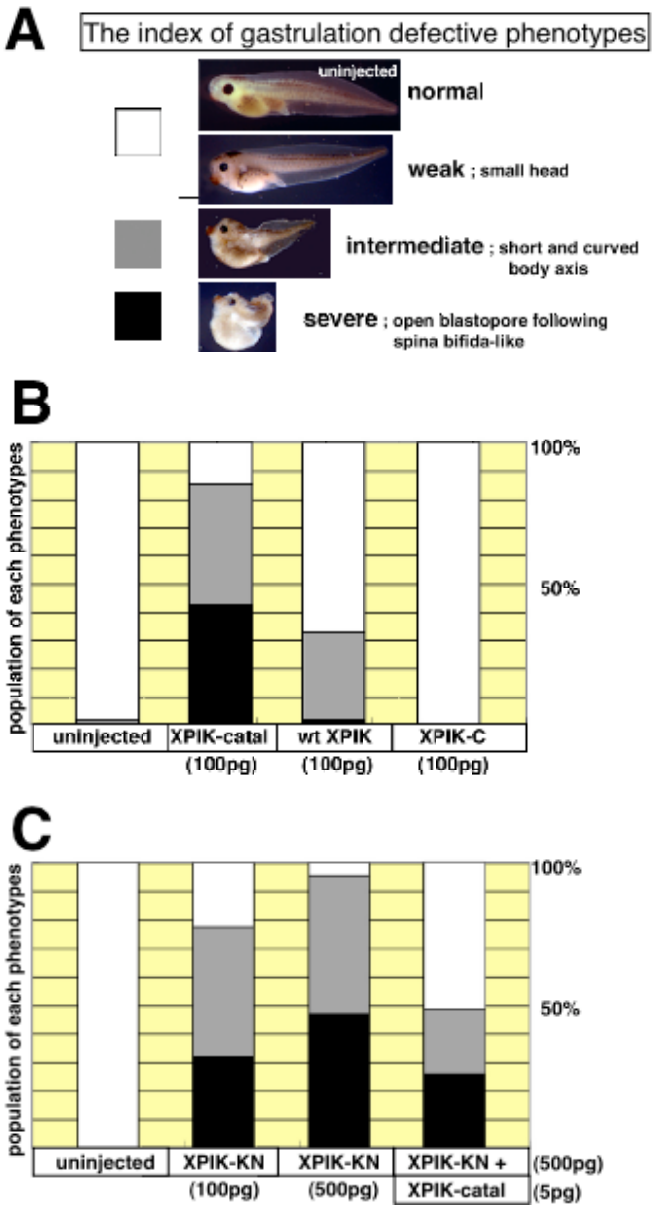


Figure 5 **Perturbation of gastrulation movements by each XPIKs overexpression into dorsal side of embryos.**

A, Index of gastrulation defective phenotypes. All constructs perturb normal gastrulation movements when injected into the dorsal embryo. Phenotypes of abnormal gastrulation were classified into 3 grades; white □, normal or weak phenotype showing a small head but nearly normal-sized trunk and tail; gray ■, intermediate phenotype showing a short and curved body axis; black ■, severe phenotype showing an open blastopore and *spina bifida*-like.

B, XPIK-catal has higher activity of gastrulation perturbation than wild-type XPIK in same dose. XPIK-C has no effects of gastrulation.

C, XPIK-KN also inhibites gastrulation movements despite of no activation of JNK. Its phenotype were rescued by XPIK-catal.

XPIK-specific antisense morpholino oligo. perturbs gastrulation movement without inhibition of mesoderm induction.

To confirm *in vivo* role of XPIK, we attempted to block endogenous translation of XPIK protein by antisense morpholino oligonucleotide, mo-XPIK designed to hybridise with the nucleotide sequence including the ATG for the initiation methionine of XPIK. Mo-XPIK inhibited gastrulation of injected embryos and led to *spina bifida* as does overexpression of XPIK-KN, supporting our hypothesis that XPIK-KN can reduce endogenous XPIK activity and thus the level of activated JNK. In these embryos, expression of a mesoderm marker *Xenopus brachyury* (*Xbra*) was not affected, suggesting that this defect is not due to the perturbation of mesodermal differentiation. As reported for *Xpk*, the negative or positive manipulation of XPIK function had indistinguishable effects on gastrulation. This is consistent with a feature of Wnt/PCP pathway; both of gain- or loss-of function of signaling results in changes of endogenous JNK activation, which reinforces our finding that XPIK together with XPK acts in Wnt/PCP signaling.

Animal cap explants elongation assay

To confirm the effect of XPIK in gastrulation, particularly in the cell movements called convergent and extension, we performed additional experiments using animal cap explants. We examined the effect of mo-XPIK or overexpression of wild-type or XPIK-KN on elongation of the animal cap stimulated with activin that mimics gastrulation cell movements via non-canonical Wnt/PCP pathway. The mo-XPIK as well as wild type XPIK and XPIK-KN mRNAs efficiently blocked the activin-induced elongation of animal caps and their effects were indistinguishable as has been observed with whole embryos. We also confirmed that expression of *Xbra*, *Xwnt11* and *Xpk*, all of which are induced by activin, were not affected (data not shown).

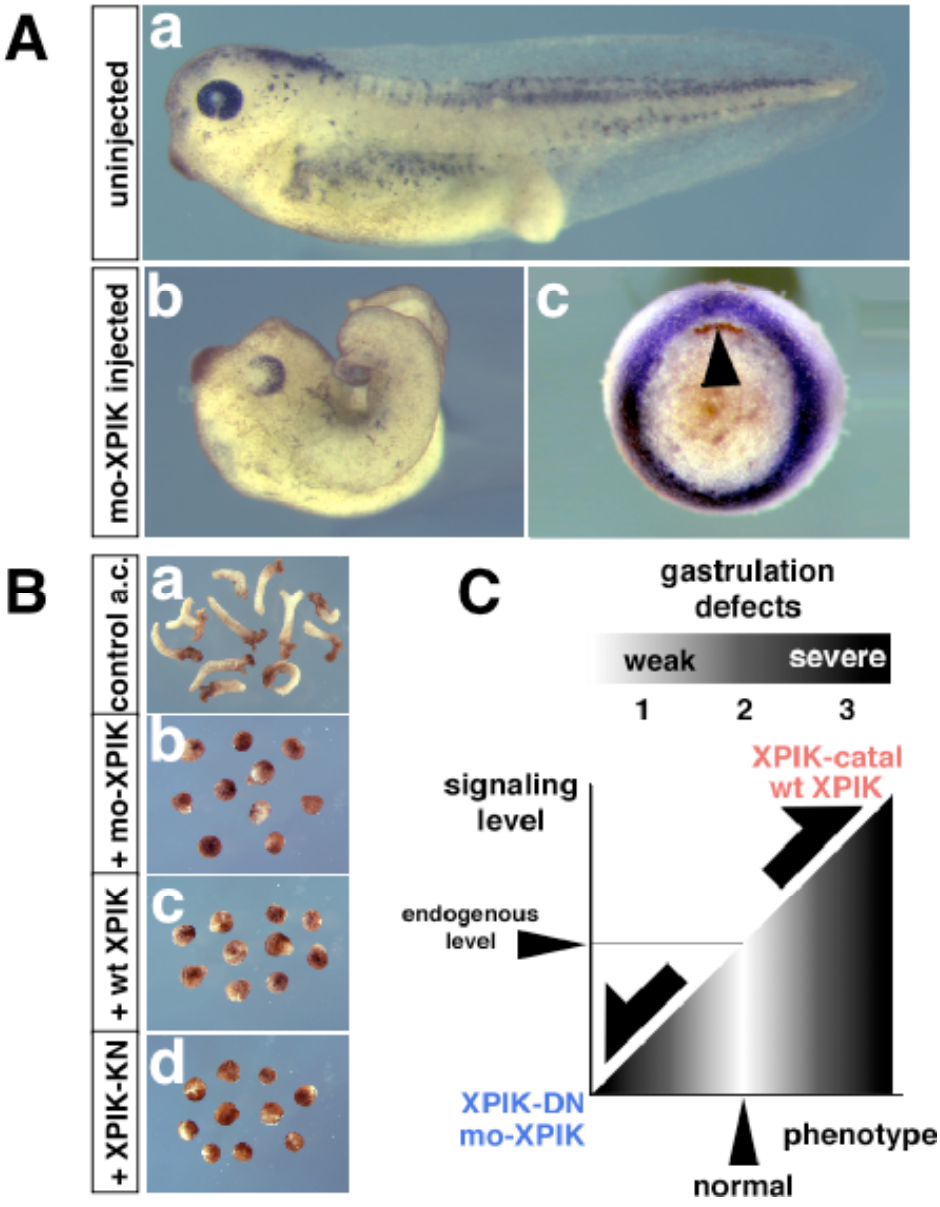


Figure 6 **mo-XPIK also inhibits gastrulation movements without inhibition of mesoderm induction.**

A, a; st. 28 uninjected embryo b; mo-XPIK phenotype in st. 28 tail bud embryo. c; Whole mount in situ hybridization of Xbra at mo-XPIK injected embryo. Arrow head pointed in injected position.

B, Animal cap elongation assay; Activin injected animal cap elongation mimics convergent extension for gastrulation cell movements (a). It was inhibited by mo-XPIK (b), wild-type XPIK (c) and XPIK-KN (d).

C, The relationship of gastrulation defects and signaling levels.

Dsh requires XPIK for JNK activation.

In HEK293T culture cells, Dsh alone could activate JNK as reported in Chapter 1. To examine functional epistasis between Dsh and XPIK, we tested whether XPIK-KN suppresses the JNK activation mediated by Dsh as well as wild-type XPIK. XPIK-KN was co-expressed with Dsh and Gal4DBD-cJun as JNK substrate in HEK293T cells. c-Jun phosphorylation in KPIK-KN expressing cells was significantly reduced compared to those only Dsh and Gal4DBD-cJun were expressed (Fig. 7A). This suggested that XPIK acts downstream of Dsh and play a role at least in part for JNK activation mediated by Dsh. As described above in this Chapter, XPIK interacts with XPK in yeast two-hybrid assay. We also tested whether XPIK binds not only to XPK but also to Dsh in culture cells. HA-tagged XPIK and flag-tagged XPK or GFP-tagged Dsh was expressed in the cells, and XPIK was co-precipitated by either anti-flag or anti-GFP antibodies. HA –epitope was detected in both precipitates, indicating that XPIK physically interacts with both XPK and Dsh in the cultured cells. In addition, it was found that increasing dose of GFP-tagged Dsh decreased the amount of XPIK co-precipitated by XPK with anti-flag antibody was decreased. It suggests that Dsh compete for the physical interaction between XPIK and XPK by in a dose dependent manner (Fig. 7B). However, it remains unclear how this regulation of binding of each component is related to the signaling including JNK activation.

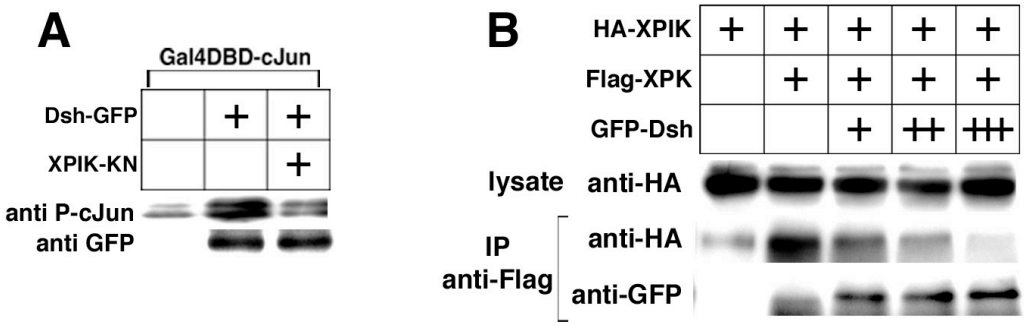


Figure 7 **XPIK-KN inhibits JNK activation mediated by Dsh in culture cell.** **A,** Only Dsh could activate to JNK, but Dsh could not activate it with XPIK-KN. **B,** Increasing dose of GFP-tagged Dsh decreased the amount of XPIK co-precipitated by XPK with anti-flag antibody was decreased. Transfected constructs indicated as + in each experiments.

Although it is known that Wnt/PCP signal activate JNK and is required for correct gastrulation cell movements including convergent extension, endogenous JNK activation could not be detected in dorsal side of embryos where convergent extension occurs. Further characterization of XPIK which is expressed in dorsal side of embryos and plays a role in gastrulation by regulating JNK, may provide a cue to understand this problem.

MATERIALS and METHODS

Cloning of Xenopus prickle

Our *Xenopus* EST database (Kitayama et al., unpublished, URL: http://xenopus.nibb.ac.jp/) was searched with the cDNA sequence of *Cipk* using BLAST. A full-length cDNA clone, xl017o12, corresponding to *XPK* was identified and subcloned into pCS2+ for functional analyses.

In situ hybridization and Northern blot analysis

In situ hybridization in *Xenopus* was carried out using digoxigenin-labeled antisense RNA probes as described previously (Harland, 1991). RNA from staged embryos was prepared with Trizol (Life Technologies). Northern blotting was performed by a standard method using polyA(+) RNA from oocytes and staged embryos.

Xpk and Zpk Morpholino oligo

Antisense morpholino (Heasman, 2002) was obtained from Gene Tools Inc. Each Morpholino oligo sequence was as follows: mo-Xpk; 5'-CTTCTGATCCATTTCCAAAGGCATG-3', 4mis mo-Xpk; 5'-CTTCAGATCGAT TTCCAAAAGCTTG-3', mo-Zpk; 5'-GCCCACCGTG-ATTCTCCAGCTCCAT-3', mo-ZpkB; 5'-TCAGTCAGAAGCGTCTGCGAGCAGT-3', 4mis mo-Zpk; 5'-GCCCGCCATGATTCTCCAACTTCAT-3'. The sequence complementary to the predicted start codon is underlined in mo-Xpk and mo-Zpk. mo-ZpkB is also specific to Zpk but does not contain the predicted start codon. The modified nucleotides are underlined in 4mis mo-Xpk and 4mis mo-Zpk.

Expression constructs

Wild-type Xpk and deletion constructs of Xpk span the following amino acids: wild-type Xpk (1-835); □PET (1-16,117-835); □LIM (1-125, 313-835); □P/L (1-16, 313-835); P/L (17-310). All constructs were fused with a FLAG sequence at their N-terminus and were inserted into expression vector pCS2+. Sense capped mRNAs were synthesized using mMESSAGE mMACHINE (Ambion) after template linearization. The full length XPIK cDNA was generated from mRNA

derived from *Xenopus* embryos using RT-PCR and 5'RACE method with XPIK specific primers. flagments encoding amino acids either 1-896 (xPIK-full), 1-215 (XPIK-catal) , and 460-896 (XPIK-C) were generated by PCR with EcoRI site. Catalitically defective xPIK was created by changing asparate 165, in the conserved DFG motif, to alanine (D165A) with PCR. The cDNAs encoding these XPIK proteins were cloned into pCS2 with a single hemmagglutinin (HA) tag at the N terminus.

Xenopus microinjection

Xenopus eggs were fertilized *in vitro*, and then capped mRNAs or a morpholino oligo was microinjected into the animal pole or marginal zone of 2- or 4-cell embryos. After injection, the embryos were cultured in 3% Ficoll/0.1x Steinberg's solution until the appropriate stage for each experiment.

Elongation assay in Xenopus animal cap explants

Capped mRNAs or a morpholino oligo were co-injected with 0.5 pg activin mRNA into the animal pole of 2-cell embryos. The animal cap was dissected manually from stage 8 embryos and cultured in 0.1% BSA/1x Steinberg's solution until sibling embryos reached stage 17 (Thomsen et al., 1990).

Phenotype scoring for structure-function analysis

The affected embryos were classified into 3 grades from 1 to 3, according to the severity of the phenotype, and the scored embryos were counted. These experiments were titrated in several dose, but the data depicting the most significant effect were shown in Figure 2b, B.

RT-PCR

RT-PCR was carried out as reported. The expression of each molecular marker was detected by PCR using the following specific primers: *Xwnt11*, forward 5'-AAGT-GCCACGGAGTGTCTGG-3'; reverse 5'-CTCAGACTCTCTC ACTGGCC-3'; *Xpk*, forward 5'-TTGGGTGTCAGCGAAGTTCC-3'; reverse 5'-TTCTCTTCAGGCAGGC-AAGC-3'. The primer sequences for *Xbra* and *histone*

H4, an internal input control, were as previously described (Yamamoto et al., 2000).

Immunocytochemistry and confocal microscopy

Each epitope-fused mRNA was injected into the animal pole of two-cell embryos. The animal cap was dissected from stage 8-10 embryos and fixed with MEMFA, followed by immunostaining by a standard method using a fluorescence-labeled secondary antibody. The localizations of FLAG-tagged Xpk, the Xpk deletion constructs, and Myc-tagged Xdsh were determined by laser-scanning confocal microscopy, using a Carl Zeiss LSM510 microscope.

Immunoprecipitation and GST pull-down analysis

293T cells were transiently transfected with the indicated constructs by the calcium phosphate method. 24-48 hours after transfection, the cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 0.1% triton-X 100, 50 mM Sodium Fluoride, 40mM beta-glycerophosphate, 200uM Sodium orthoranadate]. Immunoprecipitation and the GST pull-down analysis were performed by incubating the extracts with the indicated antibody coupled to protein A Sepharose CL 4B and with glutathione Sepharose 4B (Pharmacia), respectively, at 4°C for 1-2 hour. The precipitates were then washed with lysis buffer and subjected to western blot analysis using indicated antibody. Using antibody is as follows; M2 Flag monoclonal antibody (Sigma), anti-HA antibody (Santa Cruz Biotechnology), anti-Myc antibody (Santa Cruz Biotechnology).

Yeas two-hybrid screening

Yeast transformation and selection in S. cerevisiae AH109 was perfomed according to manufacture's instructions (Clontech). A Xenipus laevis oocyte cDNA library constructed with the pACT2 plasmid (comprising 3.5 x 10^6 independent clones) was used to screen for proteins interacting with xPK in the AH109 yeast strain. The clones growning on SD/-His/-Ade medium were recovered. Among these, a cDNA insert of xPIK (corresponding to amino acids 460-896) was identified. To examined the interactions between

xPK and xPIK, the flagment of xPIK was cloned into pGAD10. DNA flagment eccording xPK PET/LIM domain was fused to the DNA-binding domain of the Gal4 protein. The transformants were tested on SD/-His/-Ade plates.

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