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A Novel Kinesin-like Protein, Surhe is Associated with Dorsalization in the Zebrafish Embryos

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Abstract: We are reporting the expression patterns and possible biological functions of a novel Kinesin-like protein, Surhe, in the zebrafish. Homology studies of derived amino acid sequences suggest that Surhe has an amino-terminal kinesin motor domain that is similar to that of the emerging MKLP-1 subfamily [Kim and Endow, 2000] and two coiled-coil domains in a central region. Cellular localization studies in mammalian cells revealed that Surhe protein is located in cytoplasm, suggesting that Surhe may be involved in the intracellular transport. During the developmental process, *surhe* transcripts are highly expressed in early embryonic stages. Overexpression of the dominant negative form of Surhe significantly down-regulates the dorsalization markers, such as *goosecoid*, *bozozok*, and *chordin*. Taken together, we postulate that Surhe may be involved in dorsalization process as a motor molecule.

Key words: kinesin-like protein, Surhe, dorsal determinant, dorsalization

Molecular motor proteins, such as kinesin, myosin, and dynein, generate the movement of a wide variety of materials in cells. Such movements are essential for many different cellular and developmental functions, including organelle movement, localization of developmental determinants, mitosis, meiosis and possibly long range signaling in neurons [Goldstein, 2001a; Guzik and Goldstein, 2004; Hamada, 2007]. The kinesin superfamily comprises a large and structurally diverse group of microtubule-based motor proteins that produce a variety of force-generating activities within cells [Vale and Fletterick, 1997]. Kinesin-like proteins (KLPs), also known as kinesin family proteins (KIFs), share a conserved motor domain of over 340 amino acids, and similarities between this domain have been used to construct molecular phylogenies as grouping the known members of the kinesin superfamily into a number of subfamilies [Moore and Endow, 1996; Kirokawa, 1998]. The members of each subfamily share a common domain organization [Vale and Fletterick, 1997]. Motor domain contains the sequence necessary for ATP hydrolysis and microtubule binding [Kull et al., 1996], driving the movements of membrane-bound organelles and vesicles toward the plus ends of microtubules. Stalk domain consisting of α-helical coiled coil motifs is responsible for mediating the homodimerization of kinesin heavy chains (KHCs) [Diefenback et al., 1998]. Tail domain, the sequences outside of the conserved motor domain, shows few similarities and happens to interact with cargo molecules directly or through adaptor proteins to be responsible for the different cellular roles of the KLPs [Setou et el., 2000; Nakagawa et al., 2000; Karcher etal., 2002, Verhey and Rapoport, 2001].

These motor proteins can be used to position signaling complexes in cells and to send signals over long distances. This is exemplified by signal initiation or transmission during development, which in some cases requires localization of essential signaling molecules to particular cellular or embryonic regions [Goldstein, 2001a]. In the case of kinesin I, it is required for proper posterior localization of *oskar* mNRA and an associated protein, staufen protein [Brenza et al., 2000], and dynein might be involved in the mechanism of apical localization of *wingless* and *pair-rule* transcripts in the *Drosophila* blastoderm embryo [Wilkie and Davis, 2001]. In the events of embryonic body

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planning, KIF3A protein, the subunit of the kinesin II motor complex, plays an important role in the earliest cellular determinative events establishing left-right asymmetry in mammalian development [Takeda et al., 1999; Marszalek et al., 1999; Goldstein, 2001b].

How the basic body plan of vertebrates is built up is not yet fully understood. In invertebrates such as *Drosophila* and *C. elegans*, their maternally induced processes lay down the information determining the embryonic axes already in the oocyte [Bowerman, 1998; Ray and Schupbach, 1996]. The same case in vertebrates is understood best in *Xenopus laevis*, where dorsal specification is maternally controlled [Wylie et al., 1996] and the cytoplasmic determinants which specify the dorsal fate are present in the eggs just after fertilization [Heasman, 1997]. The yolk, an extraembryonic structure, is essential for the induction of both dorsal and marginal cell fates [Alexander, 2001]. The Embryos that are removed the vegetal yolk mass during the 1-cell stage present strong ventralized phenotypes as missing of axial structures [Mizuno et al., 1999; Ober and Schulte-Merker, 1999]. These results suggest that dorsal determinants are located in the vegetal pole of yolk cell after fertilization and then transported to the future dorsal side of the embryo. The results of embryological manipulation data using cold treatment, UV-irradiation, or treatment of nocodazole which causes a depolymerization of microtubules show cortical microtubule arrays are required for the transport of determinants from the vegetal pole into the future dorsal side of the embryo and proper translocation of â-catenin into nuclei [Jesuthasan and Strahle, 1996]. The molecular nature of the dorsal determinant is still unknown, but the stabilization and nuclear translocation of the â-catenin, a component of the Wnt signaling pathway that contains dorsalizing activity, in both *Xenopus* and zebrafish embryos at the early blastula stage seems to be involved in dorsal determinants [Alexander, 2001; de Robertis et al., 2000; Schneider et al., 1996; Larabell et al., 1997; Fuentealba et al., 2007]. The more detail studies in *Xenopus* demonstrated that β-catenin colocalizes with subcortical microtubules at the dorsal side of the egg and these microtubules which extend from the sperm entry point to the dorsal side of the embryo mediate the transport of key molecules toward their dorsal blastomeres during cleavage stages [Rowning et al., 1997; Weaver et al., 2003].

In this paper, we report the identification of a novel Kinesin-like protein, termed Surhe, in the zebrafish. Homology studies of derived amino acid sequences suggest that Surhe has an amino-terminal kinesin motor domain that is similar to that of the emerging MKLP-1 subfamily [Kim and Endow, 2000] and two coiled-coil domains in a central region. Cellular localization studies in mammalian cells revealed that Surhe protein is located in cytoplasm, suggesting that this motor protein may be involved in the intracellular transport. During the developmental process, *surhe* transcripts are highly expressed in early embryonic stages. Finally, we evaluate Surhe as a candidate of dorsal determinants carrier in early cleavage stages based upon the alteration in both phenotypes and expression pattern of several dorsal markers by overexpression of the dominant-negative *surhe* mRNA.

MATERIALS AND METHODS

Northern blotting and RT-PCR

For northern blot analysis, 30 μg total RNA from four different embryo stages was separated in formaldehyde agarose gels, transferred overnight to Hybond-N membranes (Amersham) in 10SSC and then cross-linked by UV irradiation. The membrane was then pre-hybridized for 1 h in ExpressHyb solution (Clontech) and then hybridized for 4 h with random primed, ^{32}P-labelled probes specific for the C-terminal coding region of Surhe. After washing (0.2SSC, 0.3% SDS) at 70°C for 30 min, the membrane was autoradiographed for 16 to 48 h at −70°C using intensifying screens. In the case of RT-PCR, total RNA was isolated from nine different embryo stages using RNAzol B (TEL-TEST, Inc.), and 3 μg total RNA was used for RT-PCR. Using *surhe*-specific primers, a 400 bp fragment was obtained by RT-PCR amplification (pre-denature 94°C, 120 sec, denaturation 94°C, 30 sec, annealing 55°C, 30 sec, elongation 72°C, 30 sec, post-elongation 72°C, 120 sec, 30 cycles). For the loading control, zebrafish β-*actin*-specific primers were used under the same condition. Two sets of primers were used to amplify *surhe*- and β-*actin*-specific products: *Surhe*, 5'-GATCTCTAGATGATTACCTATCGG AGG-3' (forward) and 5'-GATCGGATCCAGAACACCG GCATTTCTG-3' (reverse); β-*actin*, 5'-GAGGAGCACCC CGTCCTGC-3' (forward) and 5'-GATGGCTGGAACAGG GCC-3' (reverse).

5'-RACE

The 5'-RACE system Version 2.0 (Gibco BRL) was used according to the manufacturer's instructions. Total RNA was extracted from the cleavage stage and two different oligonucleotides were used for this assay, GSP-1 (TTCTA CGCTCTGCCAAGAGC) and GSP-2 (CACCAACTCCT CTTGGGGTAATATG).

Protein homology search with phylogenetic tree

The overall homology was searched using the BLAST program. The multiple alignment of amino acid sequences of N-terminal motor domain of Surhe and other MKLP-1 members were made by the CLUSTALW program. Based on the alignment, a phylogenetic tree using the maximum likelihood method was obtained.

Expression plasmid constructs for mammalian cells

To perform GST pull-down, a PCR fragment corresponding to the full (1-810), Nt plus coiled coil domain 1 (1-691), and Ct (493-810) of Surhe was generated by PCR using the PFU polymerase, and cloned into XbaI-BamHI of pEBG and EcoRI-XbaI of pFlag to generate GST-Surhe-full (1-810), FLAG-Surhe-full (1-810), FLAG-Surhe-ΔCt (1-691), and FLAG-Surhe-ΔNt (493-810). Plasmid encoding His-tagged Surhe-full was cloned into pcDNA3 (Invitrogen) with adding 6 X His into its forward primer.

Cell lines and transfection

Human epithelial kidney BOSC 23 cells and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone). FuGene 6 (Roche) was used for transfections according to the manufacturer's instructions. Briefly, BOSC 23 cells were seeded in 100-mm dishes at a density of 15×10^5 cells/dish, and after culture for 24 hours, cells were transfected with the indicated constructs. NIH 3T3 confluent cells were split 2×10^5 cells onto coverslips placed in 6-well plates, at least 24 hr before transfection. After transfection using FuGene 6, cells were grown for an additional 4-30 hr before observation.

GST pull-down assay

GST-Surhe fusion protein in pEBG vector was produced in BOSC 23 cells, and affinity-purified by glutathione-Sepharose 4B (Pharmacia Biotech). GST-Surhe bound to glutathione agarose beads was incubated with various flag-tagged proteins in 150 μL lysis buffer for 2 hours at 4°C. Unbound proteins were saved, and the beads washed three times with lysis buffer, separated by SDS-PAGE (10%), and transfected onto PVDF membrane. Various Flag epitope-tagged Surhe was detected with anti-Flag antibodies and ECL solution.

Immunofluorescence and DAPI staining

For immunofluorescence, fully confluent NIH3T3 cells were split 1:2 onto coverslips and allowed to grown 24 hours before fixation. Cells were washed in 70% PBS, fixed with 4% paraformaldehyde at RT for 100 min. Antibodies were diluted in PBS with 5% bovine serum albumin (BSA). Primary antibodies were applied for overnight at 4°C. The coverslips were washed three times with blocking solution (3% BSA) for 5 min. After secondary antibodies were then applied for 1 hour at RT, DAPI were treated for 5 min at RT. The converslips were washed 5 times with PBS for 15 min and mounted in VECTASHIELD medium (Vector Lab. Inc.). Conforcal images were acquired using a confocal laser microscope from Carl Zeiss.

Whole-mount in situ hybridization

The C-terminal *surhe* was PCR amplified then subcloned into the multiple cloning site of pcDNA3 (Invitrogen) vector. Antisense digoxigenin-labeled riboprobes were generated from the linearized pcDNA3-*surh*, according to the instructions provided from the DIG labeling kit (Roche). Antisense *goosecoid* (*gsc*) and *chordin* (*chd*) riboprobes were constructed using T7 RNA polymerase, *bozozok* (*boz*) and *no tail* (*ntl*) by SP6 RNA polymerase. To prevent pigmentation, embryoswere raised in 1-phenyl-2-thiourea solution at starting to somitogenesis before harvested. In situ hybridiation analysis followed the protocol of Westerfield (1995) with minor modification. Proteinase K treatment (10 μg/mL) was performed for 3 to 20 min depending upon the stages of embryos. The hybridized probes were detected using preabsorbed anti-digoxigenin-AP Fab fragments (Roche) diluted (1:2000) in blocking solution (PBS, 0.1% Tween-20, 5% sheep serum). After 4-10 staining, embryos were mounted in a 2:1 mixture of benzylbenzoate:benzylalcohol, then examined by microscopy.

Microinjection of synthetic mRNAs

Plasmids for the microinjection were using the pcDNA3/β-globin 3' UTR and generated by subcloning Surhe lacking motor domain (Surhe-Nt) and lacZ NLS into the pcDNA3/β-globin 3' UTR. mRNAs for injection were synthesized from the plasmid constructs linearized with the appropriate restriction enzymes, using the mMESSAGE mMACHINE T7 kit (Ambion Inc.) according to the manufacturer's instruction. After purification as following the recommendation, mRNAs were dissolved in diethypyrocarbonate (DEPC)-treated 0.1 M KCl. Before injection, the mRNAs were diluted to various concentrations and 1 μL of mRNA was used to inject about 400 embryos. The mRNA was pressure-injected into the yolk of 1-2 cell stage of embryos and injected embryos were raised in 1/3 Ringer's solution. RNA injection was performed over three times and then the data were pooled.

RESULTS

Protein sequence and structure organization of zebrafish Surhe

We previously screened a zebrafish cDNA library (Clontech, Cat # QL 4000AB) with yeast two-hybrid system using *siaz* (Ro et al., 2004) as a bait and obtained a partial cDNA, 1 Kb, of kinesin-like protein. To obtain full-length of the clone, we performed Northern blot analysis and 5' RACE. After performing a Northern blot using total RNA isolated from zebrafish embryos at different stages of development, we determined the full length size and temporal expression pattern of the cDNA (Fig. 1A). DNA sequence analysis of

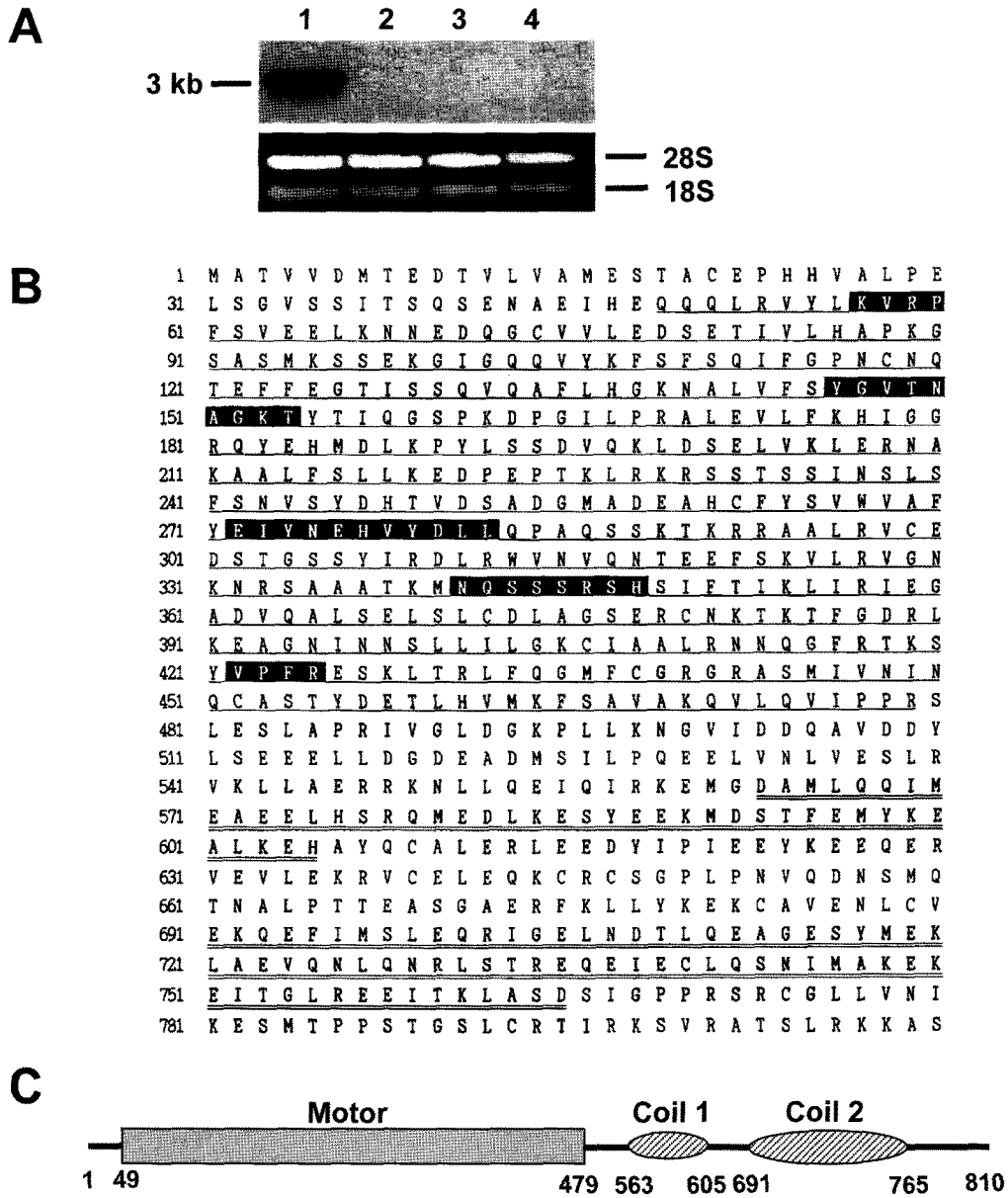

Fig. 1. Molecular cloning of zebrafish Surhe. (A) Northern blot hybridization analysis. Total RNA was extracted from zebrafish embryos at different developmental stages; lane 1, 16 cells; lane2, 80% epiboly; lane 3, 20 somite; lane 4, primordia -11. The blot was probed with ^{32}P-labeled partial cDNA and the 3 Kb transcript was detected at the 16 cells stage. (B) Putative amino acid sequence of zebrafish Surhe. Motor domain (single underline) of Surhe contains the the consensus sequences for microtubule binding and ATPase activity (black boxes) Double underlined regions indicate two coiled-coil domain. (C) Schematic illustration of the domain structure of Surhe. Surhe contains a motor domain located in the N-terminal, two -helical coiled-coil structures stalk domain in the middle of the protein and a putative tail domain in the C-terminal region.

the 5' RACE product showed a putative full-length clone with a size of 2.8 Kb encoding a novel protein of 810 amino acids, which we have termed Surhe (Accession number; AY307110). Surhe mRNA were abundant at the early cleavage stage but not detected at 80% epiboly and thereafter, indicating that Surhe expression is tightly regulated in a developmental stage specific manner. The predicted protein showed that Surhe is a kinesin-like protein (KLP) with an amino-terminal kinesin motor domain, a central stalk domain and a carboxyl-terminal tail domain, which is the most common feature shared among the N-terminal type KLPs (Fig. 1B, C) [Miki et al., 2001]. Sequence alignment of the Surhe motor domain sequence with other KLPs sequence indicated that Surhe is closely related to human KRMP1 [Kamimoto et al., 2001] and human or mouse Rabkinesin-6 (RB6K) [Echard et al., 1998; Lai et al., 2000]. These data suggest that Surhe be able to be classified as a member of MKLP-1 subfamily (Fig. 2A, B) [28]. The consensus sequences for a motor domain and ATP catalytic domain, P-loop (SGKT) and

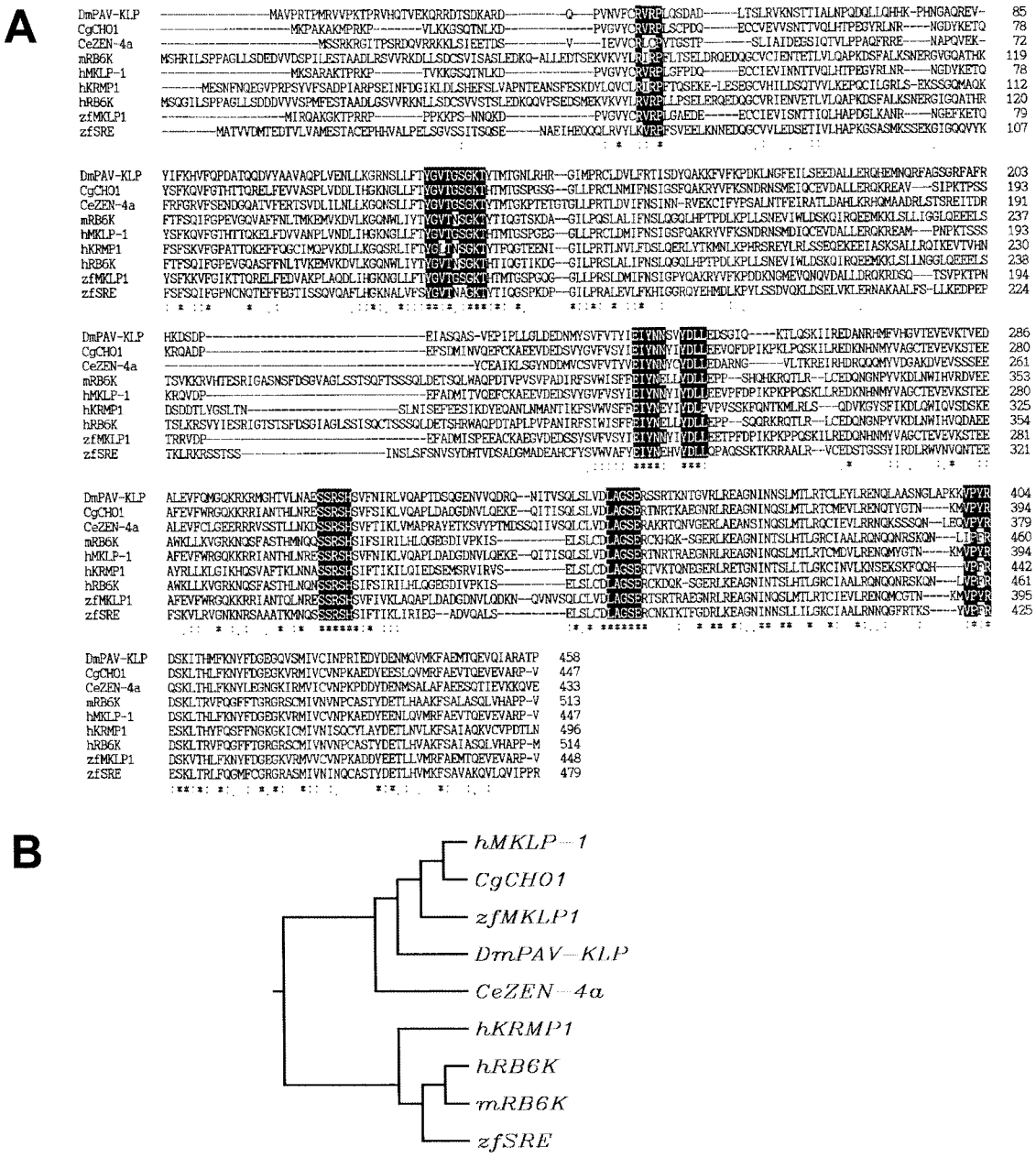

Fig. 2. Amino-terminal domain of Surhe is a kinesin-like protein. (A) Sequence comparison of the motor domain of Surhe with other members of the MKLP1 subfamily. The alignment was done using ClustalW algorithm; gaps were inserted as denoted by dashes and black boxes indicate a conserved domains in kinesin and kinesin-like proteins. The P-loop sequence, GSGKT, is dotted line and two oligopeptides common to most KLPs, SSRSHS and DLAGSE, are indicated by solid lines. (B) Phylogenetic tree representing the relation between members of the MKLP-1 subfamily. The GenBank™ accession numbers are as follows: human MKLP-1, X67155; Chinese hamster CHO1, X83575; zebrafish MKLP-1, AF139990; *D. melanogaster* PAV-KLP, AJ224882; *C. elegans* ZEN-4a, AF057567; human RB6K, AF153329; mouse RB6K, Y09632; human KRMP1, AB033337.

nucleotide-binding motif (SSRSHS, LAGSE), are located in the N-terminus (Fig. 1B, 2A) [Hirokawa, 1998; Kull et al., 1996]. Taken together, Surhe belongs to the family of kinesin-like proteins, specifically to MKLP-1 subfamily, as evidenced by the presence of the kinesin feature in the amino-terminal motor domain. In addition, it contains a P-loop sequence characteristic for ATP- or GTP-binding proteins and the consensus sequence of the nucleotide binding motif for kinesins. A phylogenetic tree generated from a CLUSTAL multiple sequence alignment of the motor domains of Surhe and other MKLP-1 subfamily identifies Surhe as a novel kinesin-like protein (Fig. 2B)

Homodimerization and cellular distribution of Surhe in mammalian cells

Like most KLPs, the structural organization of Surhe also contains two α-helical coiled-coil domains (531-605, 691-765) in central stalk region (Fig. 1B, C). Thus, to define

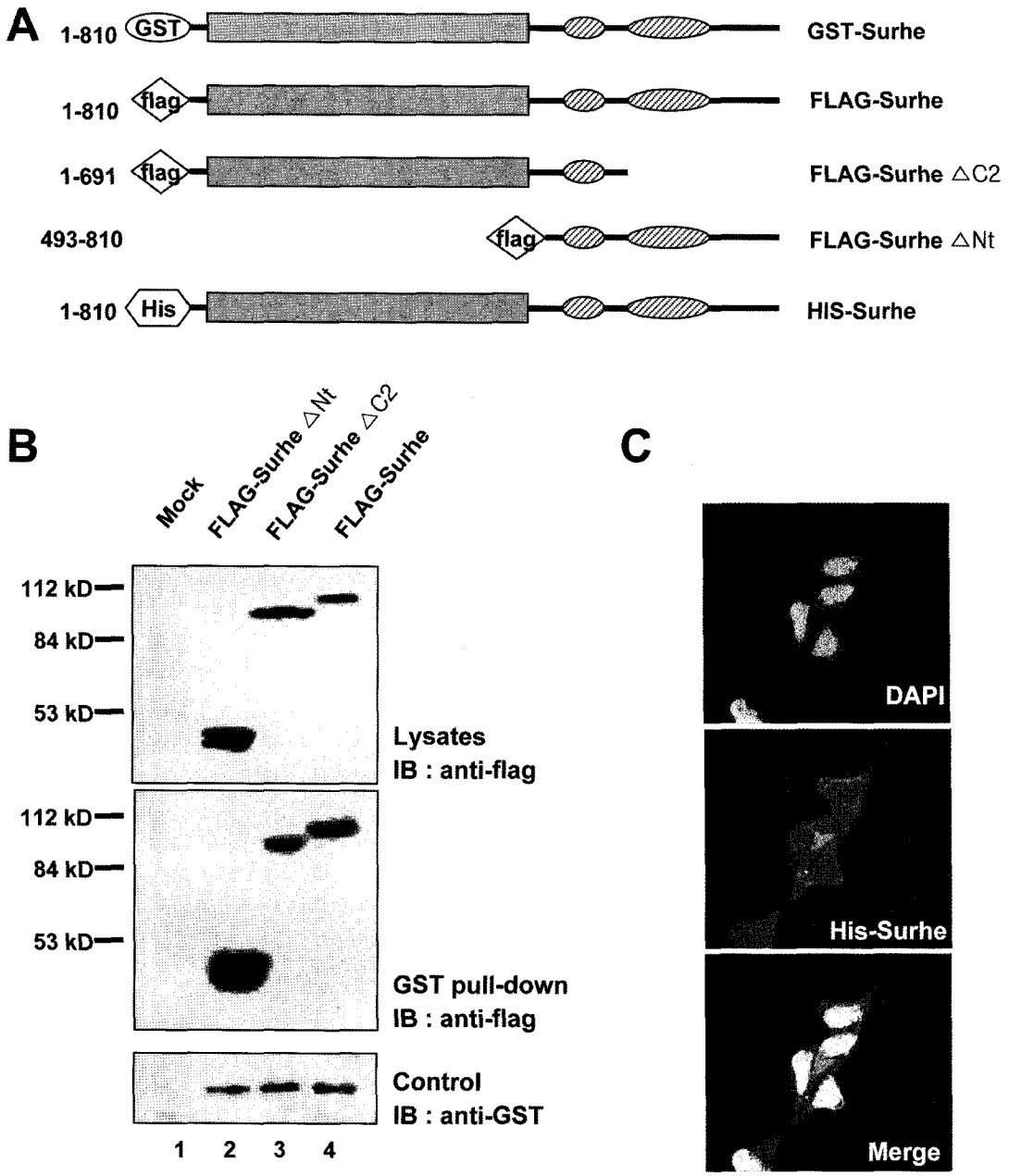

Fig. 3. Homo-dimerization of Surhe via the coiled-coil domain and its localization in mammalian cells. (A) Schematic view of the protein constructs, full-length Surhe, the second coiled coil domain deletion form of Surhe, Surhe containing only two coiled coil domains and the His-tagged construct. (B) Anti-FLAG tag western blots of GST pull-down in vitro binding experiments. GST-tagged full-length Surhe (1-810) was transfected into BOSC 23 cells together with Flag-tagged full-length Surhe (1-810) and two Flag-tagged deletion mutants Surhe (1-691, 493-810). GST binding experiment was examined by immunoblotting using anti-Flag antibody (top and middle panel), showing that GST-tagged full-length Surhe bound to Flag-tagged full-length Surhe and deletion mutants Surhe. The amounts of GST-tagged full-length Surhe in cell lysates were monitored using anti-GST antibody (bottom panel). (C) Immunofluorescence on NIH3T3 cells with anti-His-tagged Surhe (green) and (a) DAPI staining for the nucleus.

whether it forms a coiled-coil homodimer through its stalk, we constructed the expression plasmids of a full-length and a series of deletion mutants Surhe to perform GST pull-down *in vitro* interaction (Fig. 3A). GST-tagged full-length (GST-Surhe) and FLAG-tagged full-length Surhe (FLAG-Surhe) were co-expressed in BOSC 23 mammalian cells. As shown in Fig. 3B, GST-Surhe but not GST alone could specifically interact with FLAG-Surhe. Further analysis with FLAG-fusion proteins containing different proteins of Surhe (FLAG-Surhe ΔNt, FLAG-Surhe ΔC2) showed that the homodimerization of Surhe proteins was achieved via their coiled-coil domains (Fig. 3B). Considering that FLAG-Surhe ΔC2 is still able to interact with GST-Surhe, we concluded that only one of the coiled-coil domains is

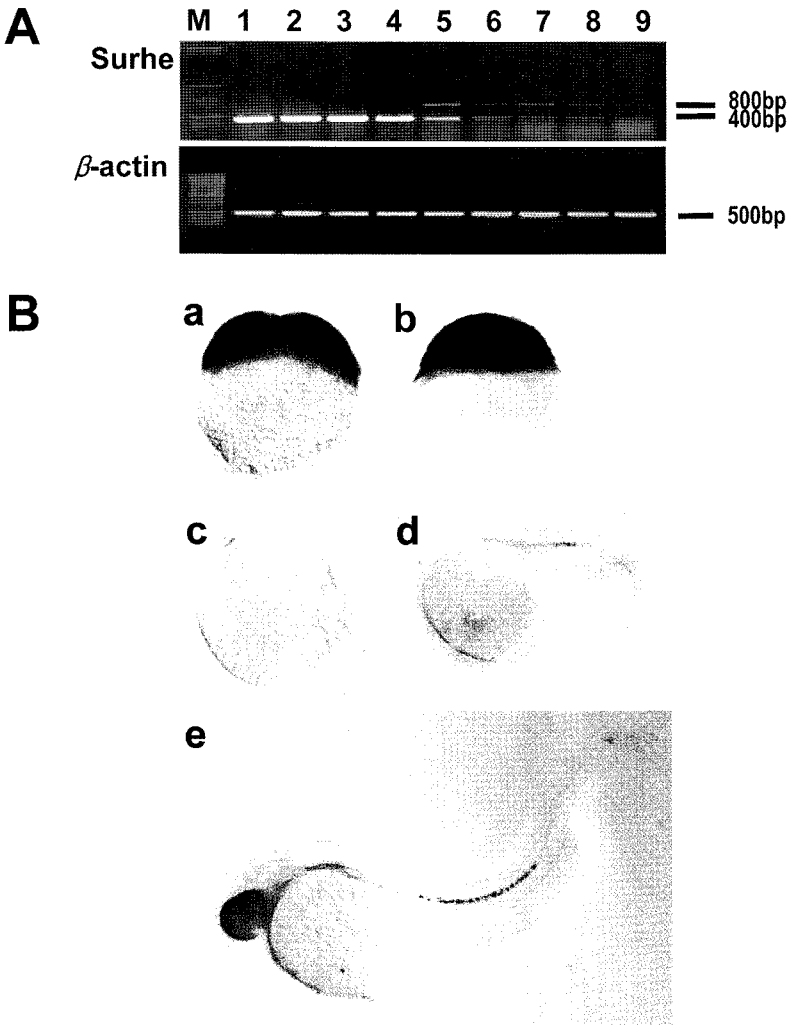

Fig. 4. Expression pattern of endogenous *Surhe*. (A) RT-PCR represents the level of C-terminal Surhe transcripts in the total RNAs from zebrafish embryos at nine developmental stages; lane 1, 1 cell; 2, 32 cells; 3, high; 4, 50% epiboly; 5, 80% epiboly; 6, 5 somites; 7, 20 somites; 8, primordia 5; 9, primordia 11. The mRNAs are highly detected in early stages, but gradually reduced after cleavage stage in 400 bp size. Alternative splicing form of *Surhe* transcripts was obtained in 800 bp size from 80% epiboly stage. Products of control PCR were amplified with primers specific for transcripts of -actin. (B) Spatiotemporal distribution of *Surhe* transcripts during the embryonic development was detected by whole-mount *in situ* hybridization. Embryos from indicated various embryonic stages were stained with a digoxigenine-labeled C-terminal *Surhe* anti-sense probe. *Surhe* transcripts are ubiquitously detected in the entire blastomeres until mid-blastula stage.

sufficient to form a homodimer between Surhe proteins, As the cellular distribution of a protein can provide crucial insights to its cellular functions, and its determination is an important part of characterizing a gene of unknown function, we determined whether Surhe is located in nucleus or cytoplasm. To examine the cellular localization of Surhe, we engineered the expression of a 6HIS-Surhe fusion protein in NIH3T3 cells (Fig. 3A). Immunofluorescence analysis with the anti-HIS monoclonal antibody revealed that Surhe is localized in the cytoplasm when co-stained with DAPI (Fig. 3C). Because we postulated Surhe to be a microtuble-based motor protein, we carried out a double immunostaining, utilizing the monoclonal antibody against α-tubulin to determine that Surhe should associate with microtubular cytoskeleton. Merged double immunostaining proposed that Surhe could possibly have a relation with microtubules in cytoplasm (data not shown). Taken together these results indicated that Surhe, forming a homodimerization, is localized in cytoplasm as a microtubule-based motor protein and remained the possibility that it might be involved in a series of transport of membranous vesicles in cytoplasm.

Spatio-temporal expression pattern of Surhe in zebrafish embryos

To prove the biological role of Surhe, Northern analysis and RT-PCR was used to examine its expression patterns at the various zebrafish embryonic stages (Fig. 1A, 4A). Because of the sequence diversity at the tail region of Surhe among KLPs, we selected C-terminus as a Northern probe. The

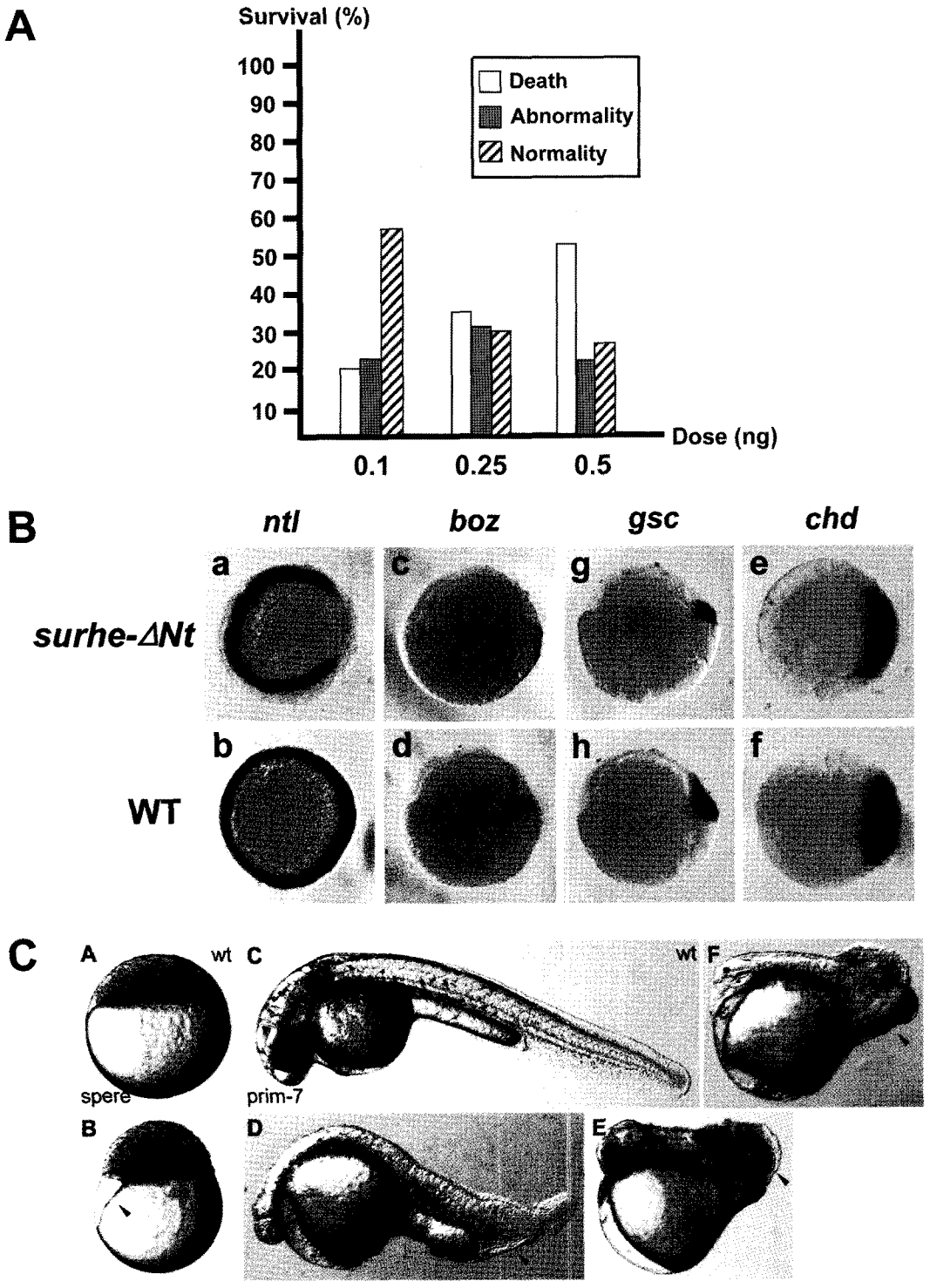

Fig. 5. Dose dependent effect and Expression of dorsal markers of dominant-negative *surhe* mRNAs injection. (A) Results of dose dependent dominant-negative injection. Zebrafish embryos at the one- or two-cell stage were injected with dominant-negative *Surhe* (ΔNt1-492) at doses of 0.1, 0.25, 0.5 ng. Histogram shows the death rate of injected embryos is increased at the high dose. (B) Analysis of the expression patterns of *no tail (ntl), bozozok (boz), goosecoid (gsc),* and *chordin (chd)* in dominant-negative *Surhe* injected embryos. (a-h) Embryos shown are at shield stage. (b) *ntl* expression in an uninjected embryo. (a) dominant-negative *Surhe* injected embryo has no *ntl* in dorsal region. (d) An uninjected embryos, with *boz* expression in the dorsal YSL. (c) Expression of *boz* is completely eliminated. (f) Expression of *gsc* in an uninjected embryo. (e) Expression of *gsc* is slightly reduced. (h) *chd* expression in an uninjected embryo. (g) *chd* expression is indistinguishable in injected embryo. Animal pole views are shown, except for c, d, e and f, which are lateral views.

transcripts of approximately 3 kb were observed and it was expressed in early cleavage stages (Fig. 1A). These data were also supported by RT-PCR performed in more fractionized stages (Fig. 4A). Even if the RT-PCR products of *surhe* (designed for amplifying 400 bp in size) were detected in all embryonic stages, its expression pattern appeared to be different for each stage. High expression of *surhe* was shown in early cleavages, but its transcripts were gradually reduced in later stages. Interestingly, the new transcripts of 800 bp was slightly presenting from 80% epiboly which starts to occur the differentiation of a neuronal system. Sequence analysis of the newly detected transcripts demonstrated that the new three portions of cDNA inserted into the transcripts of 400 bp, suggesting that more than one type of alternative splicing variants of Surhe exist after early cleavages (data not shown). Moreover in the 80% epiboly stage, the relative levels of the 400 bp and 800 bp RT-PCR products were nearly equivalent, suggesting that about half of the transcripts were alternatively spliced. Therefore, it is proposed to generate different carboxyl terminus ends for additional cargo binding sites [Khodjakov et al., 1998; Verhey et al., 2001]. To assess both the temporal and spatial distribution of *surhe* mRNAs during the zebrafish embryogenesis, digoxygenin-labelled antisense RNAs were generated from subclones and mRNA expression were analyzed by *in situ* hybridization on whole mount embryos. Surhe transcripts appeared to be distributed uniformly in all blastomeres of embryos from cleavage through early blastula (Fig. 4B), as predicted from the Northern and RT-PCR data.

Dose-dependent effect and altered expression of the dorsal makers in the dominant-negative *surhe* mRNAs injected embryos

To investigate function of the Surhe in embryogenesis, we employed a dominant-negative strategy by overexpressing the Surhe lacking motor domain. We initially attempted injection of synthesized *surhe* mRNAs with the complete ORF into 2-cell stage embryos, but injected RNAs did not cause any significant phenotypic changes even at the highest dosage (data not shown). Therefore, we explored the loss-of-function analyses for Surhe by using dominant-negative construct, missing motor domain, to interfere with endogenous wild type Surhe. This idea was supported by the previous GST pull-down data that the full-length Surhe (GST-Surhe) could be associated with the motor domain deletion form of Surhe (FLAG-Surhe-ΔNt) (Fig. 3B). Injection with the various amounts (100-500 pg) of Surhe-ΔNt demonstrated that the embryos injected with the dominant-negative Surhe mRNAs showed embryonic defects in dosage dependent manner (Fig. 5A). Most of the injected embryos showed an enlarged yolk syncytial layer (YSL) at 4 hpf and severe defect in trunk and tail, and abnormal axis formation at 24 h and thereafter (data not shown). As it was reported that the formation of an enlarged YSL or abnormal axis results from the disruption of microtubules [Jesuthasan and Strahle, 1996], we postulated that the observed phenotypic defects might be due to malfunctional Surhe rather than the depolymerizing microtubules, and consequently presented by the failure of dorsal organizer formation. In Xenopus, formation of the organizer depends on maternally encoded β-catenin required for embryonic axis formation [Sokol, 1999]. β-catenin protein starts to accumulate in the dorsal cytoplasm as early as the 2-cell stage and in dorsal nuclei by the 16-cell stage [Larabell et al., 1997]. By the midblastula stage, nuclei stain positively for β-catenin in the entire dorsal side, including regions that later give rise to endoderm, mesoderm and ectoderm [Schneider et al., 1996]. Moreover, it was reported that zebrafish blastomeres appear to require substances which are transported from vegetal pole by microtubule in order to form an organizer [Jesuthasan and Strahle, 1996] and there were abnormal axis formation and enlarged YSL in *surhe-ΔNt* injected embryos. Therefore, to examine whether Surhe is involved in activation of the several genes related with or located in downstream from β-catenin needed for axis establishment, we performed *in situ* hybridization with various dorsal markers. In the *surhe-ΔNt* injected embryos at shield stage, *ntl*, a pan-mesodermal marker, is normally expressed in ventrolateral marginal cells (Fig. 5B-a), but not detected in dorsal region (Fig. 5B-b). A direct downstream molecule activated by β-catenin, *boz* is normally expressed in dorsal YSL of wild type embryo at this stage (Fig. 5B-c), but was completely eliminated in the *surhe-ΔNt* injected embryos (Fig. 5B-d). Another dorsal mesodermal marker, *gsc*, was slightly decreased upon Surhe-ΔNt overexpression (Fig. 5-e). But *chd* expression in Surhe-ΔNt embryos is indistinguishable from wild-type embryos (Fig. 5B-g). This is consistent with the previous report that expression level of *chd* in *boz* mutants is comparable to wild type embryos [Sirokin et al., 2000].

DISCUSSION

Kinesin is the most abundant motor in many cell types and is responsible for the movement of many different cargoes [Goldstein, 2001; Vale and Fletterick, 1997; Moore and Endow, 1996; Hirokawa, 1998]. We cloned a cDNA encoding a new kinesin-like motor from one-month old zebrafish cDNAs and designated the cDNA as *surhe*. Structural analysis demonstrated that Surhe contains a predicted domain organization composed of conserved motifs including nucleotide-binding consensus motif within motor domain and á-helical coiled-coil domains in a central stalk domain, which is identical to that of other KLPs (Fig. 1C). The

primary sequence of Surhe motor domain was classified into the MKLP-1 subfamily based on the sequence similarity within the motor domain (Fig. 2A), whereas the C-terminus lacks any discernible homology. We also found that the central domain predicted to form coiled-coil structure is capable of interacting with each other, even if it contains the only one coiled-coil domain (Fig. 3B). Moreover, the result that C-terminus of Surhe excluding N-terminal motor domain is able to associate with full-length Surhe protein brings about the dominant-negative *surhe* construct for microinjection. *surhe* transcripts appeared in early embryonic stages and contained alternative splicing variants. Although we do not know yet the specific cargo of Surhe, it does not exclude that there are motor protein interacting with various cargoes (Fig. 4A). In particular, additional cargo binding sites could be regenerated in the variable C-termini for motor proteins, which arise from alternative splicing (Verhey et al., 2001). Moreover, Whole mount *in situ* hybridization, using C-terminal anti-sense probes of *surhe*, revealed that *surhe* transcripts were presented in entire blastomeres of cleavage stage embryo. Zebrafish Mklp1, known as another motor protein in zebrafish, contains potential nuclear localization signals (NLS) in both N- and C-terminal regions and therefore is involved in embryonic cytokinesis (Schneider et al., 1996). In the case of Surhe, we confirmed that it does not contain any nuclear targeting signals through the primary sequence analysis and is localized in cytoplasm, associated with microtubule-based on cytoskeleton. These data suggest that Surhe may participate in the movement of membrane-bound organelles and vesicles toward the plus ends of microtubules, rather than cell division in cytoplasm.

We have shown that injection of dominant-negative *surhe* mRNA resulted in various embryonic defects in a dose-dependent manner, while wild type surhe overexpression did not. As dominant-negative proteins must bind to the same proteins as wild type, we confirmed that SurheΔNt interferes with the function of the endogenous Surhe to give rise to various phenotypic defects. This result is presumed that endogenous Surhe might be essential molecule, but there is no necessity for normal embryogenesis to be sufficient. However, overexpression of wild type Surhe together with SurheΔNt did not rescue the phenotypic defects induced by SurheΔNt overexpression (data not shown). This might be caused by non-specific interaction of the SurheΔNt with other molecules essential for embryonic development, because coiled-coil motifs could be a kind of conserved domain in cellular properties. The microtubules in the yolk cell of zebrafish embryo during early development was reported to act in axis specification by transporting molecules required for the formation of an axis, from the vegetal hemisphere into marginal blastomeres. Therefore, early blastomeres of the zebrafish embryo including the disrupted microtubules do not contain all the molecules necessary for the formation of an axis, and develop into the state with the premature formation of an enlarged YSL [Jesuthasan and Strahle, 1996]. The enlarged YSL formed in the embryo injected with *surheΔNt* is similar to the phenotypic defects of the embryo where microtubules were disrupted. This particular correlation suggests that Surhe, as a microtubule-based motor protein, may participate in a transport system to establish the formation of an axis. Recent progress has been made to show that a kinesin-like protein in fact plays critical roles in embryonic development. Costal2 functions as a kinesin-like protein in the hedgehog signal transduction pathway [Farzan et al., 2008]. The hedgehog signaling pathway initiates an evolutionalarily conserved developmental program required for the proper patterning of many tissues [Varjosalo and Taipale, 2008]. Motility of Costal2 is required for its biological function, and may be regulated by Hedgehog signal [Farzan et al., 2008].

Dorsal determinants, the key molecule of early dorsal axis establishment, are located in membrane vesicles in the vegetal pole of the embryo and transported toward the dorsal side by cortical microtubules [De Robertis et al., 2000]. This event is correlated with the activation of the Wnt signaling pathway that is mediated by â-catenin which is translocated into the nuclei of cells on the dorsal side of both *Xenopus* and zebrafish embryos at the early blastula stage [Larabell et al., 1997; Schneider et al., 1996; Fuentealba et al., 2007]. Altered expression pattern of the dorsal marker genes indicates that Surhe might be involved in the transport of substances like dorsal determinants as a potential carrier along microtubules. It will be of great interest to look for proteins associated with Surhe protein, which may contain activity of dorsal determinant.

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