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Cluap1 localizes preferentially to the base and tip of cilia and is required for ciliogenesis in the mouse embryo

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ABSTRACT

Qilin is one of several genes in zebrafish whose mutation results in cystic kidney. We have now studied the role of its mouse ortholog, *Cluap1*, in embryonic development by generating *Cluap1* knockout (*Cluap1*$^{-/-}$) mice. *Cluap1*$^{-/-}$ embryos died mid-gestation manifesting impairment of ciliogenesis in various regions including the node and neural tube. The basal body was found to be properly docked to the apical membrane of cells in the mutant, but the axoneme failed to grow. Cluap1 is a ciliary protein and is preferentially localized at the base and tip of cilia. Hedgehog signaling, as revealed with a *Pacthed1-lacZ* reporter gene, was lost in *Cluap1*$^{-/-}$ embryos at embryonic day (E) 8.5 but was ectopically expanded at E9.0. The *Cluap1* knockout embryos also failed to manifest left–right asymmetric expression of *Nodal* in the lateral plate, most likely as a result of the loss of Hedgehog signaling in node crown cells that in turn leads to pronounced down-regulation of *Gdf1* expression in these cells. Crown cell-specific restoration of *Cluap1* expression rescued *Gdf1* expression in crown cells and left-sided *Nodal* expression in the lateral plate of mutant embryos. Our results suggest that Cluap1 contributes to ciliogenesis by regulating the intraflagellar transport (IFT) cycle at the base and tip of the cilium.

Introduction

Cilia are present in various cell types of vertebrates and play essential roles in developmental and physiological processes (Oh and Katsanis, 2012). Dysfunction of cilia in humans is responsible for diverse disorders including obesity, retinal degeneration, renal failure, and heterotaxy or situs inversus (Fliegauf et al., 2007). Whereas motile cilia are required for motility of cells or for the generation of extracellular fluid flow, immotile cilia act as antennae that sense various extracellular signals, both chemical and mechanical.

Given the essential roles of cilia in development and physiology, the mechanism of ciliogenesis has received much attention. Cilium formation is dependent on the cell cycle (Nigg and Stearns, 2011). As a cell undergoes G_0 phase, the centrosome moves to the apical cell membrane and the mother centriole is transformed into the basal body, from which the axoneme extends to form the cilium (Kobayashi and Dynlacht, 2011). Biogenesis of cilia requires intraflagellar transport (IFT) machinery, particles which mediates the bidirectional (anterograde and retrograde) movement of cargoes needed for extension and maintenance of the cilium (Rosenbaum and Witman, 2002). At the base of the cilium, IFT particles are assembled from kinesin motors, IFT-A and IFT-B complexes, and cargo. At the tip of the cilium, IFT particles undergo a remodeling process to change the motor from kinesin to dynein and to release and reload cargo. However, how IFT particle assembly and turnaround are regulated remains unknown.

Qilin is one of several genes in zebrafish whose mutation results in cystic kidney (Sun et al., 2004), with the encoded protein being essential for cilium assembly and kidney development in this species (Li and Sun, 2011). The mammalian ortholog of *Qilin*, *Cluap1*, was initially identified as a gene that encodes a clusterin-associated protein that is frequently up-regulated in human cancer cells (Takahashi et al., 2004). *Qilin*/*Cluap1* encodes a coiled-coil domain-containing protein that is expressed in ciliated cells of multiple organisms including *Chlamydomonas reinhardtii* (Stolc et al., 2005), *Caenorhabditis elegans* (Murayama et al., 2005), and mouse (Pasek et al., 2012) in addition to zebrafish (Sun et al., 2004; Li and Sun, 2011) and human (Marshall, 2004). The Cluap1 protein is up-regulated during flagellum regeneration in *C. reinhardtii*, is required for assembly of sensory cilia and undergoes IFT-dependent motion in *C. elegans* (Ou et al., 2005), interacts with the IFT machinery in zebrafish (Omori et al., 2008) and associates with B complex in mammals (Boldt et al., 2011). Its role in ciliogenesis in mammals has remained unclear, however.

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We have now generated and characterized a *Cluap1* mutant mouse in order to determine the role of this gene in mammalian development. Our results suggest that Cluap1 is required for ciliogenesis during embryonic development.

Materials and methods

Generation of Cluap1 knockout and knockin alleles

Mouse *Cluap1* knockin and knockout targeting vectors were both generated by recombineering with bacterial artificial chromosome (BAC) clone RP23-171I6 (Copeland et al., 2001; Liu et al., 2003). For generation of the knockout targeting vector, a single LoxP site was inserted 180 bp upstream of *Cluap1* exon 1 (Fig. S1). A cassette consisting of *Frt-Pgk-em7-neo-Frt-LoxP* from the PL451 vector was then inserted 1.5 kb downstream of *Cluap1* exon 3. The knockin targeting vector was generated by insertion of an *Ires-lacZ* sequence linked to a similar *neo* selection cassette immediately upstream of the stop codon in exon 12 of *Cluap1*. The linear constructs were introduced into G4 mouse embryonic stem (ES) cells (C57BL/6 and 129sv hybrid). After selection of transformant clones with G418, homologous recombinants were detected by Southern blot hybridization with a series of specific probes that target external or internal sites relative to the vector sequence. Chimeric mice were generated by aggregation of ES clones with ICR morulas, and germline transmission was monitored on the basis of the coat color of pups. The null and functional knockin alleles were then generated by mating of pups with *CAG-Cre* or *CAG-Flp* transgenic mice, to remove *Cluap1* exons 1 to 3 or the *neo* cassette, respectively. Analyses were performed with mice on a 129/B6 hybrid background unless indicated otherwise.

Kif3a$^{+/-}$ mouse was purchased from The Jackson Laboratory (Strain Name: B6.129-Kif3a < tm1Gsn > /J).

Transgenic mice

For the generation of a transgene that confers *Cluap1* expression specifically in node crown cells, mouse *Cluap1* cDNA and a downstream *Ires-lacZ* cassette were placed under the control of two copies of a 0.7-kb DNA fragment containing the node-specific enhancer (NDE) of mouse *Nodal* (Krebs et al., 2003) and one copy of the mouse *Hsp68* promoter. A *Patched1-lacZ* transgenic line was generated by insertion of *lacZ* into *Patched1* exon 1 in BAC clone RP24-139N24. The transgenes were injected into the pronucleus of fertilized eggs obtained by crossing B6C3F1 females with *Cluap1*$^{+/-}$ males. Specific LacZ expression was monitored by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) according to standard procedures.

Whole-mount in situ hybridization

Whole-mount in situ hybridization with digoxigenin-labeled RNA probes was performed according to standard procedures (Wilkinson, 1999).

Embryo culture and lipofection

Embryos at embryonic day (E) 7.5 were recovered in Hepes-buffered Dulbecco's modified Eagle's medium (DMEM), and an expression vector for mouse *Nodal* was mixed with the Lipofectamine 2000 reagent (Invitrogen) and injected into the right lateral plate mesoderm (LPM) with the use of a glass needle, as described previously (Nakamura et al., 2006). The embryos were then cultured under 5% CO_2 with rotation at 37 °C in DMEM supplemented with 75% rat serum. *Nodal* expression was monitored by whole-mount in situ hybridization at 10 h after lipofection, when the cultured embryos had achieved the five- to six-somites stage.

Antibodies

Polyclonal antibodies that recognize a COOH-terminal sequence of mouse Cluap1 were recovered from an injected rabbit and purified by affinity chromatography (Immuno-Biological Laboratories). Antibodies to acetylated tubulin were obtained from Sigma (T6793), those to outer dense fiber protein-2 (Odf-2) were kindly provided by S. Tsukita (Osaka University) or obtained from Abcam (ab43840), and those to phosphorylated Smad2/3 were obtained from Cell Signaling Technology (#3101). Alexa Fluor-conjugated goat secondary antibodies were obtained from Molecular Probes.

Immunofluorescence analysis

Cells and embryos were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), after which nonspecific sites were blocked with a solution containing 0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl, and 0.5% TSA blocking reagent (Perkin Elmer). The samples were then incubated overnight at 4 °C with primary antibodies diluted in the blocking solution, washed with PBS containing 0.1% Triton X-100, and incubated overnight at 4 °C with Alexa Fluor-conjugated secondary antibodies diluted in PBS, 0.1% Triton X-100 before observation with an Olympus FV1000 confocal microscope. For observation of transverse sections, frozen sections were first autoclaved for 5 min at 121 °C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Subsequent blocking and antibody incubations were performed in PBS containing 0.1% Triton X-100 and 3% dried skim milk. To stain the node cell membrane, embryos were soaked 3 min in 1% Vybrant DiI dye (Molecular probes, V-22885) in PBS prior to observation. Phosphorylated Smad2/3 was detected as described previously (Kawasumi et al., 2011).

Establishment and culture of MEFs

Mouse embryonic fibroblasts (MEFs) were isolated from E8.75 embryos and cultured in high-glucose DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1 mM sodium pyruvate, streptomycin (0.05 mg/ml), and penicillin (0.05 U/ml). Growth of cilia was induced by changing the culture medium of confluent cells to DMEM supplemented with 0.5% fetal bovine serum and incubation for 48 h.

Luciferase assay

MEFs were seeded at a density of 2×10^5 cells/cm^2 in 24-well plates and transfected for 24 h with 500 ng of DNA, consisting of 8 × 3′Gli-BS firefly luciferase (Sasaki et al., 1997) and pRL-TK *Renilla* luciferase (Promega) vectors in a mass ratio of 4:1, with the use of Lipofectamine LTX (Sigma). The growth of cilia was induced for 48 h, and the cells were then exposed to the active NH_2-terminal fragment of Sonic hedgehog (ShhN) (R&D Systems, 461-SH) at 10 nM for an additional 72 h. The cells were then lysed, and luciferase activities were measured with the use of a Dual-Luciferase Reporter Assay (Promega) and a TD 20/20 luminometer (Turner Designs). Firefly luciferase activity was normalized by that of *Renilla* luciferase.

Electron microscopy

Embryos between E7.5 and E8.5 were recovered in Hepes-buffered DMEM, washed with PBS, and fixed overnight with 1% glutaraldehyde in PBS. They were then incubated for 30 min on ice with 1% osmium tetroxide in PBS, dehydrated with ethanol,