Left-Right Asymmetry and Kinesin Superfamily Protein KIF3A: New Insights in Determination of Laterality and Mesoderm Induction by kif3A−/− Mice Analysis

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Abstract. KIF3A is a classical member of the kinesin superfamily proteins (KIFs), ubiquitously expressed although predominantly in neural tissues, and which forms a heterotrimeric KIF3 complex with KIF3B or KIF3C and an associated protein, KAP3. To elucidate the function of the kif3A gene in vivo, we made kif3A knockout mice. kif3A−/− embryos displayed severe developmental abnormalities characterized by neural tube degeneration and mesodermal and caudal dysgenesis and died during the midgestational period at ~10.5 dpc (days post coitum), possibly resulting from cardiovascular insufficiency. Whole mount in situ hybridization of Pax6 revealed a normal pattern while staining by sonic hedgehog (shh) and Brachyury (T) exhibited abnormal patterns in the anterior-posterior (A-P) direction at both mesencephalic and thoracic levels. These results suggest that KIF3A might be involved in mesodermal patterning and in turn neurogenesis. Furthermore, homozygotes for kif3A showed randomization of laterality in heart looping. In contrast to wild-type embryos where almost all have a D-loop heart (situs solitas), ~40% of homozygous embryos exhibited cardiac L-loops (situs inversus). Moreover, their pericardial space was filled with effusion resulting from/in circulatory insufficiency. Scanning electron microscopy (SEM) and video-enhanced contrast DIC and fluorescent images (VEC-DIC/FL) microscopy revealed the absence of motile monocilia on mutant nodal pit cells, which could not generate leftward nodal flow of extraembryonic fluid and are considered to be responsible for initial determination of the left-right (L-R) asymmetry. These results collectively suggest the important role of the KIF3A protein in determination of embryonic body planning, particularly for the laterality.

Key words: microtubule • kinesin • kif3A • gene targeting • left-right asymmetry

Intracellular trafficking is one of the fundamental mechanisms for maintaining cellular organization by distributing the molecules properly to the lieu where they are required. In the case of neurons, this transportation system is highly differentiated and is termed axonal transport (Grafstein and Forman, 1980; Hirokawa, 1993) in accordance with their highly polarized structure. To carry out these tasks, three major kinds of cytoskeleton-associated motor proteins are recognized today: kinesin superfamily proteins (KIFs)1 (Aizawa et al., 1992; Hirokawa, 1996, 1998), dynein superfamily proteins (Gibbons et al., 1994; Tanaka et al., 1995; Gibbons, 1996), and unconventional myosins (Fath and Burgess, 1994; Wang et al., 1996). All of these motor proteins exert their force by hydrolysis of ATP, although the track for the former two is microtubules (MTs) while the latter one slides along the microfilaments composed of actin. Among them, KIFs comprise several dozens of molecules that take part in various processes including axonal transport (Kondo et al., 1994; O'kada et al., 1995; Yamasaki et al., 1995), intracellular traffic such as intraciliary-flagellar transport (IFT; Kozminski et al., 1995; Morris and Scholey, 1997; Cole et al., 1998), and cell division (Boleti et al., 1996).

1. Abbreviations used in this paper: AP, alkaline phosphatase; dpc, days post coitum; ES, embryonic stem; IFT, intraciliary-flagellar transport; MTV, microtubule; RT, room temperature; VEC-DIC/FL, simultaneous observation of video-enhanced contrast green fluorescent images.

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The Journal of Cell Biology, Volume 145, Number 4, May 17, 1999 825–836
http://www.jcb.org
KIF3A protein is one of the authentic murine KIFs discovered by our PCR screening (Aizawa et al., 1992) that takes on heterotrimers with KIF3B (Kondo et al., 1994; Y amazaki et al., 1995) or KIF3C (Muresan et al., 1998; Yang and Goldstein, 1998) and an associated molecule KA P3 (Y amazaki et al., 1995, 1996), which are in toto called KIF3 complex. Both KIF3A/B have an ATPase activity at their NH2-terminal head region followed by a coiled-coil stalk region assumed to be responsible for dimerization and a COOH-terminal tail domain. Previous biochemical studies (Kondo et al., 1994) have revealed that KIF3A is expressed ubiquitously but predominantly in brain, testis, and adrenal medulla. Furthermore, it has been biochemically revealed that only KIF3A can exist in a soluble form in the absence of its counterpart (Y amazaki et al., 1995) and can form heterodimers with either KIF3B or KIF3C (Y amazaki et al., 1995; Muresan et al., 1998; Yang and Goldstein, 1998), whereas KIF3B aggregates under physiological conditions in the absence of KIF3A in the baculovirus expression system and does not dimerize with KIF3C. Moreover, developmental expression of the KIF3A protein preceded that of KIF3B. Recently, we have made ki f3B knockout mice (N onaka et al., 1998) and revealed that KIF3B is essential in early developmental stages, especially for left-right (L-R) determination. From another standpoint, KIF3 has diverse orthologues among species, such as sea urchin kinesin II (Cole et al., 1993), Chlamydomonas FLA 10 (Walther et al., 1994), C. elegans O sm3 (Shakir et al., 1993), and a fish photoreceptor KIF3 antigen (Beech et al., 1996). Considering all the aforementioned properties of KIF3A, this molecule proper must therefore have essential and regulatory roles in various animals and is anticipated to play a more severe phenotypic role than that observed in the kif3A gene-targeting mice. To pave the way into the investigation of murine KIF3A in situ, we disrupted the murine kif3A gene by homologous recombination. The resulting kif3A+/− mice displayed severe developmental deficits at the mid-gestation stage and died before 10.5 dpc (days post coitum). The defects were mainly localized to the neural tube, heart, brachial arches, and lower truncal mesoderm, leading to highly underdeveloped posterior structures. Surprisingly, the cardiac looping was randomized, i.e., ~40% of all homzygote embryos exhibited a L-loop pattern (situs inversus). Because it has been predicted that the node plays an important role in L-R determination, we examined the node of kif3A−/− and compared it with that of wild-type embryos. Since Chlamydomonas orthologue FLA10 has been reported to be localized in the motile flagella as a motor for the IFT (Kozminski et al., 1995; Cole et al., 1998; Rosenbaum et al., 1999), we also focused our attention on the monocilia of the embryonic node. In the wild-type embryos, the monocilia of nodal pit cells are motile and generate a leftward flow of extraembryonic fluid (nodal flow). However, formation of cilia on the surface of nodal pit cells was severely affected and no flow was recorded in kif3A−/− homzygous embryos. These data suggest the possibility that KIF3A molecules are involved in the initial determination of the L-R asymmetry by contributing to formation of cilia, which generates leftward flow of the extraembryonic fluid. In addition, the lack of nodal cilia might bring about abnormal distribution of mesoderm-inducing activity such as sonic hedgehog (shh), and homozygous embryos displayed disturbed mesodermal structures.

Materials and Methods

Generation of the Targeting Vector

A 20-kb genomic fragment containing full-length kif3A was selected from a murine genomic library of embryonic stem (ES) cell line J1 (Tanaka et al., 1998) and probed using a mouse cDNA fragment encoding the P-loop exon of kif3A (Kondo et al., 1994). The two independent phase clones obtained were cloned into pBS (Stratagene) vector. These clones were subsequently subcloned into pBS and mapped by a combination of several restriction enzymes and partial sequencing. We applied the conventional positive-negative selection (Yagi et al., 1993) together with the poly(A) trap strategy. As shown in Fig. 1A, we applied a 1-kb-long HindIII-SalI fragment as a 5′-homologous arm and a 5-kb-long fragment as a 3′-homologous arm. Then poly(A)-less pGK neo positive selection cassette was flanked by these arms and cloned into a modified multiple cloning site in the DT-A cassette B vector (GIBCO BRL). Finally, this construct was prepared on a large scale with a QIA GEN miniprep kit. This plasmid was linearized by NotI and an appropriate concentration of vector (25 μg/ml) was prepared for the subsequent transfection into ES cells.

Transfection and Screening of Recombinant ES Clones

We have performed the electroporation of targeting vector to J1 cell line basically according to Harada et al. (1994) with a slight modification in G418 concentration. A few electroporation, the transfected cells were selected in the presence of G418 (GIBCO BRL) at a concentration of 175 μg/ml. A bout 10 d after electroporation, the growing positive selected colonies of ES cells were picked up in duplicate 96-well plates, one for cell stock and the other for genomic DNA preparation. Screening of recombinants was performed by genomic Southern blotting with32P-radioabeled external probe (generated by PCR ~0.4 kb) as indicated in Fig. 1. Then the identity of candidate clones was further confirmed with an internal and neo probes (data not shown). Three independent recombinants out of ~450 ES colonies were obtained by this poly(A) trap targeting strategy. These recombinant clones were injected into the blastocysts recovered from superovulated C57BL/6J/n mouse and raised chimeric mice from two independent cell lines. We identified chimera mice by contribution of agouti coat color. Male chimeras were bred with C57BL/6J to obtain heterozygous offspring, from the mating of which we could obtain kif3A null mutants.

PCR Genotyping

The generated mice were routinely probed by PCR for the identification of their genotype as described previously (Tanaka et al., 1998). In brief, a small portion of mouse tail was incubated in a tail lysis buffer (10 mM Tris·HCl, 25 mM EDTA, 1% SDS, and 75 mM NaCl, pH 8.0) supplemented with 100 μg/ml proteinase K (Merck). The Eppendorf tubes containing these samples were shaken in an air incubator maintained at 55°C overnight. Phenol-extraction ethanol and ethanol precipitation were performed before the PCR reaction. About a hundredth amount of each purified DNA was routinely used for PCR. The following two pairs of primer sets were used for genotyping offsprings: (a) neo primers, neoF (1620–1649) 5′-TGG GCA CAA CAG ACA ATC GG-3′, neoR, (1841–1820) 5′-ACT TGG CCC AAT AAT GAG CAG-3′; (b) internal primers, kif3A-F4, (346–369) 5′-TGT TCC ATA TAG CCC AAG GGA-3′, kif3A-B1, (545–526) 5′-GAT GGT CCC TGA A A TGG TGC-3′. To determine the embryonal genotype, we collected amniotic membranes. They were dissolved in aqua destillata supplemented with 100 μg/ml proteinase K under shaking at 55°C for 1 h. A small amount (2 μl) of the lysate was used for PCR amplification.

Western Blotting of the Embryos

The whole embryos ranging from 9.5 to 10 dpc were killed and minced using a pair of ophthalmological scissors in PBS supplemented with protease inhibitor cocktails (5 mM PM SF, 10 ng/ml leupeptin, pepstatin, benzamidine, and 100 mM DTT; Wako Pure Chemicals Co.) on ice. By using a Potter’s homogenizer, small pieces of embryos were completely de
prehybridized, and then hybridized with appropriate markers (overnight, treated with RNase to remove unbound or nonspecific binding protein concentration of each sample was adjusted to 10 μg/ml and used for standard Western blotting procedure as described previously (Tanaka et al., 1998). Here we used the following antibodies to ascertain the expression level of KIF3A and KIF3B proteins in the mutant mice: both monoclonal and polyclonal anti-KIF3A antibodies (Kondo et al., 1994), and polyclonal anti-KIF3B antibody (Yamaizaki et al., 1995).

Preparation of Embryos for Light Microscopy

The embryos dissected from the pregnant mice were processed for the light microscopic observation by the following method. As soon as the embryos were extirpated from the uterus, a small portion of extraembryonic membranes was retained for the determination of genotype by PCR and the embryonic proper was soaked into Bovin’s fixative (75% pycric acid, 5% glacial acetic acid, and 25% formaldehyde; Wako Pure Chemicals Co.). A stereomicroscope for fixing embryos by 2 h at room temperature (RT), the samples were dehydrated by a graded series of ethanol solutions, followed by substitution of xylene. Finally, the whole mount embryos were placed in melted Parafla (Oxford Labware) at 65°C for ~90 min with two changes of this embedding material. The samples were embedded in fresh Parafla after complete penetration of the samples by the embedding material. The blocks were cut by using a rotary microtome (HM 355; Carl Zeiss) into 7-μm-thick serial sections, then the sections were mounted onto glass slides, which were in turn deparaffinized and stained with hematoxylin and eosin according to Kaufman (1995) with slight modifications. These sections were observed and photographed by a Nikon Optiphot-2 microscope.

Preparation of Embryos for Scanning Electron Microscopy

The dissected embryos were fixed by using half Karnovsky solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) for 2 h at RT. Then we soaked these embryos into 10% sucrose solution buffered by 0.1 M cacodylate for 20 min, followed by postfixation with 1% osmium tetroxide in cacodylate buffer on ice for 15 min. After washing the embryos extensively with aqua destillata, dehydration with a graded series of ethanol solutions was performed. A stereomicroscope completing the dehydration process, the media was substituted with isoamyl acetate and stained with hematoxylin and eosin according to Kaufman (1995) with slight modifications. Half-thickness sections were observed and photographed by a Nikon Optiphot-2 microscope.

Transmission Electron Microscopy of the Nodal Monocilia

Embryos at 7.5 dpc were dissected out from the uterus and were fixed by using half Karnovsky solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) for 2 h at RT. Then we soaked these embryos into 10% sucrose solution buffered by 0.1 M cacodylate for 20 min, followed by postfixation with 1% osmium tetroxide in cacodylate buffer on ice for 15 min. After washing the embryos extensively with aqua destillata, dehydration with a graded series of ethanol solutions was performed. A stereomicroscope completing the dehydration process, the media was substituted with isoamyl acetate and stained with hematoxylin and eosin according to Kaufman (1995) with slight modifications. These sections were observed and photographed by a Nikon Optiphot-2 microscope.

Whole Mount In Situ Hybridization

Whole mount in situ hybridization of the mouse embryos other than lefty-2 was carried out principally according to Conlon and Rossant (1992) with several modifications. In brief, the embryos were dissected in sterilized RNase-free PBS and fixed with 4% paraformaldehyde not equilibrated with NaOH at 4°C. A fter incubation for 2 h, they were washed twice with PBS (0.05 M sodium phosphate buffer, pH 7.2; 200 mM NaCl) for 20 min with 200 mM sodium pyruvate) and sealed it by simply placing a coverslip on it. Fluorescent beads (Fluspheres, carboxylate-modified microspheres; Molecular Probes) with a diameter of ~220 nm were added to the assay solution to ~5% for visualization of flow generated by the motile cilia. The preparations were viewed under VEC-DIC/FL microscopy (Olympus Inc.) and a series of motility assay was videotaped simultaneously. The embryos under investigation were genotyped after observation.

Nodal Cilia Motility Assay

Nodal cilia can be observed during the nodal formation and bulging at 7.5 dpc (Nonaka et al., 1998). The lower halves of the embryos dissected from the uterus were eliminated by using a pair of electrolytically sharpened tungsten needles. The half-egg like structure was embedded into a small hole of silicon-rubber membrane (thickness ~300 μm) attached onto the surface of a glass slide. A nodule silicon membrane having a hole at its center (diameter ~5 mm) was overlaid onto it. We filled the hole with motility solution (50% heat-inactivated rat serum, 49% D M E M and 1% 1 mM sodium pyruvate) and sealed it by simply placing a coverslip on it. Fluorescent beads (Fluspheres, carboxylate-modified microspheres; Molecular Probes) with a diameter of ~220 nm were added to the assay solution to ~5% for visualization of flow generated by the motile cilia. The preparations were viewed under VEC-DIC/FL microscopy (Olympus Inc.) and a series of motility assay was videotaped simultaneously. The embryos under investigation were genotyped after observation.

Immunocytochemistry of the Nodal Cilia and the Embryo Section

Embryos were fixed with 4% paraformaldehyde and processed by a standard cryosectioning method. The blocks were sectioned with a Leica cryo-microtome, and extended on the surface of glass slides. The samples were permeabilized with 0.1% Triton X-100 and blocked with 5% skim milk followed by a standard immunocytochemical procedure as described elsewhere (Nonaka et al., 1998). For the staining by anti-axonal dynein antibody, we generally followed the method of Umeda et al. (1995) with slight modifications. The stained preparations were examined under a confocal laser scanning microscope (Bio-Rad).

Results

Targeting Strategy and Confirmation of Homologous Recombination

Initially, we tried to obtain homologous recombinants by using a standard positive-negative selection vector and screened ~800 ES cell colonies. However, we could not successfully obtain any recombinant clones, which led us to adopt the poly (A) less pGK-neo cassette with a A-T rich/pausing signal (Fig. 1 A). Under this condition, three independent clones out of ~450 colonies were isolated, all of which have been used for blastocyst injection. A s shown in Fig. 1 B, wild-type embryos displayed a single 3.2-kb fragment when digested with EcoRI, while an additional 2.1-kb band was observed in heterozygotes by genomic Southern blotting. A nother restriction enzyme (X bal) being diagnostic for targeting events, also verified successful homologous recombination (data not shown). G enotypes were also identified by PCR where primer pairs explicitly detect the targeted events. A s represented in Fig. 1 D, almost all littermates of heterozygous mothers intercrossed with male heterozygotes revealed proper separation of genotype according to Mendel’s law. We made a necropsy
of 20 pregnant heterozygotes expected to have homozygote offsprings during 7.5–11.5 dpc, which revealed that no homozygotes were observed after 10.5 dpc (Fig. 1 D, Table I), suggesting the midgestational lethality of homozygous embryos. Western blotting analysis of all littermates revealed a complete absence of KIF3A protein from homozygous mice. Interestingly, KIF3B protein, being a counterpart in the KIF3 complex, was expressed at a normal level (Fig. 1 C, lane 3).

**Severe Developmental Abnormalities in kif3A<sup>−/−</sup> Mice**

kif3A homozygotes were generally smaller compared with heterozygous and wild-type littermates at 9.5 dpc. Furthermore, no living embryos were obtained when cesarean section of pregnant mice was carried out after 11 dpc (Table I). At 9.5 dpc, the homozygotes displayed severe malformation characterized by swelling and degeneration of the neural tube (Fig. 2 and see Fig. 5, B and D) mimicking the phenotype of hydrocephalus. Furthermore, extreme distension of the pericardium due to pericardial effusion was noted. A regular array of somites as seen in wild-type embryos (see Fig. 5 A) could not be observed in homozygote embryos (see Fig. 5 C), simply showing degenerated bulk in the lower truncal part and abnormal somitegenesis, i.e., sirenomelia. In most cases, embryonic turning was not completed at 9.5 dpc, suggesting growth retardation/inhibition of the homozygotes. Staggering of the neural tube
was prominent at this incomplete turning region. These phenotypes are suggestive of disturbed mesodermal development resulting in failure of neural tube induction. In addition, the severity of the phenotype observed at 9.5 dpc was almost correlated with the expression level of the KIF3A molecule, which revealed relatively strong signal intensity in the neural tube (see Fig. 4 B, Nt) and the heart (idem, Cor).

Moreover, we encountered abnormal heart looping. In wild-type cases (Fig. 3, A and D), almost 100% of embryos displayed a D-loop pattern while a few atypical phenotype also existed. On the contrary, in homozygotes, ~40% of the embryos revealed a L-loop type heart (Fig. 3, C and F). In some cases (~20%), incomplete cardiac looping, i.e., a tuba rectae, was observed. These results suggested that randomization of the heart looping occurred in kif3A homozygous mice. To address this point more precisely, we scrutinized these embryos with lefty-2 RNA probe, to see whether it involves abnormal lefty-2 expression, as lefty-2 product is considered to be one of the earliest left-right determinant in early murine development (Meno et al., 1997, 1998). As has been observed in our previous report for kif3B−/− (Nonaka et al., 1998), the expression of lefty-2 was bilateral or downregulated in kif3A−/− embryos (see Fig. 6 B). In some cases, the expression was extended to the contralateral paraxial mesoderm (Fig. 6, the right two) while normal pattern still existed (Fig. 6 B, the left-most embryo). On the contrary, almost all wild-type embryos and heterozygotes displayed normal left-side restricted pattern (Fig. 6 A). Regarding a cardiogenesis, hypoplasia of the myocardium was another striking feature of kif3A homozygous embryos. A sagittal section of the heart wall indicated fairly underdeveloped myocardium (Fig. 6 E) compared with normal embryos (Fig. 6 F), which might be a cause of circulatory insufficiency in utero.

**Table I. Genotype Separation of Offspring after Mutual KIF3+/+ Mating**

| Age  | Wild-type | +/- | −/− | ND | Total |
|------|-----------|-----|-----|----|-------|
| E7.5 | 12        | 23  | 12  | 4  | 51    |
| E8.0–8.5 | 20  | 41  | 18  | 8  | 87    |
| E9.5 | 9         | 21  | 12  | 0  | 42    |
| E10.5 | 14  | 29  | 6   | 0  | 49    |
| E11.5 | 9   | 17  | 0   | 0  | 26    |
| Adult| 52        | 90  | 0   | 0  | 142   |

Allelic separation of embryos at various stages. Until 9.5 dpc, clear Mendelian separation was encountered while homozygous mice started to die at ~10.5 dpc. No homozygotes could be encountered after 11 dpc.

No Cilia Were Formed in the Node of 7.5 dpc kif3A−/− Embryos

The node is normally formed at 7.5 dpc and is essential for further induction of mesodermal anlage and in turn for neural tube formation. As Chlamydomonas orthologues of the KIF3 complex have been reported to engage in IFT (Kozminski et al., 1995; Cole et al., 1998; Rosenbaum et al., 1999), we focused on the ciliated mesodermal cells which normally accommodate monocilia (Sulik et al., 1994). As shown in Fig. 7, A and B, the nodal region forms a small dimple at the base of the dorsal part of embryo where the cell density is quite high. Since the cells constituting the node are smaller than surrounding endodermal cells at this developmental stage, this region could readily

![Figure 2. Macroscopic overview of both wild-type and homozygous embryos at 9.5 dpc. (A) Lateral view of a wild-type embryo. Normal development of somites and the nervous system can be observed. (B) Same view of a homozygous embryo. Note the severe degeneration of the lower half of the body accompanied by thin neural tube wall. (C) Dorsal view of a homozygous embryo, exhibiting a staggered midline structure with disorganized somites. (D) Extensive pericardial effusion (arrow) resulting in circulatory insufficiency. Bar, 1 mm.](image-url)
be discriminated from other parts of the embryonic body. At a glance, the wild-type/heterozygous cells normally exhibited cilia ranging from 2 to 4 μm in length and having pinpoint-like ends (Fig. 7 C). However, in the case of kif3A +/- mice (Fig. 7 D), the surface structure displayed quite striking differences. There were almost no mature cilia, although there existed some very short rudimentary processes. This phenotype was invariably observed among

Figure 3. Scanning electron microscopy of the embryos at 8.5 dpc. Wild-type embryo displays normal body turning (A) and heart loop (D, D-type) while homozygotes (B and C) did not make turning. Some homozygotes exhibited inverted heart looping (F, L-type). In addition, seemingly normal heart of homozygote (E) display irregular cardiac surface as seen in F. Bars: (A-C) 500 μm; (D-F) 100 μm.

Figure 4. Expression pattern of KIF3A molecule in 9.5 dpc embryos (B). Expression of the KIF3A molecule is almost ubiquitous although we can recognize relatively strong accent in the neural tube (Nt) and the heart (Cor). Orl, oral cavity; Caud, caudal region. (A) Control panel stained with preimmune serum. Bar, 1 mm.
homozygous mouse littermates, suggesting the possible role of the KIF3A molecule in constructing and maintaining the monocilia structure.

The KIF3A-null Mutant Embryos Were Defective of the Leftward Extraembryonic Nodal Flow as Revealed by Fluorescent Beads

To decipher the degree of dysfunction of nodal pit cell cilia in kif3A−/− embryos, we have carried out a motility assay. A n embryonic body harboring a node was placed under the V E C-D I C/FL microscope and fluorescent beads were added to the solution surrounding the embryos. By using this method, we can simultaneously observe both ciliary movement and nodal flow. In wild-type and heterozygous embryos, distinct flow generated by the rotating cilia was recorded and this flow was directed leftward with reference to the presumed body A-P axis (Fig. 8 A). This regular flow was especially evident in the posterior part of the node. On the contrary, in KIF3A-deficient embryos, we could identify no such a regular flow, while rather Brownian movement of beads was observed (Fig. 8 B). This result clearly indicates that the cilia of nodal pit cells is involved in gradient formation within the nodal groove, implying some important roles of the KIF3A molecule in the determination of early body planning.

We have carried out immunostaining of nodal cilia to determine whether KIF3A molecules are really installed in the motile cilia. Cryosection of the node stained with anti-α-tubulin (DM1A) indicates the presence of cilia containing MT cytoskeleton (Fig. 8, B and D). Double immunostaining of the same embryo with an anti-KIF3A antibody revealed the localization of KIF3A molecule within the cilia (Fig. 9 A), suggesting that the KIF3A molecule might be engaged in the construction and maintenance of the ciliary structure. These results are almost the same as those obtained from the KIF3B-deficient embryos (Nokawa et al., 1998), strongly suggesting that KIF3A is really a counterpart of KIF3B in vivo. Collectively, KIF3A molecules may be essential for ciliary formation, determining the subsequent developmental cascades.

Moreover, staining with an anti-outer axonemal dynein antibody (A D 2; Yoshida et al., 1989) and anti-axonemal dynein intermediate chain (I C 1 4 0; Yang and Sale, 1998) also revealed the presence of this molecule within the monocilia (Fig. 9, C and E). Because the nodal area is composed of several optical sections by confocal laser
scanning microscopy while that for cilia is made of a few sections, the background fluorescence in the nodal area seems to be higher. However, as there are no positive staining of cilia in the negative control panel (Fig. 9 G), we could state that the localization of ciliary axonemal dynein is specific. We could also identify both inner and outer arm dynein localizing on the surface of doublet MTs within the monocilia (arrows for inner arm dynein and for outer arm dynein, Fig. 9, I and J) by a conventional electron microscopy. These results collectively imply that axonemal dynein is a candidate cargo of KIF3A that may in turn be engaged in the movement of monocilia.

**Shh Expression Was Somewhat Reduced in Mutant Rostral Portion**

As both development of the mesodermal structure and the determination of L-R asymmetry were highly affected in the kif3A null mice, we also examined the expression pattern of some developmental markers for delineating the role of the KIF3A molecule in this process. Pax6 (Jordan et al., 1992) expression in 9.5 dpc embryos did not exhibit any apparent changes in the eyes except for slightly reduced expression in the forebrain (Fig. 10, A and B). Brachyury (T, see review by Willison, 1990) is a mesodermal marker that is normally expressed in the midline structures with a strong accent at the tail bud region. At 8.5 dpc, its expression patterns in both embryos (wild-type; Fig. 10 C, homozygote; Fig. 10 D) were very similar, but midline staining corresponding to the notochord was fainter in the case of homozygous embryos. Of noteworthiness, another midline mesodermal marker, sonic hedgehog (shh; Conlon et al., 1995), exhibited the most dramatic change in homozygote embryos even at early developmental stage (7.5 dpc, Fig. 10 G). Normally, this marker is expressed in the node, notochord and subsequently in the floor plate. Furthermore, the ventral half of the mesencephalon also expresses it resulting from induction by the underlying floor plate at this region (Ekker et al., 1995). In the case of homozygous embryos (Fig. 10, E and F, lower embryos), the expression of shh at the thoracic notochord was significantly reduced and the staining per se became staggered to a certain extent. Moreover, the expression in the mesencephalon was completely absent in homozygous embryos.
mice (Fig. 10 E, lower embryo) while abundant expression could be encountered in wild-type embryos (Fig. 10 E, upper embryo). This absence of shh expression in brain structures might result in abnormal development of the neural tube, leading to the hydrocephalus-like phenotype and neural tube hypoplasia. Actually, exencephaly (Fig. 3 C) was relatively frequently observed in our kif3A null mutants under the genetic background with lesser contribution of C57BL/6J, suggesting the possibility that some modifier loci of this phenotype are present in the 129/Sv strain. In addition, reduced expression of shh in the rostral region together with reduced T expression in the truncal part collectively suggest the influence of the KIF3A molecule in the formation of A-P axis possibly through transporting inductive signals or their receptors.

**Discussion**

In summary, this is one of the first reports describing the general function of the KIF3 complex in vivo. The null mutant embryos displayed smaller body size and early developmental failure characterized by mesodermal hypoplasia and degeneration followed by the development of neural tube defects. Furthermore, determination of L-R asymmetry was randomized, exhibiting the phenotype represented by L-loop formation of the cardiac tube (situs inversus). This phenotype was presumably due to defective nodal cilia as identified in our previous kif3B mutant (Nonaka et al., 1998). These results reinforce our biochemical data that KIF3A is a counterpart of KIF3B (Yamazaki et al., 1995). A new idea on the function of KIF3A is that it is involved in mesodermal formation, which then regulates the development of neural tissues. Here we should like to discuss the possible role of the KIF3A molecule with special reference to (a) the early developmental events and (b) the determination of laterality.

**KIF3A and Early Developmental Sequella**

KIF3A is reported to be already expressed in ES cells and precedes that of KIF3B. Considering the biochemical data that KIF3A could remain soluble in the absence of its counterpart (Yamazaki et al., 1995), the earlier death of kif3A−/− embryos than that of kif3B homozygous embryos (no surviving embryos after 11.5 dpc; Nonaka et al., 1998) is quite reasonable. Although the macroscopic phenotype was not so evident until 8.5 dpc in kif3A null mutants, microscopical changes in kif3A homozygotes were even evident early in the node formation stage (7.5 dpc). The node is generally believed to be responsible for the induction of further midline mesodermal structure. The absence of cilia on the surface of nodal pit cells in kif3A null mutants and the normal distribution of KIF3A molecules within these cilia in wild-type embryos strongly suggest that the nodal cilia are to some extent constructed and maintained with the aid of KIF3A molecules. Although a previous report suggested the presence of axonemal outer arm dynein in primary cilia (9 + 0 structure, two basal bodies; Umeda et al., 1995) of mammalian sperm and lung ciliated epithelium, and it might be responsible for actual motility, our data presented here and in a previous study (Nonaka et al., 1998) indicated KIF3A and KIF3B are essential for transporting these ciliary components. A axonemal outer arm dynein per se is probably transported down within the cilia as a transporting complex for the following two reasons. On the first line, our immunocytochemical staining clearly demonstrated the colocalization of KIF3A and outer axonemal dynein within monocilia (Fig. 9, C and E). Second-
Figure 10. Whole mount in situ hybridization. The wild-type embryos (A and C) and homozygous embryos (B and D) were probed with Pax6 (A and B) and Brachyury (C and D) at 9.5 dpc. In the case of sonic hedgehog, lateral view (E) and dorsal view (F) of both wild-type embryos (upper embryos) and homozygous embryos (lower embryos) are presented at 8.5 dpc. Although normal expression pattern of Pax6 was almost preserved in kif3A-deficient embryos while its expression level was downregulated (see text). Expression patterns of shh and T were significantly altered. Note the absence of shh at the mesencephalic region (arrowhead). Some nonspecific deposition of signals can be observed in the head region of the panel (D), which might result from precipitate flow through the distended canalis in mutants. At 7.5 dpc (G), shh expression is dramatically reduced without strong localization within node (arrow) compared with wild-type embryo (Node). Bars: (A–F) 1 mm; (G) 150 μm.
arily, mutations in a putative axonemal dynein heavy chain (Ird), being abundantly expressed in these ciliated nodal cells (Supp et al., 1997), de facto resulted in situs inversus. These results are in good agreement with the case of Chlamydomonas FLA 10, where large IFT complexes are transported by this KIF3 orthologue and depletion of this motor protein resulted in the absence of normal motile flagella (Cole et al., 1998; Rosenbaum et al., 1999).

Then by what mechanism are the early developmental events disorganized, giving rise to multysystem failure? Although there is no direct evidence interfacing the function of KIF3A and early inductive events, it is supposable that the defective nodal cilium could not properly distribute some presumably soluble substances to their proper destinations. Alternatively, intracellular transport of some kinds of receptors to proper direction might be affected. Especially, the abnormal expression pattern of Brachury (T) and sonic hedgehog (shh) could partly explain the hypoplasia of midline structure and degeneration of posterior body structure. The generally accepted idea holds that shh is secreted as a complex bound to cholesterol (Cooper et al., 1998). Considering the size of this complex and cilia, it is reasonable that the lack of cilia resulted in disturbance of orthotopic shh expression in kif3A−/− homozygote mice. In addition, spontaneous mutation of shh, cyclopia (Chiang et al., 1996), and targeted disruption of shh (Lanske et al., 1996) partly share the phenotypes observed in this kif3A null mutant. These ideas collectively imply the relationship between the KIF3 molecule and transport of the Shh molecule.

From another standpoint, as neural induction depends on the mesoderm, abnormal neural tube formation could be attributed to this general failure of anterior development. Indeed, the lack of shh staining at the ventral half of the mesencephalon implies that the neural phenotype is altered than that of wild-type embryo, displaying very faint trace amount with no nodal accentuation. However, considering that neuronal morphogenesis also involves cellular movement and assortment by themselves, the neural phenotype might be explained independently by genuine neuronal failure brought about by kif3A disruption. In addition, as some reports suggested the presence of cilia on the neuron (Vigh-Teichmann et al., 1980), the neural phenotype may partially be attributable to the lack of neuronal cilia.

**Ciliary Movement in Node: Implications for Left-Right Asymmetry**

As has been discussed in the previous section, KIF3A might transport materials required for the cilia assembly and maintenance, so that the absence of KIF3A molecules results in ciliary disorganization, leading to several developmental defects. One of the most remarkable phenotypes in these mutant mice was randomization of L-R asymmetry. A s we have previously reported in kif3B null mutant mice (Nonaka et al., 1998), these null mutant mice exhibited randomized heart loop formation. Importantly, failure of cardiac looping to occur in neither direction was also encountered in kif3A null mutant embryos (~20%).

Then what mechanism is responsible for left-right determination? Our experiment by using fluorescent beads clearly indicated that the nodal cilia propelled certain kinds of substances in the extraembryonic fluid in the vicinity of the node from right to left. A concentration gradient that reaches the threshold for switching on lefty expression is probably formed and it might further destine the L-R asymmetry. This conclusion is further supported by our recent unpublished work showing randomness of left-right asymmetry occurs in mutant whose nodal pit cells harbor primary cilia, but they are immotile (O'kada, Y., S. Nonaka, and N. Hirokawa, unpublished data). A s a candidate of determinants, we expect that N-Shh protein might be involved since the cholesterol-conjugated form of N-Shh (Cooper et al., 1998) could not diffuse out a significant distance from a source of secretion and be concentrated to where it should be localized. There is no direct evidence for the relationship between lefty and shh, but it is now generally accepted that shh expression in chick is restricted to the left side before that of lefty-2 (Levin et al., 1995).

Furthermore, although the expression of shh is bilateral in mammals, it is most probable that the leftward flow of the extraembryonic fluid generated by nodal cilia produce the concentration gradient of secreted morphogens including Shh which are upstream of the L-R determinants such as lefty-1, -2. The absence of this flow in kif3A null mutant mice (Fig. 8 B) might result in disturbance of the correct topology of determinants.

A s a next step in delineating the total function of KIF3 complex in vivo, conditional gene targeting is now under way. Furthermore, we could also examine the genetic interaction between these molecules by making double knockout mice for KIF3A/B. From another standpoint, by using the cell biological disciplinary, now we are also conducting experiments to unveil the function of KIF3 complex in mesodermal induction and neuronal development.

We would like to express our gratitude to Dr. Toshimichi Yoshida (Mie University, Japan) for the anti-axonemal outer arm dynein antibody (A D2); Dr. Winfield Sale (Emory University, Georgia) for the anti-dynein intermediate chain antibody (IC140); Dr. Hiroshi Hamada (O'saka University, Japan) for the lefty-2 and shh probe; Drs. Y. umiko Sako (National Institute of Health Science, Japan) and Bernhard G. Herrmann (Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany) for the Brachury probe; Dr. Peter Gruss (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany) for the Pax6 probe. We appreciate Dr. Mika Karasawa (Chiba University, Japan) for providing us with a good protocol for whole mount in situ hybridization. We are also grateful to Drs. Yoshimitsu Kanai and Kihiro H ara, M s. Chunjie Zhao, and M s. Noriko H omma for their assistance in transgenic technology, and to M s. H ayuo Fukuda, M s. H iromi Sato, and M. N obuhsa Ono-uchi for their technical and secretarial assistance.

This work was supported by a grant for Center of Excellence (COE) from the Ministry of Education, Science, Sports and Culture of Japan to Nobutaka Hirokawa.

Received for publication 14 January 1999 and in revised form 24 March 1999.

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