RESEARCH ARTICLE

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Nip2/Centrobin May Be a Substrate of Nek2 That Is Required for Proper Spindle Assembly During Mitosis in Early Mouse Embryos

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SUMMARY

Nek2 is a mitotic kinase with multiple cellular functions involving phosphorylation of diverse substrates. Suppression of *Nek2* in early mouse embryos has been shown to arrest development at the 4-cell stage with defects in mitotic spindle assembly as well as in interphase nuclear morphology. In the present study, we suppressed expression of two Nek2 centrosomal substrates, Nip2 and C-Nap1, in early mouse embryos. The development of the *Nip2*-suppressed embryo was arrested at the 4-cell stage with mitotic defects in the blastomeres. In contrast, *C-Nap1* suppression did not produce a visible phenotype. The phenotypic similarities of the *Nip2*- and *Nek2*-suppressed embryos suggest that Nip2 may be a substrate of Nek2 that is required for mitotic spindle assembly in early mouse embryos.

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INTRODUCTION

Phosphorylation is an important regulatory mechanism for execution of mitotic events. A single mitotic kinase can participate in multiple stages of mitosis by phosphorylating multiple substrates. For example, Plk1 is critical for mitotic entry, centrosome maturation, spindle assembly, the DNA damage checkpoint, and cytokinesis (reviewed in Petronczki et al., 2008). Plk1 phosphorylates diverse proteins to exert its regulatory functions at the centrosome, kinetochore and mid-body during the cell cycle.

Nek2 is a mitotic kinase whose activity oscillates during the cell cycle (Fry et al., 1995; reviewed in Fry, 2002). Subcellular distribution of Nek2 is dynamic during the cell cycle. Nek2 is associated with chromosomes up to metaphase, dissociates from them at the onset of anaphase, and is then detected at the midbody of the cytoplasmic bridge during telophase (Rhee and Wolgemuth, 1997; Kim et al., 2002). Nek2 has also been observed to localize to the centrosome throughout the cell cycle (Fry et al., 1998a; Kim et al., 2002).

A number of studies have indicated that Nek2 participates in multiple regulatory functions through phosphorylation of specific groups of substrates. Perhaps the best-characterized function of Nek2 is induction of centrosome separation, because overexpression of Nek2 induced premature centriole splitting (Fry et al., 1998a). C-Nap1 is thought to be a Nek2 substrate that contributes to centriole splitting since it is located at the proximal end of the centriole and its suppression also resulted in centriole splitting (Fry et al., 1998b; Mayor et al., 2000). Recently, another centrosomal protein, called Rootletin, was identified as a candidate linker between the mother and daughter centrioles, and it is also a substrate of Nek2 (Bahe et al., 2005).

In addition to centrosome separation, Nek2 is also critical for centrosome integrity and activity. In *Xenopus* embryos, depletion of Nek2 caused abortive cleavage with fragmented or dispersed centrosomes (Uto and Sagata, 2000).

Developmental defects were also observed in early mouse embryos, including abnormal nuclear morphology due to aberrant mitosis (Sonn et al., 2004). It was suggested that Nek2 may function as a priming kinase of Plk1 for activation of Nlp, a microtubule nucleation factor at the centrosome (Rapley et al., 2005). Involvement of Nek2 in regulation of the spindle checkpoint has also been suggested (Chen et al., 2002; Lou et al., 2004). However, substrates of Nek2 that participate in these processes remain to be identified.

We recently characterized a centrosomal protein called Nip2/Centrobin which we determined to be a substrate of Nek2 (Jeong et al., 2007). Nip2 was characteristically located at daughter centrioles but was also detected on nascent cellular microtubules and spindles (Zou et al., 2005; Jeong et al., 2007). Since its overexpression resulted in formation of microtubule bundles, we proposed that Nip2 may stabilize nascent microtubules (Jeong et al., 2007). Knockdown of *Nip2* resulted in defects in centrosome duplication (Zou et al., 2005) and reduction of the activity of the microtubule organizing center (MTOC) in both interphase and mitotic cells. As a result, *Nip2*-suppressed cells had reduced cellular surface area and defects in mitotic spindle assembly (Jeong et al., 2007).

In the present study, we determined the expression and subcellular localization of two Nek2 substrates, Nip2 and C-Nap1, in early mouse embryos. Furthermore, we compared knockdown phenotypes of *Nip2* and *C-Nap1* with those of *Nek2*. The results showed that *Nip2* suppression induced defects in mitosis whereas *C-Nap1* suppression did not produce a visible phenotype.

RESULTS

Expression and subcellular localization of Nip2 and C-Nap1, two known substrates of Nek2, were examined in early mouse embryos. RT-PCR analysis revealed that both the *Nip2* and *C-Nap1* mRNAs were present at constant levels during early embryogenesis (Fig. 1A). Centrosomal localization of the Nip2 and C-Nap1 proteins in early mouse embryos was also confirmed. Immunostaining analysis revealed localization of Nip2 and C-Nap1 to the acentriolar centrosomes of interphase blastomeres (Fig. 1B). In mitotic blastomeres, both Nip2 and C-Nap1 were localized at the spindle poles (Fig. 1C,D). This localization pattern in early mouse embryos is consistent with previous findings in tissue culture cells (Fry et al., 1998a; Jeong et al., 2007).

We suppressed embryonic expression of *Nip2* and *C-Nap1* by microinjecting specific dsRNAs into one-cell mouse embryos. dsRNA specific to GFP was injected as a negative control. Nip2 and C-Nap1 protein levels were undetectable within 3 days, as determined by immunoblot

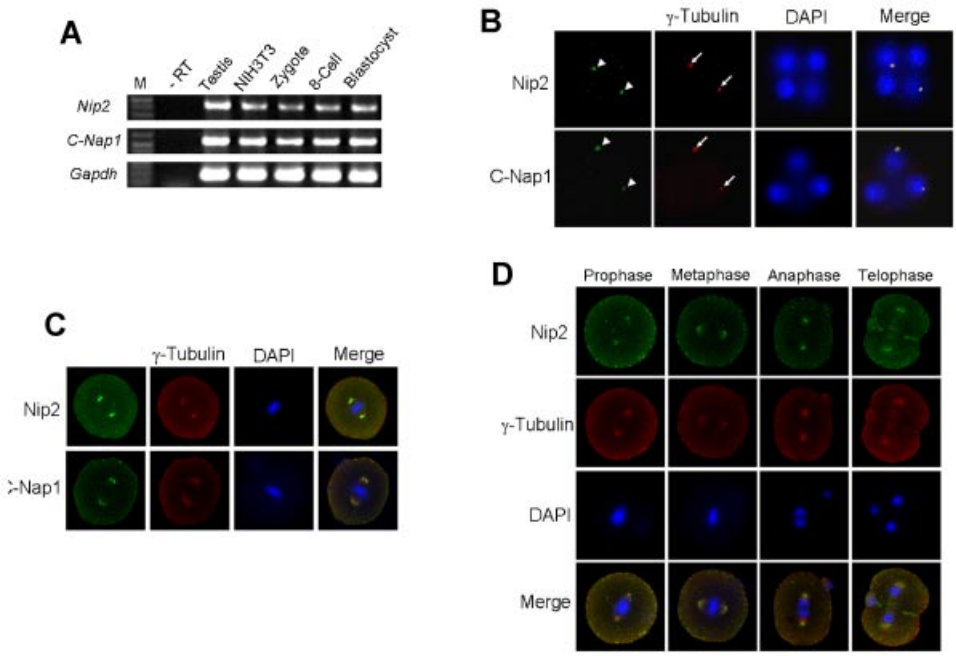

Figure 1. Expression and subcellular localization of Nip2 and C-Nap1 in early mouse embryos. **A**: RT-PCR analysis. Total RNA isolated from the mouse testis, NIH3T3 cells, and the indicated stages of embryos was reverse-transcribed followed by PCR-amplification with primers specific for *Nip2* and *C-Nap1*. *Gapdh* was detected as a control. **B**: Interphase blastomeres from 3- or 4-cell stage embryos were co-immunostained with antibodies specific to Nip2 or C-Nap1, along with γ-tubulin. DNA was stained with DAPI. **C**: A zygote at metaphase was immunostained with antibodies specific to Nip2 or C-Nap1. γ-Tubulin was co-immunostained and DNA was stained with DAPI. **D**: Immunostaining of the Nip2 protein during the first mitosis in mouse embryos. Zygotes at the first mitotic division were co-immunostained with antibodies specific to Nip2 and γ-tubulin. DNA was stained with DAPI. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com]

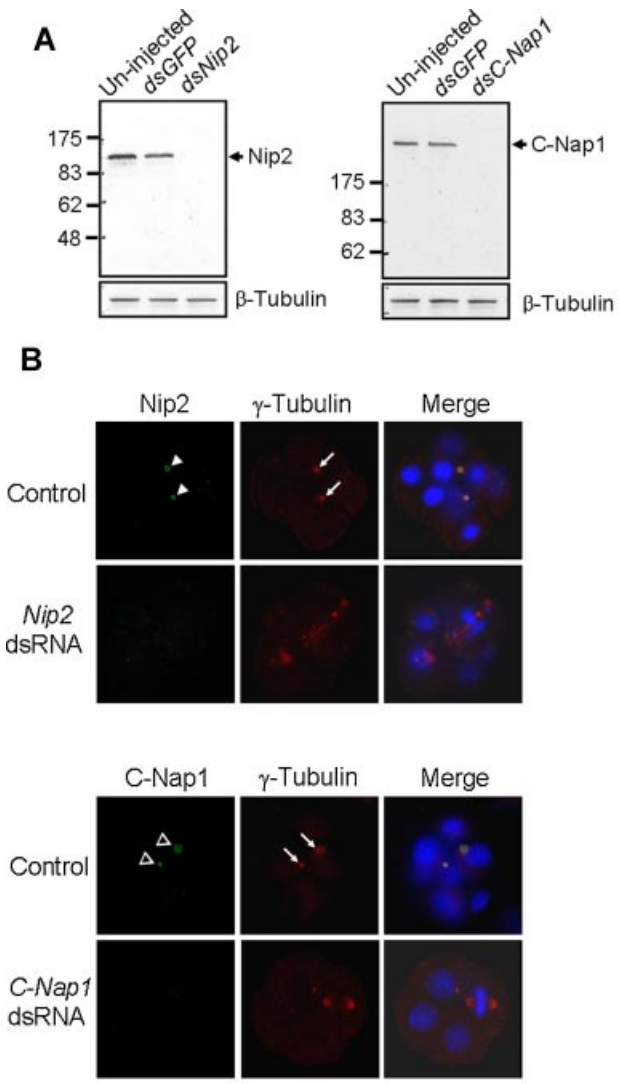

Figure 2. Suppression of *Nip2* or *C-Nap1* expression in the mouse early embryos. **A**: Double-stranded RNA specific to *Nip2* or *C-Nap1* was microinjected into mouse one-cell embryos. After being cultured for 3 days, the embryos were subjected to immunoblot analyses for detection of the Nip2 or C-Nap1 proteins. Un-injected embryos were used as a control, and β-tubulin was detected as a loading control. **B**: One-cell embryos microinjected with dsRNA specific to *Nip2* or *C-Nap1* were cultured for 72 hr and immunostained with antibodies specific to Nip2 and C-Nap1. Un-injected embryos were used as a control. DNA was stained with DAPI. Nip2, C-Nap1 and γ-tubulin are marked with closed arrowheads, open arrowheads and arrows, respectively. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com]

analysis (Fig. 2A). Immunostaining analysis showed that the Nip2- or C-Nap1-specific signals disappeared from the centrosomes of the interphase blastomeres, and from the spindle poles of the mitotic blastomeres (Fig. 2B). Nonetheless, the acentriolar centrosome appeared intact when immunostained with γ-tubulin antibody (Fig. 2B).

We observed the development of the *Nip2*- and *C-Nap1*-suppressed embryos. As expected, more than 80% of *GFP* dsRNA-injected embryos, as well as un-injected embryos, developed to the blastocyst stage after 4 days in culture (Fig. 3A). As reported previously, only 25% of the *Nek2*-suppressed embryos reached blastocyst stage (Fig. 3A; Sonn et al., 2004). Development of the *Nip2*-suppressed embryos was arrested at a rate comparable to that of *Nek2*-suppressed embryos (Fig. 3A). Suppression of *C-Nap1* expression did not affect embryo development (Fig. 3A). These observations suggest that Nip2 is required for early mouse embryogenesis, while C-Nap1 is not.

We analyzed the phenotypes of the *Nek2*- and *Nip2*-suppressed embryos by immunostaining using antibodies specific to β-tubulin and γ-tubulin (Fig. 3B). As reported previously, most of the *Nek2*-suppressed embryos did not develop beyond the 4-cell stage (Fig. 3A; Sonn et al., 2004). The blastomeres in the *Nek2*-suppressed embryos were arrested in either mitotic or interphase stages (Fig. 3B,C). Defects in spindle assembly and chromosome segregation were common in mitotic blastomeres of the *Nek2*-suppressed embryos, while abnormalities such as dumbbell-like nuclei and micronuclei were observed in interphase blastomeres (Fig. 3B,C).

Development of the *Nip2*-suppressed embryos was also arrested at the 4-cell stage (Fig. 3B). We observed mitotically arrested blastomeres in the *Nip2*-suppressed embryos but very few abnormalities in the interphase blastomeres (Fig. 3B,C). Multiple spindle poles with disorganized or extended spindles were observed most frequently in the *Nip2*-suppressed blastomeres (Fig. 3B).

DISCUSSION

In the present study, we observed that both Nip2 and C-Nap1 were localized to the centrosome in early mouse embryos. Furthermore, we compared the knockdown phenotypes of *Nip2* and *C-Nap1* with the knockdown phenotype of *Nek2*. Development of the *Nip2*-suppressed embryo was arrested at the 4-cell stage, with mitotic defects in the blastomeres. The *Nek2*-suppressed embryos were also arrested at the 4-cell stage. Many of the blastomeres were blocked at mitosis with defects in spindle assembly. The interphase blastomeres also revealed abnormal nuclear morphology, including dumbbell-like nuclei, nuclear bridges and micronuclei. These nuclear abnormalities in the *Nek2*-suppressed embryos most likely resulted from improper completion of the previous mitosis (Sonn et al., 2004). Phenotypic similarities of the *Nip2*- and *Nek2*-suppressed embryos indicate that Nip2 may be a substrate of Nek2, and may contribute to proper spindle assembly in mitosis of early mouse embryos.

Nip2 suppression in tissue culture cells produced phenotypes in both mitotic and interphase cells. In mitotic cells, defects in spindle assembly were typically observed, consistent with the results of *Nip2* knockdown in early mouse embryos (Jeong et al., 2007). In interphase cells, *Nip2* knockdown resulted in defects in centriole duplication (Zou et al., 2005). Furthermore, MTOC activity in the *Nip2*-suppressed cells was reduced, resulting in reduced cell size (Jeong et al., 2007). In the present study, we did not observe

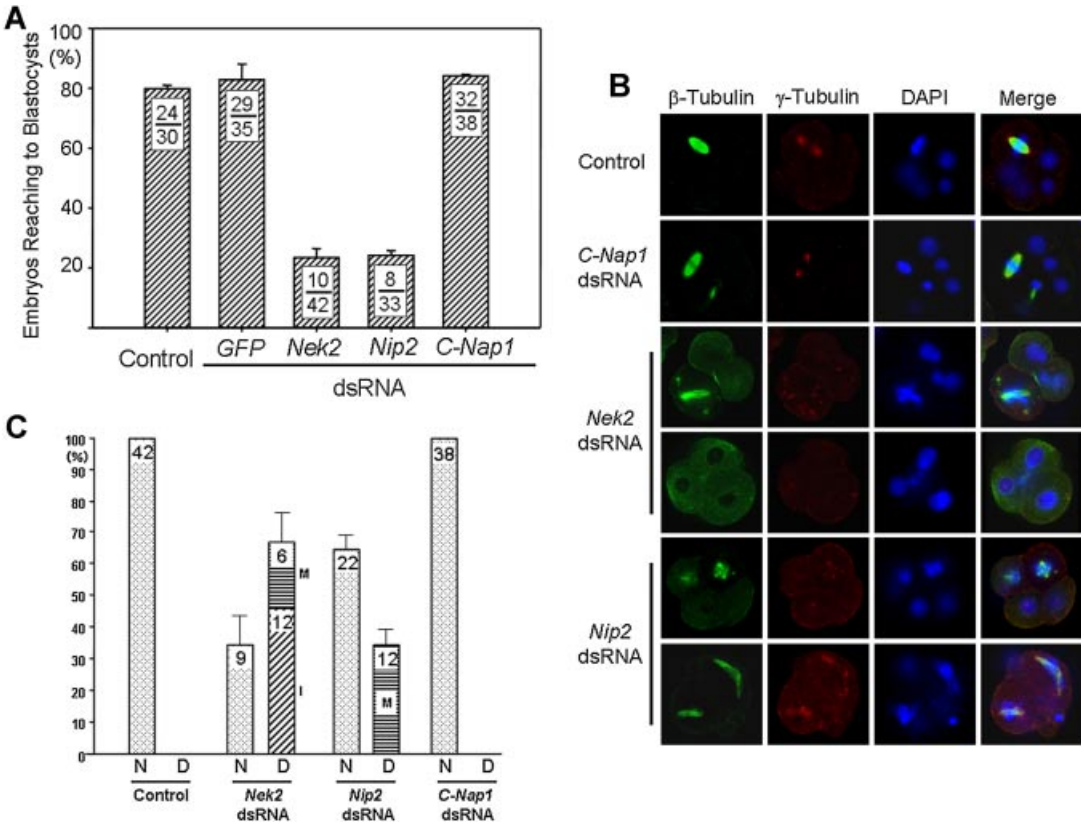

Figure 3. Effects of *Nip2* or *C-Nap1* suppression on development of early mouse embryos in culture. **A**: One-cell embryos microinjected with dsRNAs specific to *GFP*, *Nek2*, *Nip2*, or *C-Nap1* were cultured for 4 days, and the embryos that reached blastocyst stage were counted. Un-injected embryos were included as a control. The total number of embryos used in the experiments and the number of embryos reached to blastocysts were indicated within the bar. The experiments were repeated three times, and the data are presented as mean and standard error. **B**: Representative morphology of the early embryos in which *C-Nap1*, *Nek2*, or *Nip2* expression was suppressed by RNAi. The embryos were co-immunostained with antibodies specific to β-tubulin and γ-tubulin. DNA was stained with DAPI. **C**: The embryos microinjected with dsRNA specific to *GFP*, *Nek2*, *Nip2*, or *C-Nap1* were cultured for 3 days and the blastomeres with normal morphology (N) or with defects (D) at mitosis (M) or interphase (I) were counted. The number of embryos in the experimental group was indicated within the bar. The experiments were repeated three times, and the data are presented as mean and standard error.

an interphase phenotype in the *Nip2*-suppressed early mouse embryos. The centrosome in early mouse embryos lacks a centriole but consists of aggregates of pericentriolar materials including γ-tubulin (Szollosi et al., 1972; Meng et al., 2004). The structure and subcellular localization of the acentriolar centrosome appeared dynamic, with dispersal of polar aggregates during the cell cycle (Houliston et al., 1987). Roles of Nip2 in such acentriolar centrosome dynamics remain to be investigated.

It is somewhat surprising to detect C-Nap1 at the centrosomes of early mouse embryos, because it was previously shown to be localized to the proximal end of the centriole, where it is thought to connect the mother and daughter centrioles (Fry et al., 1998b; Bahe et al., 2005). We did not observe a noticeable phenotype in the *C-Nap1*-suppressed embryos. *C-Nap1* expression was knocked down sufficiently, since the C-Nap1 protein levels in the knockdown embryos were below detection levels in both immunoblot and immunohistochemical analyses. Rather, C-Nap1 may be involved in a novel cellular function that is dispensable for early embryonic development of the mouse. In fact, our preliminary work revealed that C-Nap1 interacts with multiple centrosomal proteins in addition to Nek2 and Rootletin (K.R., unpublished work). We are currently investigating novel functions of C-Nap1 by analyzing the C-Nap1 interaction network.

MATERIALS AND METHODS

Embryo and Oocyte Collection, Microinjection and Culture

Superovulated FVB female mice were mated and checked for vaginal plugs in the next morning. The one-cell zygotes were collected from oviducts by tearing ampulla region with fine forceps in CZB medium containing hyaluronidase (1 mg/ml, Sigma, St. Louis, MO) to remove the cumulus cells. Microinjection was performed following a standard procedure (Nagy et al., 2002). The one-cell embryos were placed in a CZB medium containing 20 mM HEPES and 5 mM sodium bicarbonate, under pre-equilibrated

washed mineral oil for 10 min prior to micromanipulation (Chatot et al., 1989). A holding pipette was used to hold the one-cell embryos stationary during manipulation. The injection pipette loaded with dsRNA solution that were microinjected into the cytoplasm of the zygotes using a constant flow system (Transjector, Eppendorf, Hamburg, Germany). Each zygote was injected with 10 pl dsRNA. Un-injected or injected embryos were then cultured in CZB medium containing 0.5% BSA (Sigma) under paraffin oil in sterile culture dishes in an atmosphere of 5% CO_2 in air at 37°C. On day 2 afternoons when embryos reached 4-cell stage, embryos were washed several times and transferred to the CZB medium without EDTA but supplemented with glucose (1 mg/ml).

Double-stranded RNA Preparation

Double-stranded RNA was prepared by annealing two complementary RNAs transcribed by T7 or SP6 RNA polymerase in vitro. The cDNA fragments for dsRNA were initially subcloned into the *pGEM-T* vector. The *Nek2* dsRNA was a 501 bp-fragment at the kinase domain common to both *Nek2A* and *Nek2B* starting from 5′-CGA ACC AAC ACA ACC CTG TA-3′ to 5′-GCC ATC AGA GTA GCG GTA GG-3′. The *Nip2* dsRNA was a 408 bp-fragment starting from 5′-GAA AGG CAG GCC TGG ATC AA-3′ to 5′-TTG GTG CTG TCC ACT CAG TT-3′. The *C-Nap1* dsRNA was a 690 bp-fragment starting from 5′-GAG GAC CTA GAA CAG ATC AA-3′ to 5′-CTC AGC ACA CCG TTG TTG AA-3′. The *GFP* dsRNA was a 443 bp-fragment starting from 5′-CAC ATG AAG CAG CAC GAC TT-3′ to 5′-ACG AAC TCC AGC AGG ACC AT-3′.

After RNAs were synthesized using the T7 and SP6 RNA polymerase (Roche, Mannheim, Germany), DNA templates were removed with the DNase I treatment. The RNA products were extracted with phenol/chloroform, and ethanol-precipitated. To anneal sense and antisense RNAs, equimolar quantities of both sense and antisense RNAs were mixed in the annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) to a final concentration of 2 μM each, heated for 1.5 min at 94°C, and incubated at room temperature for several hours. To remove leftover single-stranded RNA, the mixture was treated with 2 μg/ml RNase T1 (Calbiochem, Darmstadt, Germany) and 1 μg/ml RNase A (Sigma) for 30 min at 37°C. The dsRNA was treated with 140 μg/ml proteinase K (Sigma), phenol/chloroform extracted, ethanol precipitated, washed in 75% ethanol and dissolved in water. The quality of dsRNA was confirmed by running an agarose gel. The dsRNA samples were diluted to a final concentration of 2–4 mg/ml and stored at −70°C before use.

Quantification of mRNA in the Embryos

Total RNAs were isolated by the acid guanidium thiocyanate–phenol–chloroform extraction method as reported previously (Khang et al., 2005). Ten embryos were added into a tube containing 300 μl of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% *N*-lauryl sarcosyl, and 0.1 M 2-mercaptoethanol) on ice, and immediately plunged into liquid nitrogen for storage until use. After thawing, 0.1 volume of 2 M sodium acetate (pH 4.0), one volume of water-saturated phenol, and 0.2 volume of chloroform-isoamyl alcohol (49:1) were added. After vortex-mixing, the mixture was incubated on ice for 10 min. Total RNA was then fractionated by centrifugation at 12,000 rpm for 15 min at 4°C and precipitated from supernatant in the presence of one volume of isopropanol. The pellet was washed with 75% ethanol, and dissolved in water.

For reverse transcription, the RNA sample was heated in the presence of 100 pmol random hexanucleotides in a final volume of 8 μl at 65°C for 5 min. After brief centrifugation at 4°C, 12 μl of master mix [200U RNaseH-MMLV reverse transcriptase, 4 μl dNTP mix (2.5 mM each), 1 μl RNasin (26 U/μl), 2 μl 0.1 M DTT, and 4 μl of 5 × RT buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol)] was added, and the reaction mixture was incubated at 37°C for 1 hr. The reaction was terminated by incubating the sample at 75°C for 15 min.

The amounts of specific mRNA were determined with reverse transcription-PCR based methods. To quantify the *Nip2* mRNA, we used a pair of the following primers that generate a 457 bp-fragment; 5′-CTG ATG ATC ACA GGG CCG AA-3′ to 5′-TGT TCC AGC CTC TTC AGG AT-3′. For quantification of *C-Nap1* mRNA, we used a primer set which resulted in a 579 bp fragment: 5′-GAG CAG TGC AGG TCA GTC TT-3′ and 5′-CGT CCT CTG ATC TTC CAG AA-3′. The PCR amplification was carried out with 2 μl of RT reaction mixture in 20 μl of PCR reaction solution containing 2 μl of 10 × PCR buffer, 1.6 μl dNTP mix (2.5 mM each), 10 pmol each of PCR primers, and 1U Ex-Taq polymerase (Takara, Seoul, Korea). The sample was subjected to a 35 cycle-amplification on Gene Amp PCR System 2400 (Perkin Elmer, Fremont, USA). Six microliters of PCR products were analyzed on 1% agarose gel electrophoresis.

Immunocytochemical Staining of Embryo and Oocyte

Embryos were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, neutralized with 50 mM NH_4Cl in PBS for 10 min, and post-permeabilized with 0.25% Triton X-100 in PBS for 10 min. Immunocytochemical staining was performed by incubating the fixed samples with the anti-Nip2 or C-Nap1 polyclonal antibodies (Jeong et al., 2007) in PBST (PBS containing 0.1% Triton X-100 and 3% BSA) for 60 min, followed by an incubation in FITC-conjugated anti-rabbit Ig antibodies (KPL) diluted 1:200 in PBST for 40 min. Likewise, immunocytochemical staining was performed by incubating the fixed samples with the anti-β-tubulin mouse monoclonal antibody or anti-γ-tubulin goat polyclonal antibody diluted 1:200 in PBST for 60 min, followed by an incubation in FITC-conjugated anti-mouse IgG antibodies (KPL) diluted 1:200 (in PBS/Triton X-100) for 40 min. Samples were observed under a Zeiss fluorescence microscope.

Immunoblot Analysis

Protein samples from embryos, testis, or cultured cells were solubilized in the Laemmli sample buffer, resolved by 8% SDS–PAGE, and blotted onto a nitrocellulose membrane. The membrane was blocked by soaking in Blotto (Tris-buffered saline with 0.3% Triton X-100 and 5% nonfat dried milk) for 1 hr 30 min, and incubated overnight with the primary antibody in the blocking solution. The membrane was then washed three times with TBST (Tris-buffered saline with 0.3% Triton X-100), incubated with a secondary antibody conjugated with horseradish peroxidase for 45 min, and washed 5 times with TBST. The signal was detected with the ECL Western blotting detection reagents (Amersham, Braunschweig, Germany) following the manufacturer's recommendations.

Statistical Analysis

Data for *Nip2* RNAi analysis were statistically evaluated using Student's *t*-test or one-way analysis of variance followed by Fisher's least significant difference test for a post hoc comparison. Statistical significance was set at $P < 0.05$.

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