

Ruvbl1 is Essential for Ciliary Beating during *Xenopus laevis* Embryogenesis

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Received: June 23, 2023

Revised: August 27, 2023

Accepted: September 16, 2023

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Conflict of interests

The authors declare no potential conflict of interest.

Acknowledgements

This research was supported by the National Research Foundation of Korea, Ministry of Science & ICT, Korea (2021R1A2C1010408) and Korea Environment Industry and Technology Institute (KEITI) through Core Technology Development Project for Environmental Diseases Prevention and management funded by Korea Ministry of Environment

Abstract

The Ruvb-like AAA ATPase1 (Ruvbl1; also known as Pontin) is an evolutionary conserved protein belonging to the adenosine triphosphates associated with diverse cellular activities (AAA+) superfamily of ATPases. Ruvbl1 is a component of various protein supercomplexes and is involved in a variety of cellular activities, including chromatin remodeling, DNA damage repair, and mitotic spindle assembly however, the developmental significance of this protein is unknown and needs detailed investigation. We investigated the developmental significance of Ruvbl1 in multiciliated cells of the *Xenopus laevis* epidermis since *ruvbl1* is expressed in the multiciliated cells and pronephros during *X. laevis* embryogenesis. The knockdown of *ruvbl1* significantly impaired cilia-driven fluid flow and basal body polarity in the *X. laevis* epidermis compared to control embryos, but did not affect cilia morphology. Our results suggest that Ruvbl1 plays a significant role in embryonic development by regulating ciliary beating; however, further investigation is needed to determine the mechanisms involved.

Keywords: Ruvbl1, Ciliogenesis, Fluid flow, Basal body polarity, *Xenopus laevis*

INTRODUCTION

Motile cilia beat in a coordinated fashion to create a fluid flow essential to development and homeostasis (Meunier & Azimzadeh, 2016). Ciliary beating is generated by a complex of axonemal dynein motors that form a bridge between adjacent double microtubules. When ATP activates the kinetic region of the dynein, it begins to travel along the adjacent double microtubules. The force generated by the motion of the dynein can induce sliding between the adjacent microtubules, but since they are connected by nexin, they cannot slide and the energy is converted into a bending motion of the microtubules (Ishikawa, 2017). Motile cilia are found in human airways, fallopian tubes, and the brain to generate mucus clearance, egg transportation, and cerebrospinal fluid circulation, respectively. Genetic mutations that impair ciliary beating are relevant to primary ciliary dyskinesia (PCD) and include mutations in axonemal dynein and factors related to dynein assembly (Fabczak & Osinka, 2019; Leigh et al., 2019).

Ruvb-Like AAA ATPase1 (Ruvbl1; also known as Pontin) is an evolutionarily conserved protein belonging to the adenosine triphosphates associated with diverse cellular activities (AAA+) superfamily of ATPases. Ruvbl1 was discovered in 1998 in large RNA polymerase II holoenzyme oligomers (Qiu et al., 1998). Ruvbl1 is associated with many cellular processes (Jha & Dutta 2009), including chromatin remodeling, transcription regulation (Jónsson et al., 2001), ribonucleoprotein complex biogenesis (Izumi

(2022003310001).

Authors' contributions

Conceptualization: Kim CY, Lee HS.
Data curation: Kim CY, Lee HK.
Methodology: Kim CY.
Writing-original draft: Kim CY.
Writing-review & editing: Kim CY, Lee HK, Lee H, Lee HS.

Ethics approval

This study was approved by Kyungpook National University (2021-0017).

et al., 2010), and mitotic assembly (Gartner et al., 2003). Ruvbl1 is highly expressed in mouse embryonic stem cells and Ruvbl1 knockout mice are lethal. It is also found in hematopoietic tissues, testes, lung, brain, and liver in adult mice and targeted knockout of Ruvbl1 in hematopoietic tissues results in bone marrow failure, suggesting that Ruvbl1 is essential for early embryogenesis as well as adults in mice (Bereshchenko et al., 2012). Various Studies have shown that Ruvbl1 and Ruvbl2 act as dynein assembly chaperones involved in cilia-associated processes (Li et al., 2017; Hartill et al., 2018). Conditional ablation of Ruvbl1 in mice results in renal disease and hydrocephalus, which suggests that Ruvbl1 is crucial for the development and function of monocilia and multicilia (Dafinger et al., 2018).

In this study, we investigated the developmental significance of Ruvbl1 in ciliogenesis using *Xenopus laevis* as an animal model. *X. laevis* embryos have several ciliated tissues, such as the gastrocoel roof plate, neural tube, and mucociliary epidermis, and are easily accessible for manipulation, observation, and analysis (Walentek & Quigley, 2017). *Ruvbl1* is strongly expressed in multiciliated cells of *X. laevis* as investigated by whole-mount *in situ* hybridization and the knockdown of *ruvbl1* significantly disrupts the basal body polarity, which affects cilia-driven fluid flow. Thus, we propose that Ruvbl1 affects basal body polarity during *X. laevis* embryogenesis and is crucial for cilia motility.

METHODS

1. *Xenopus laevis* growth conditions and *in vitro* fertilization

Mature *X. laevis* were obtained from the Korean *Xenopus* Resource Center for Research and maintained in plastic aquarium tanks circulated with dechlorinated water at 18°C (recommended by the Institutional Review Board of Kyungpook National University, Korea). Ovulation was induced in females by injecting of 1,000 IU of human chorionic gonadotrophin (hCG) into the dorsal lymph sac during the evening before the experiment. After 16 hours, the eggs were collected in 1 X MBS (88 mM NaCl, 5 mM Hepes, 2.5 mM NaHCO₃, 1 mM KCl, 1 mM MgSO₄, and 0.7 mM CaCl₂; pH 7.8) by squeezing the female. The eggs were fertilized after several washes with 0.1 X MBS using a sperm suspension solution from isolated testes. Following fertilization, the jelly coat was removed by swirling the embryos in 2% L-cysteine in a 0.5 X MBS solution. The embryos were washed several times with 0.5 X MBS and cultured in 0.5 X MBS at 15°C–18°C.

2. mRNA synthesis, morpholinos (MO) design, and microinjections

To visualize the basal bodies, mRNA for *centrin*-RFP and *clamp*-GFP were synthesized using the SP6 mMessage mMachine kit (Invitrogen, Carlsbad, CA, USA). For the knockdown experiment, morpholinos (MO) were synthesized to the following base composition: 5'-[CTC CTC GAT TTT CAT GGT GGA AG]-3' (Gene Tools, Philomath, OR, USA). Finally, mRNAs or MOs were injected into one-cell staged embryos, then the embryos were incubated at 18°C–23°C until the development reached the desired stages.

3. Whole-mount *in situ* hybridization (WISH)

The embryos were fixed at the desired stages in MEMFA (4% paraformaldehyde, 0.1 M MOPS (pH 7.4), 1 mM MgSO₄, 2 mM EGTA) overnight at 4°C. An antisense probe for *Ruvbl1* was synthesized using the T7 mMessage mMachine kit (Invitrogen). The probe was detected using an alkaline phosphatase-labeled anti-digoxigenin antibody (1:1,000, Roche, Basel, Switzerland) and NBT/BCIP (Roche).

4. Reverse transcription – polymerase chain reaction (RT-PCR)

Total RNA was extracted from embryos using the TRIzol™ Reagent (Invitrogen). cDNA was synthesized via reverse transcription using a Primescript™ 1st strand cDNA synthesis kit (Takara, Shiga, Japan) from RNAs. PCR was performed using specific primer pairs (Table 1) and EmeraldAmp® PCR Master Mix (Takara). The PCR products were separated on a 1% agarose gel and images were captured using WiseCapture I-1000 (Daihan Scientific, Wonju, Korea). The following primers were used:

5. Immunofluorescence and confocal microscopy

mRNA and MO-injected embryos were fixed at stage 32 in MEMFA. Acetylated tubulin (1:1,000, Sigma-Aldrich, St. Louis, MO, USA) was used as the primary antibody to observe the motile cilia in the embryos. The secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG (1:2,000, Invitrogen) and Alexa Fluor 568 goat anti-mouse IgG (1:2,000, Invitrogen). Imaging was performed with an Olympus FV1200 confocal microscope (Olympus, Shinjuku, Japan).

6. Velocity measurement of fluid flow and statistical analysis

The embryos were anesthetized with 0.1 X benzocaine. Then, fluorescent microbeads were added along the surface of the embryos and videos were recorded. The velocity of the fluorescent beads was measured using Tracker 6.0. Statistical analyses were performed by Prism 9 (GraphPad software). The results are means±SD of three different experiments. The student *t*-test was used to identify the level of significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS AND DISCUSSION

1. *Ruvbl1* is expressed in multiciliated cells during *Xenopus laevis* embryogenesis

To elucidate the developmental role of *Ruvbl1*, we analyzed the temporal and spatial expression patterns of *ruvbl1* using reverse transcription – polymerase chain reaction (RT-PCR) and whole-mount *in situ* hybridization (WISH) during *X. laevis* embryogenesis. *Ruvbl1* was a maternal gene and expressed generally through all stages of embryonic development, with particularly high expression at stages 8 (blastula), 25 (early tailbud), and 35–40 (late tailbud) (Fig. 1A). *Ruvbl1* was expressed as regular dots in the epidermis at stages 17, 20, 25, and 30 and was localized in the branchial arches, eyes, and brain at stages 25, 30, and 35. It was weakly expressed in the pronephros but highly expressed in the three nephrostomes at stage 35 (Fig. 1B). Based on the *ruvbl1* spatial expression in the epidermis and ciliated tissues (e.g., pronephros and brain), we hypothesized that *ruvbl1* is specifically expressed in multiciliated cells and performed WISH and immunofluorescence using acetylated tubulin antibodies in the same embryos. The WISH-IF data showed that *ruvbl1* was localized in the multiciliated cells of the *X. laevis* epidermis (Fig. 1C). These results suggest that *Ruvbl1* may play a crucial role in *X. laevis* embryogenesis and be involved in ciliogenesis.

2. *Ruvbl1* is required for ciliary-driven fluid flow in *Xenopus laevis* epidermis

Ruvbl1 was expressed in ciliated cells during *X. laevis* embryogenesis. *Ruvbl1* and *Ruvbl2* are outer dynein arm subunits crucial for ciliary movement (Lee et al., 2020). Ciliary movement

Table 1. Primer sequences for RT-PCR

Gene	Forward primer	Reverse primer
<i>odc</i>	5'-CAG CTA GCT GTG GTG TGG-3'	5'-CAA CAT GGAAAC TCA CAC C-3'
<i>ruvbl1</i>	5'-ATG GCG CTG AGC GAC-3'	5'-CGG AAG TTC CTG GAC-3'

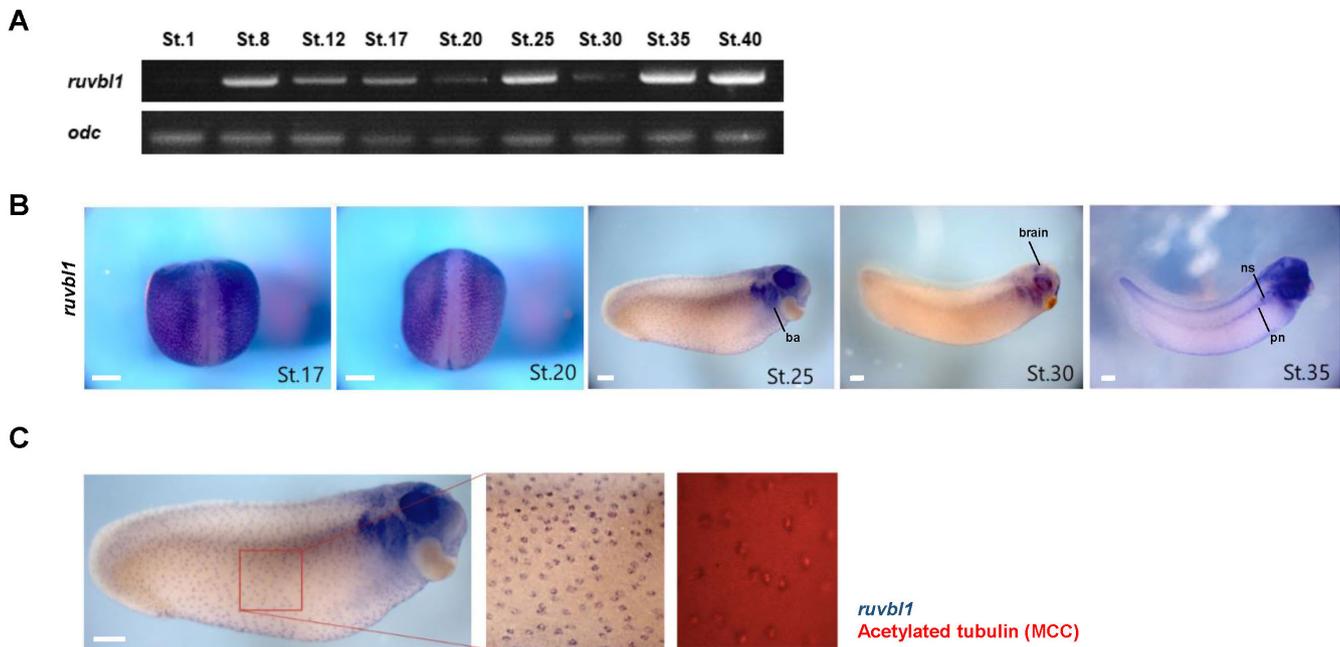


Fig. 1. Spatio-temporal expression patterns of *ruvbl1* during *Xenopus laevis* embryogenesis. (A) Temporal expression patterns by RT-PCR. *Ruvbl1* is expressed maternally throughout all stages. Ornithine decarboxylase (*odc*) is used as a loading control. (B) Spatial expression patterns via whole-mount *in situ* hybridization. Digoxigenin-labeled antisense probes for *ruvbl1* were used. At the late neurula stage (St. 17 and 20), *ruvbl1* is expressed as dots in the epidermis. At the tailbud stage, *ruvbl1* is expressed in the epidermis, branchial arches, eyes, and brain. At stage 35, *ruvbl1* is expressed in the pronephros and nephrostome (scale bar=250 μ m). (C) Double staining with WISH of *ruvbl1* (purple) and immunofluorescence for multiciliated cells using acetylated tubulin (red) on the same embryos (scale bar=250 μ m). ba, branchial arch; pn, pronephros; ns, nephrostomes.

generates the directed fluid flow essential for development and physiological processes, such as brain development and kidney function (Werner & Mitchell, 2012). The fluid flow in *X. laevis* epidermis functions in the defense against infections and is easily visualized using fluorescent microbeads. To investigate Ruvbl1 association with ciliary-driven fluid flow, we injected *ruvbl1* morpholino into *X. laevis* embryos at the one-cell stage to block translation and used fluorescent microbeads to observe the fluid flow in the epidermis. We found that *ruvbl1* morphants had a much slower fluid flow compared to the control MO-injected embryos (Fig. 2A and B), suggesting that Ruvbl1 is required for ciliary beating in the *X. laevis* epidermis.

3. Knockdown of *ruvbl1* disrupts basal body polarity

Defective fluid flow may be caused by a decreased number of cilia in multiciliated cells. To evaluate the role of Ruvbl1 in ciliogenesis during *X. laevis* embryogenesis, we stained *Ruvbl1* morphants using acetylated tubulin and observed multicilia in the epidermis. The knockdown of *ruvbl1* did not affect the length and amount of the cilia (Fig. 3A). Dafinger et al. (2018) reported that Ruvbl1 deletion in mice disrupts cilia formation and leads to renal disease and hydrocephalus. In this study, we examined the multiciliated cells only in the epidermis of *ruvbl1* morphants. Thus, additional studies of cilia in the pronephros and brain are needed to reveal whether Ruvbl1 in *X. laevis* ciliogenesis is consistent with that of mice.

Impaired fluid flow may also be due to disorganized basal body polarity within each multiciliated cell (Werner & Mitchell, 2012). Thus, we injected *centrin* - RFP and *clasp* - GFP with *ruvbl1* MO to examine the effect of *ruvbl1* knockdown on basal body polarity. In the control MO-injected embryos, the basal bodies were all polarized in a similar manner, but the *ruvbl1* morphants

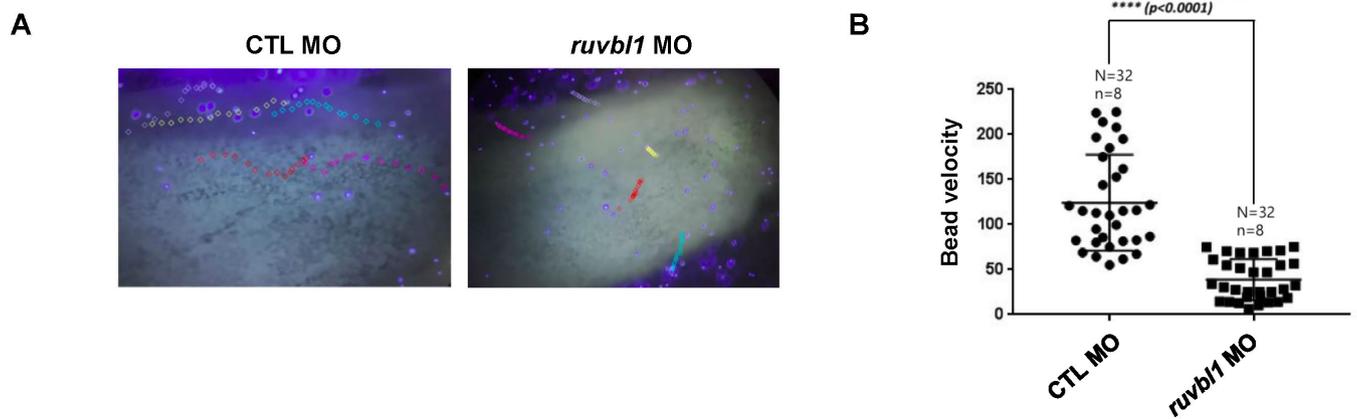


Fig. 2. Knockdown of *ruvbl1* perturbed ciliary movement in *Xenopus laevis* epidermis. (A) Control MO (32 ng) or *ruvbl1* MO (32 ng) was injected into one-cell staged embryos. Injected embryos were collected at stage 28 and treated with fluorescent microbeads on their head side. The microbead flow from head to tail was recorded using the Tracker 6.0 program. (B) Bead velocity graph. A total of 32 beads from 8 embryos for each control MO or *ruvbl1* MO-injected embryo. *Ruvbl1* morphants had significantly reduced microbead flow. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

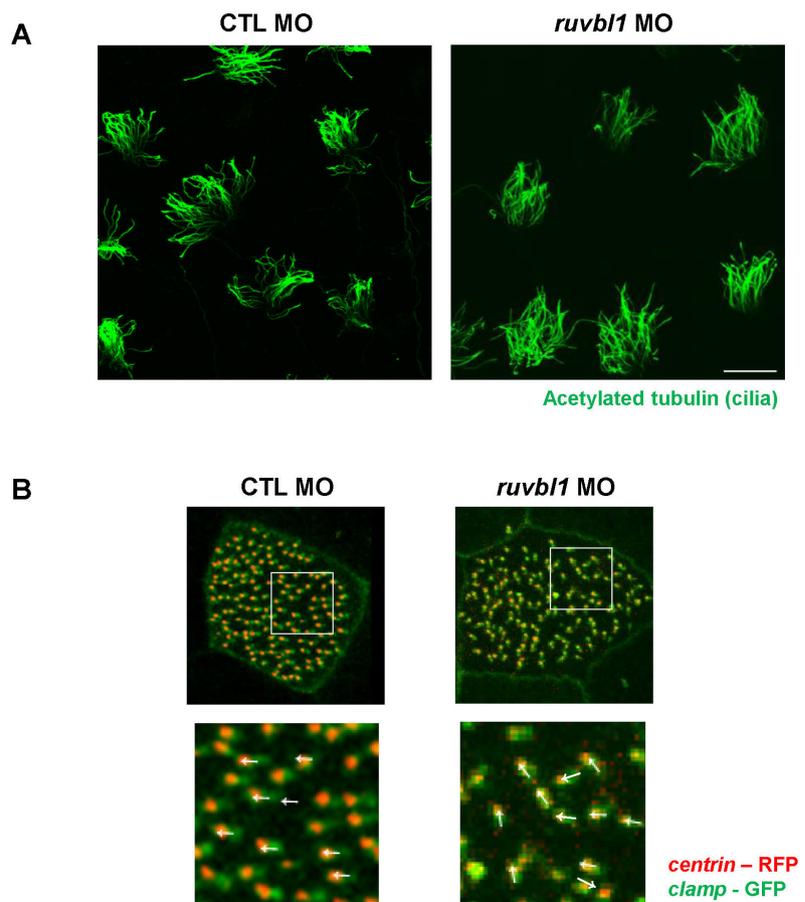


Fig. 3. Knockdown of *Ruvbl1* led to defective basal body polarity in *Xenopus laevis* embryos. (A) Embryos were injected with control MO or *ruvbl1* MO and fixed in MEMFA at stage 32. Motile cilia were stained green with acetylated tubulin and observed via confocal microscopy. The depletion of *ruvbl1* did not affect the number of cilia in multiciliated cells (scale bar=20 μ m). (B) Embryos were injected with *centrin*-RFP and *clamp*-GFP with each MO to visualize basal body polarity, which is illustrated by white arrows. *Ruvbl1* morphants had defective basal body polarity compared to the control MO-injected embryos.

displayed disruptive basal body polarity (Fig. 3B). These experimental results suggest that *ruvbl1* knockdown does not affect cilia length or shape, but does disturb basal body polarity, which can lead to defective fluid flow in the epidermis. A recent study in zebrafish has shown that the depletion of Ruvbl1 reduced both their inner and outer dynein (Li et al., 2017). Although we did not check the axonemal dyneins in the *ruvbl1* morphants, we determined that *ruvbl1* knockdown disrupts basal body polarity. This suggests a novel function of Ruvbl1 in multiciliated cells. Interactome analysis of Ruvbl1 identified several ciliary proteins and the involvement of Ruvbl1 in cilia-related processes via its interactions with several ciliary proteins, such as DNAAF1 (Dafinger et al., 2018; Hartill et al., 2018). Previous studies combined with our data prompted us to hypothesize that Ruvbl1 may be involved in both axonemal dynein assembly and cilia-related processes, including basal body polarization, via interacting ciliary proteins. Further studies are needed to determine the accuracy of this hypothesis. In conclusion, this study showed that Ruvbl1 is specifically localized in multiciliated cells and plays a crucial role in multicilia motility during *X. laevis* embryogenesis.

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