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TPRA40/GPR175 Regulates Early Mouse Embryogenesis Through Functional Membrane Transport by Sjögren's Syndrome-Associated Protein NA14

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TPRA40/GPR175 is an orphan receptor whose physiological functions have not been found to date. In an attempt to generate transgenic mice that express an shRNA of TPRA40, we observed that the cell division of early mouse embryos that injected the short hairpin RNA expression vector was significantly accelerated compared with the control vector. The regulation of cell division by TPRA40 was also observed in HeLa cells. Since the C-terminal region of TPRA40 has been shown to be essential for the regulation of cell division, we performed yeast two-hybrid screening using the C-terminal region as bait. Nuclear antigen of 14 kDa (NA14), an autoantigen of Sjögren's syndrome, was identified as a binding protein to the C-terminal region of TPRA40. The binding of TPRA40 and NA14 was confirmed by GST pull-down assay and co-immunoprecipitation assay. FLAG-TPRA40 is transported from the cytosol to the plasma membrane in time-dependent manner and the translocation was inhibited by GFP-NA14ΔN, an N-terminal deletion mutant that cannot bind to microtubules but binds to TPRA40. TPRA40ΔC, which cannot bind to NA 14, shows impaired transport to the plasma membrane. Finally, we found that the effect of TPRA40 on mouse embryogenesis is strengthened by GFP-NA14, but not by GFP or GFP-NA14ΔN. These observations indicate that the functional plasma membrane transport of TPRA40 that regulates cell division of mouse embryos is mediated by NA14.

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G protein-coupled receptors (GPCRs) comprise a large superfamily of genes accounting for about 1% of the human genome, and have attracted much attention because approximately 50% of drugs target GPCRs (Drews, 1996). GPCRs transmit signals from the extracellular environment to the intracellular cytoplasm through activation by their cognate ligands (Marchese et al., 1999; Civelli et al., 2001). Although large numbers of orphan GPCRs were discovered during the course of the genome project (Horn et al., 1998, 2003), the cognate ligands and physiological functions of many of these orphan receptors remain unknown. GPCRs regulate intracellular signal transduction pathways, at least in part, through the generation of reactive oxygen species (ROS). For example, the generation of ROS is required for the activation of vascular smooth muscle cell proliferation by angiotensin II (Griendling et al., 1994) and thrombin (Patterson et al., 1999). Angiotensin II and thrombin also activate Jak/STAT pathways through the activation of Rac small GTPase and the subsequent generation of ROS (Pelletier et al., 2003). During oxidative stress caused by ischemia/reperfusion, we have demonstrated that mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) translocates from the cytoplasm to the nucleus, and is activated in the nucleus in rat heart (Mizukami and Yoshida, 1997). The nuclear translocation and activation of ERK are dependent on the activations of phosphoinositide-3 kinase (PI3K) and protein kinase C (PKC)ζ, suggesting that the PI3K/PKCζ/ERK pathway is activated during oxidative stress (Mizukami and Yoshida, 1997; Mizukami et al., 1997, 2000). ERK maintains contractility and survival during reperfusion in rat cardiomyocytes through the c-Myc-mediated up-regulation of glycolytic α-enolase, as revealed by a proteomic approach (Mizukami et al., 2004). Since the PI3K/ERK pathway is activated by GPCRs (Stephens et al., 1994; Cantrell, 2001), it is possible that GPCRs might be responsible for the activation of the PI3K/PKCζ/ERK and the subsequent cellular responses during oxidative stress.

We have screened GPCRs whose expressions are regulated during hypoxia and reoxygenation, and identified two orphan GPCRs. The expression of TPRA40 (a 40 kDa transmembrane protein regulated in adipocytes; corresponds to mouse GPR175) declines rapidly during hypoxia and reoxygenation (Fujimoto et al., 2001), whereas GPR30 is up-regulated during reoxygenation after hypoxia (Kimura et al., 2001). Although we have revealed that GPR30 activates a p53-dependent apoptotic pathway during oxidative stress caused by ischemia/reperfusion (Kimura et al., 2001), no physiological functions for TPRA40

Abbreviations: TPRA40, trans-membrane protein of 40 kDa regulated in adipocytes; NA14, nuclear autoantigen of 14 kDa; SS, Sjögren's syndrome; MT, microtubule; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; shRNA, short hairpin RNA; DAPI, 4′,6-diamidino-2-phenylindole; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ROS, reactive oxygen species; GST, glutathione *S*-transferase; RT, reverse transcription; PCR, polymerase chain reaction.

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have been revealed to date. TPRA40 is a seven transmembrane domain protein whose expression is induced during the differentiation of 3T3-L1 cells from fibroblasts to adipocytes (Yang et al., 1999). We have demonstrated that TPRA40 contains a longer (84 amino acids) cytoplasmic region (Fujimoto et al., 2001), compared with the relatively short (13 amino acids) C-terminal putative cytoplasmic region of the initially identified TPRA40 (Yang et al., 1999). The additional 71 amino acids include several consensus sequences for post-translational modifications including PKC phosphorylation, *N*-glycosylation, and myristoylation, indicating the possible importance of the C-terminal region for the functional expression of TPRA40 (Fujimoto et al., 2001). TPRA40 shows low homology to other orphan receptors. Although a combined phylogenetic/ligand analysis showed that each GPCR can be assigned to one of four classes of GPCRs (A, B, C, and F/S), TPRA40 is classified into a fifth GPCR group that cannot be assigned to any of the known classes (Vassilatis et al., 2003). It has been reported that the expression of TPRA40 is increased in the epididymal fat of 24-month-old mice as compared with young controls, and is also increased in obese and diabetic mice as compared with age-matched controls (Yang et al., 1999), suggesting that TPRA40 expression is regulated by environmental conditions. Despite the lack of structural as well as physiological clues to uncover the function of TPRA40, the regulation of TPRA40 expression during oxidative stress, aging, and under pathophysiological conditions suggests that TPRA40 should play important roles in cellular responses related to diseases.

During an attempt to generate knockdown mice with a reduced expression of TPRA40, we found that TPRA40 regulates the cell division of early mouse embryos. Using a yeast two-hybrid system, nuclear autoantigen of 14 kDa (NA14), an autoantigen of Sjögren's syndrome, an inflammatory disease (Ramos-Morales et al., 1998), was identified as a TPRA40 binding protein. The functional plasma membrane expression of TPRA40 is mediated by NA14, and TPRA40 involved in the regulation of cell division in early mouse embryos.

Experimental Procedures

Materials

Anti-FLAG mouse monoclonal antibody was purchased from Sigma (St. Louis, MO). Anti- green fluorescent protein (GFP) mouse monoclonal antibody was purchased from Santa Cruz (Santa Cruz, CA). Peroxidase-conjugated anti-mouse and -rabbit IgG antibodies were purchased from Promega (Madison, WI). Anti-actin antibody and anti-FLAG mouse monoclonal antibody-immobilized beads and glutathione Sepharose 4B were purchased from Sigma and Amersham-Pharmacia (Buckinghamshire, UK), respectively. Streptavidin magnet particles were from New England Biolabs (Beverly, MA). Peroxidase-conjugated streptavidin was from Invitrogene (Carlsbad, CA). All other reagents were commercially available.

Production of polyclonal antibody

A rabbit polyclonal antibody against TPRA40 was generated using a peptide corresponding to a region of rat TPRA40 (amino acids 139–149) as an antigen. Sera were collected and the antibody was purified by ammonium sulfate precipitation.

Plasmids

To create TPRA40 short hairpin RNA (shRNA) expression vectors, two pairs of synthesized oligo DNAs (type I; sense strand 5′-TCCCAAATGCCAAGTGGACGAGGCTTTCAAGAGAAGCCTCGTC-CACTTGGCATTTTT-3′ and antisense strand 5′-CAAAAAAATGCCAAGTGGACGAGGCTTCTCTTGAAAGCCTCGTCCACTTGGCATTT-3′, type II; sense strand 5′-TCCCAAGATCCTGTGGGAAATCACCTTCAAGAGAGGTGATTTCCCACAGGATCTTTT-3′ and antisense strand 5′-CAAAAAAAGATCCTGTGGGAAATCACCTCTCTTGAAGGTGATTTCCCACAGGATCTT-3′) were annealed and ligated into psiRNA-Hh1neo-CX-EGFP, which was constructed by ligating the pCX-EGFP gene (Hasuwa et al., 2002) as a marker gene into psiRNA-Hh1neo (Invivogene, San Diego, CA). The mutation 9 and 10 for TPRA40 shRNA-II were created with a point mutation at the position 9 and 10 of the targeting sequence for the shRNA-II, respectively, as indicated in boldfaces (Pusch et al., 2003) (type II mutation 9; sense strand 5′-TCCCAAGATCCT**A**TGGGAAATCACCTTCAAGAGAGGTGATTTCCCA**T**AGGATCTTTT-3′ and mutation 9; antisense strand 5′-CAAAAAAAG-ATCCT**A**TGGGAAATCACCTCTCTTGAAGGTGATTTCCCA**T**AGGATCTT-3′, type II mutation 10; sense strand 5′-TCCCAAGATCCTG**C**GGGAAATCACCTTCAAGAGAGGTGATTTCCC**G**CAGGATCTTTT-3′ and mutation 10; antisense strand 5′-CAAAAAAAGATCCTG**C**GGGAAATCACCTCTCTTGAAGGTGATTTCCC**G**CAGGATCTT-3′). An N-terminal FLAG-tagged TPRA40 (FLAG-TPRA40) expression vector was created by ligating the full-length rat TPRA40 cDNA, which was cloned previously (Fujimoto et al., 2001), into p3xFLAG-CMV10 (Sigma). A C-terminal deletion mutant of FLAG-TPRA40 (FLAG-TPRA40ΔC) was created by PCR using the sense primer 5′-GCGAATTCAATGGCCAGCCTG-3′, antisense primer 5′-CGGGATCCTCAGGGTTCAGA-3′, and FLAG-TPRA40 as a template. Gateway Technology (Invitrogene) was used to create the GFP- and GST-tagged NA14 expression vectors. Human NA14 cDNA was amplified from human kidney cDNA with sense primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACCCAGCAGGGCGCG-3′ and antisense primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACACACTTGCTGGGGCTGAG-3′. The PCR product was cloned into pDONR207 to create the entry clone (pDONR207NA14). Then, N-terminal-tagged FLAG-NA14, GFP-NA14, and glutathione *S*-transferase (GST)-NA14 expression vectors were created using a site-specific recombination reaction according to the instruction manual. A N-terminal GFP-tagged deletion mutant of NA14, GFP-NA14ΔN, was created by ligating a N-terminal deletion fragment of human NA14 (amino acids 22–129) to pEGFP-C1 (Clonetech, Palo Alto, CA). To create pT7-NA14, PCR was carried out with sense primer 5′-TAATACGACTCACTATAGGATGACCCAGCAGGGCGC-3′, antisense primer 5′-TAATACGACTCACTATAGGACACTTGCTGGGGCGAG-3′, and pDONR207NA14 as a template. The PCR product was ligated to pUC13 and used as a template for in vitro transcription. The nucleotide sequences were confirmed by sequencing.

Injection to mouse embryos

The microinjection of vector DNA into mouse embryos was performed using standard methods (Hogan et al., 1994). In brief, fertilized one-cell embryos were collected from the oviducts of superovulated C57BL/6 female mice (8 weeks old). The purified vector DNA (7 ng/μl) was injected into the pro nuclei of the zygotes. The surviving embryos were cultured in a microdrop of M16 medium in an atmosphere of 95% air/5% CO_2 at 37°C, and were observed under a microscope for 4 days after injection. The significant variation was unobserved in no injection control. A few empty vectors may be influenced in the cell division. The development of embryos was determined simultaneously in the comparative group (empty vector vs. expression vector).

Author Proof

Yeast two-hybrid screening

The C-terminal fragment of rat TPRA40 (amino acids 286–369) was subcloned into a bait vector pGBKT7 to fuse the GAL4 DNA binding domain. The yeast strain AH109 was first transformed with the bait vector and was subsequently transformed with a prey vector of a human kidney cDNA library (pACT2) fused with the GAL4 transcriptional activation domain. Approximately 2×10^6 transformants were screened for histidine prototrophy in the presence of 5 mM 3-aminotriazole.

Cell culture and transfection

COS7 cells and HeLa cells were maintained in DMEM supplemented with 10% FBS at 37°C under a 5% CO_2 atmosphere. All plasmids were transfected using LipofectAMINE2000 (Invitrogen) according to the instructions provided by manufacturer as described previously (Mizukami et al., 2004).

FACS analysis

Cells harvested with PBE (5 mM EDTA in phosphate buffered-saline with 0.5% bovine serum albumin) were fixed with 70% ethanol and incubated with phosphate-buffered saline containing 5 μg/ml propidium iodide and 5 μg/ml RNase for 30 min at room temperature. At least, 10,000 cells were counted per samples. FACS analyses were performed using a BD Bioscience flow cytometer (FACSCalibur).

GST pull-down, immunoprecipitation, and immunoblotting

For GST pull-down experiments, COS7 cells grown to approximately 80% confluence on 10 cm diameter dishes were transfected with 10 μg GST-NA14 or FLAG-TPRA40 for 48 h. Then, the cells were lysed in lysis buffer [1% Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride], and cellular extracts were mixed and incubated with glutathione Sepharose 4B. Then, the beads were precipitated and washed three times with lysis buffer, and the co-precipitated proteins were subjected to immunoblotting. Immunoblotting was carried out as described previously (Mizukami et al., 2004). For co-immunoprecipitation experiments, COS7 cells were grown to approximately 80% confluence in 3.5 cm diameter dishes and transfected with 2 μg each of FLAG-TPRA40 vector and GFP-NA14 vector. Forty-eight hours after transfection, the cells were washed with PBS and lysed in lysis buffer. The cellular extracts were then incubated with anti-FLAG antibody-immobilized beads overnight at 4°C. Then, the beads were collected by centrifugation and washed three times with lysis buffer. The precipitates were eluted from the beads by incubation in SDS–PAGE sample buffer at 95°C for 3 min, and were used for immunoblotting. In vivo GST pull-down experiments were performed as the co-immunoprecipitation experiments except that the GST-NA14 vector and glutathione Sepharose 4B were used instead of GFP-NA14 and anti-FLAG antibody-immobilized beads, respectively.

Selection of transfected-cells by magnet particles

To select cells, the pMACS4.1 vector, which expresses a truncated CD4 cell surface marker, was co-transfected with FLAG vectors as described previously (Mizukami et al., 2004). Briefly, 24 h after transfection, the cells were selected with a magnet beads-bound antibody for CD4, followed by the selection by G418 (final concentration: 750 μg/ml). The selected cells were observed under a bright field microscope on days 4–7 after transfection, and the numbers of cells were counted.

Fluorescence microscopy

For immunofluorescence experiments, cells were grown to approximately 50% confluence on coverslips and transfected with vectors as described previously (Kimura et al., 2001). After transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and made permeable by incubation in 0.5% Triton X-100 in PBS for 5 min. Then, the cells were blocked with 10% FBS in PBS for 30 min, and incubated with anti-FLAG antibody overnight at 4°C. After incubation with a Cy3-conjugated anti-mouse IgG antibody and DAPI (final concentration: 1 μg/ml) for 30 min, the cells were observed under an inverted fluorescence microscope (IX70, Olympus, Japan[Q1]). Images were processed using MetaMorph software (Universal Imaging Corp[Q2].).

RNA interference

A small interference RNA (siRNA) mixture for NA14 was created using a Short Cut RNAi kit (New England Biolab) according to the instructions. In brief, both the sense and antisense full-length RNAs of NA14 were created by in vitro transcription using T7 RNA polymerase and pT7-NA14 plasmid as a template. The sense and anti-sense RNAs were annealed to create double-stranded RNA, which was then digested to 21–23 base pair fragments by Short Cut RNase. Complete digestion was checked by polyacrylamide gel electrophoresis followed by ethidium bromide staining. The siRNA mixture was transfected into cells at a concentration of 4 nM.

Cell surface labeling of FLAG-TPRA40

Cells were incubated with 4 μM sulfo-NHS-biotin (Pierce) in PBS for 30 min at 4°C, and inactivated by washing three times in 4 mM glycine in PBS. Cells were lysed by sonication in lysis buffer and incubated with streptavidin magnet beads at 4°C for 1 h. Then, the beads were washed with lysis buffer three times and the biotinylated proteins were eluted from the beads by incubation in SDS–PAGE sample buffer at 95°C for 3 min.

Reverse transcription (RT)-polymerase chain reaction (PCR) method

Fertilized one-cell embryos were collected from the oviducts of superovulated C57BL/6 female mice (8 weeks old), and were cultured in a microdrop of M16 medium in an atmosphere of 95% air/5% CO_2 at 37°C for 2 days. The embryos were resuspended in lysis buffer in Cell-to-Signal kit (Applied Biosystems, Foster City, CA) after removal of zona pellucida. First-strand cDNA was synthesized directly using chemicals from the Cells-to-Signal kit and primed by pd (N)10 primers. mRNA expressed in mouse brain was directly extracted by using MagNA Pure LC system with MagNA Pure LC mRNA Isolation Kit II (tissue), according to the manufacturer's instructions (Roche Diagonistics, Mannheim, Germany). RT was performed with mRNA and random primer using SUPERSCRIPT II (Invitrogene). The products of the RT reaction were amplified by 40 cycles of PCR using KOD dash (TOYOBO, Osaka, Japan) at 94°C for 30 sec, 62°C for 30 sec, and 74°C for 30 sec for NA14, and 94°C for 30 sec, 62°C for 2 sec, and 74°C for 30 sec for TPRA40 and G3PDH. The 5′-ACCGGACCATCGCAGAAAC-3′ (sense) and 5′-TCAGCTGTCCTTGCCTCCA-3′ (antisense) primers for NA14, 5′-CACTGAAGGAGCGTGTATC-3′ (sense) and 5′-ACAGCAGAGCCCCACAAT-3′ (antisense) primers for TPRA40, or 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTG-3′ (antisense) primers for G3PDH were used to observe the expression. The

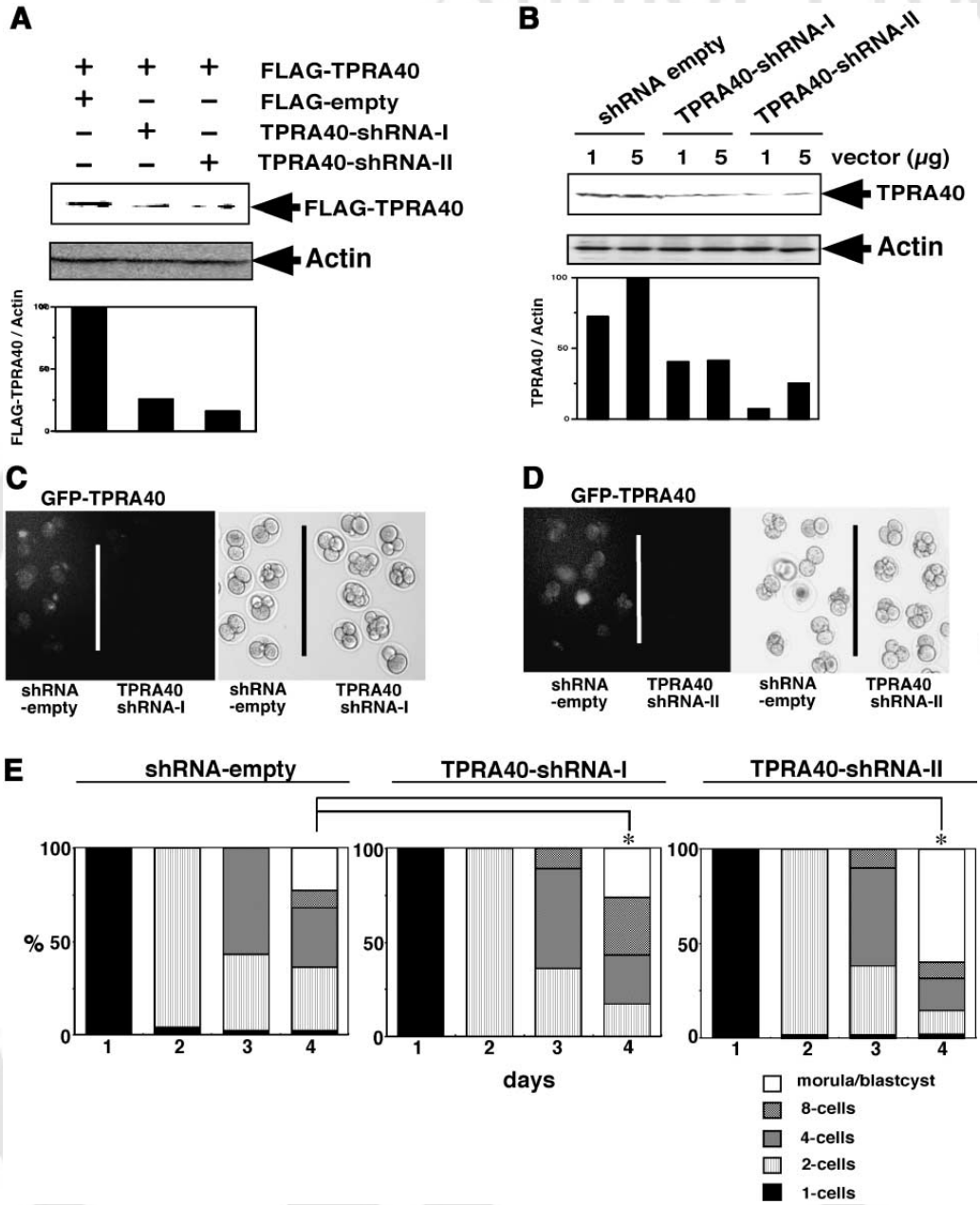

Fig. 1. Effect of short hairpin RNA-mediated knockdown of TPRA40 on cell division of mouse embryos. A,B: Short hairpin RNA expression vectors, TPRA40-shRNA-I and -II, were transfected into COS7 cells with the FLAG-TPRA40 expression vector (A) or into H9c2 cells without the FLAG-TPRA40 expression vector (B). Forty-eight hours after transfection, cell extracts were prepared and subjected to immunoblotting with an anti-FLAG antibody (A) or an anti-TPRA40 antibody (B). Similar results were obtained in two separate experiments. C,D: The GFP-TPRA40 expression vector and shRNA-I (C), -II (D), or shRNA-empty vector removed short hairpin sequences (C,D) as the indicated, were simultaneously injected into mouse embryos 1 day after fertilization, were incubated in a drop of M16 medium, and were observed at 48 h after the injection under a fluorescence microscopy (left parts). Similar results were obtained in three separate experiments. The embryos were also observed under a bright field (right parts). E: A short hairpin RNA expression vector, TPRA40-shRNA-I, TPRA40-shRNA-II, or shRNA-empty vector removed the short hairpin sequence, was injected into mouse embryos 1 day after fertilization and the embryos were incubated in a drop of M16 medium. Forty-six (shRNA-empty vector), 50 (TPRA40-shRNA-I), 53 (TPRA40-shRNA-II) embryos were used in each group (*$P < 0.05$ for shRNA-empty vector; Mann–Whitney's *U*-test). F: The GFP-TPRA40 expression vector and shRNA-II vector, mutation 9 with a point mutation at the position 9 of the targeting sequence for shRNA-II, or mutation 10 with a point mutation at the position 10 were simultaneously injected into mouse embryos 1 day after fertilization, were incubated in a drop of M16 medium, and were observed at 48 h after the injection under a fluorescence microscopy (upper parts). The embryos were also observed under a bright field (lower parts). Similar results were obtained in three separate experiments. G: TPRA40-shRNA-II, mutation 9 or mutation 10, was injected into mouse embryos 1 day after fertilization and the embryos were incubated in a drop of M16 medium. Fifty-three (shRNA-II for TPRA40), 50 (mutation 9), 50 (mutation 10) embryos were used in each group (*$P < 0.05$ for TPRA40-shRNA-II; Mann–Whitney's *U*-test). The development of the embryos was observed at the indicated times under a bright field microscope during the 4 days after fertilization.

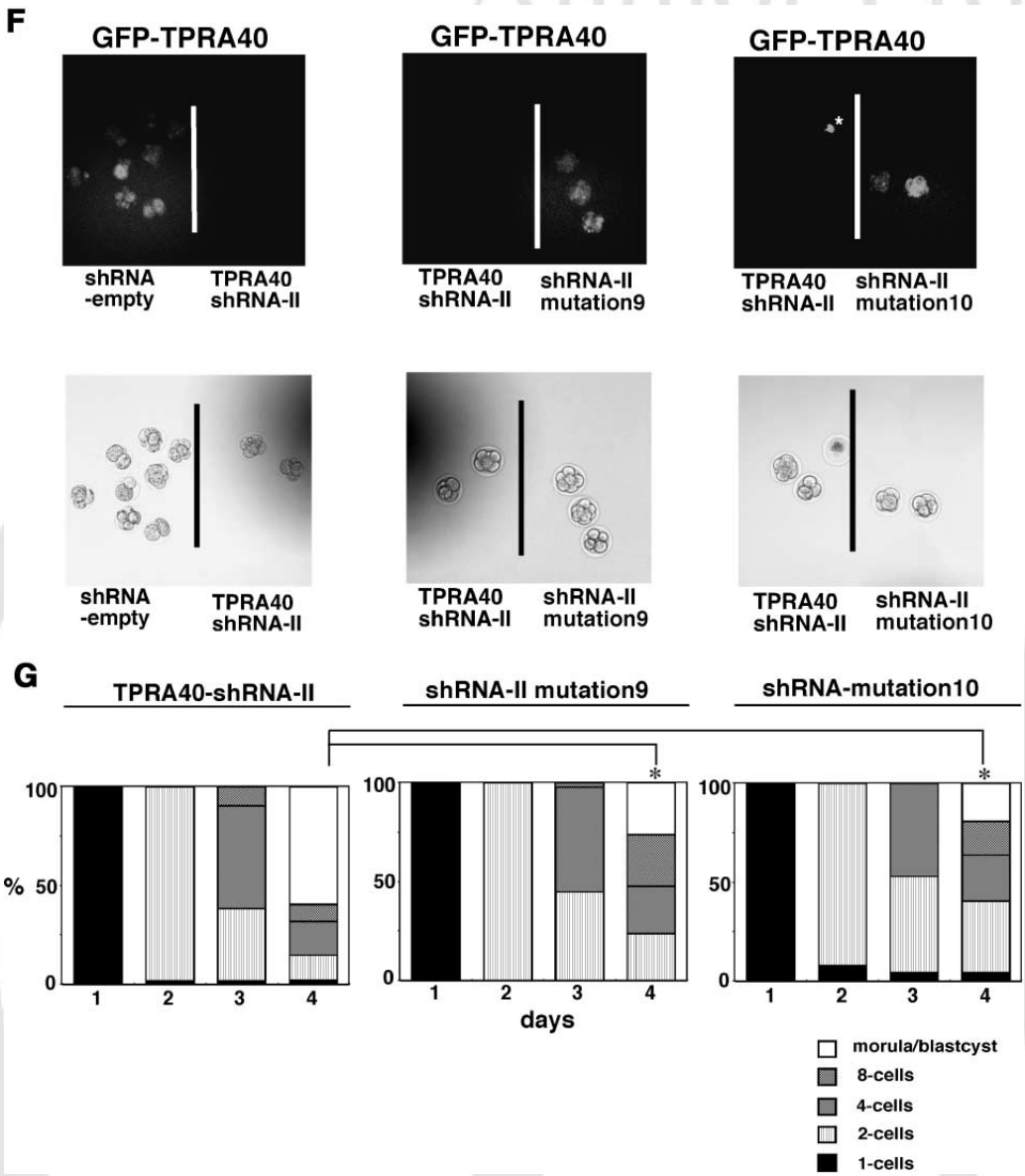

Fig. 1. (*Continued*)

amplified fragments were separated by electrophoresis on a 1.8% agarose gel.

Results

Knockdown of TPRA40 by RNA interference accelerates cell division of early mouse embryos

To investigate the physiological role of TPRA40, we first tried to generate transgenic mice that express a shRNA of TPRA40. Two candidate sequences for the shRNA were selected from the region in which the nucleotide sequences are perfectly conserved in the mouse and rat TPRA40 cDNAs. The effects of these shRNAs on TPRA40 expression were examined by immunoblotting using anti-FLAG and anti-TPRA40 antibodies for exogenous and endogenous TPRA40 expression. As shown in Figure 1A, both vectors (TPRA40 shRNA-I and -II) suppressed FLAG-TPRA40 expression more than approximately 50% in COS7 cells. In H9c2 cells, endogenous TPRA40 expression was also suppressed by these shRNAs (Fig. 1B). To confirm that shRNAs work in mouse early embryos, TPRA40 shRNA-I or -II was injected into the embryos with GFP-TPRA40. ShRNAs for TPRA40 inhibited the GFP-TPRA40 expression, indicating the shRNAs were useful for the embryos (Fig. 1C,D). We next examined the effect of microinjecting the shRNA expression vector on the development of mouse embryos. Of 46 embryos injected with shRNA-empty vector, 14 embryos developed to the 8-cell stage by 4 days after fertilization (Fig. 1E). On the other hands, of 50 embryos and 53 embryos injected the TPRA40 shRNA-I and shRNA-II vector, respectively, more than 26 (56%) embryos developed to the 8-cell stage or more by the same time (Fig. 1E, $P < 0.05$ vs. vector control). To confirm the findings, we prepared the mutant 9 and 10 with a point mutation at the position 9 and 10 of the target sequence for shRNA-II, respectively. The both mutants for shRNA-II entirely restored in GFP-TPRA40 expression to control level (Fig. 1F), and the

cell divisions in early embryos injected the mutants were considerably slow as compared with those injected shRNA-II for TPRA40, and were similar to the data in the embryos injected shRNA-empty vector (Fig. 1G). These results suggest that TPRA40 negatively regulates the cell division of the early mouse embryos.

The C-terminal region of TPRA40 is required for the regulation of mouse embryogenesis

To determine the region essential for the effect of TPRA40 on cell division, the proliferation of HeLa cells expressing FLAG-TPRA40 or its C-terminal deletion mutant, FLAG-TPRA40ΔC (Fig. 2A), was examined by counting cell numbers for 4 days. As shown in Figure 2B, TPRA40 significantly decreased the proliferation of HeLa cells compared with the control ($P < 0.05$ vs. vector control) at days 2 and 3. The proliferation of cells expressing TPRA40ΔC was unchanged for 4 days compared with the control (Fig. 2B). These results suggest that TPRA40 regulates HeLa cell proliferation through its C-terminal region. To determine at which stage of the cell cycle is involved in the proliferation by TPRA40, we performed the cell cycle analysis upon serum stimulation in HeLa cells expressing TPRA40. As shown in Figure 2C,D, overexpression of FLAG-TPRA40 induced a decrease in cell population in G2/M phase and an increase in cell population in G1 phase compared with HeLa cells expressing empty vector. In contrast, there was no difference in profile of cell population between empty vector- and TPRA40ΔC-expressing cells (Fig. 2C,E). These results suggests that overexpression of TPRA40 may participates in G2/M arrest. We next examined the effects of TPRA40 and TPRA40ΔC on the development of mouse embryos. Of the TPRA40-injected 32 embryos, 11 (35%) developed to the 8-cell stage by 4 days after fertilization (Fig. 3), while approximately 51% of the control vector-injected embryos developed to the 8-cell stage (Fig. 3). These observations indicate that the development in the embryos injected FLAG-TPRA40 cDNA was delayed as compared with that in FLAG-empty vector, consistent with the results in HeLa cell transfected with FLAG-TPRA40. The TPRA40ΔC-injected embryos developed at the same rate as vector control by 4 days after fertilization (Fig. 3). These observations indicate that TPRA40 regulates the proliferation/division of cells through its C-terminal region.

Screening of TPRA40-interacting proteins by the yeast two-hybrid system

To identify binding proteins for the putative C-terminal cytoplasmic tail of TPRA40, we carried out yeast two-hybrid screening with a cDNA library derived from human kidney using the C-terminal fragment of TPRA40 as bait. Of ~200,000 transformants, 31 clones that grew in the absence of histidine, tryptophan, adenine and leucine were isolated. Among them, 3 clones turned out to be positive in the α-galactosidase spot assay (data not shown). Clone 6 encodes an N terminal-deleted partial fragment of human NA14 (Nuclear Autoantigen for Sjögren's syndrome of 14 kDa), a protein identified as an antigen recognized by the autoimmune serum of a patient with Sjögren's syndrome (Ramos-Morales et al., 1998). The characterizations of the other clones are now in progress.

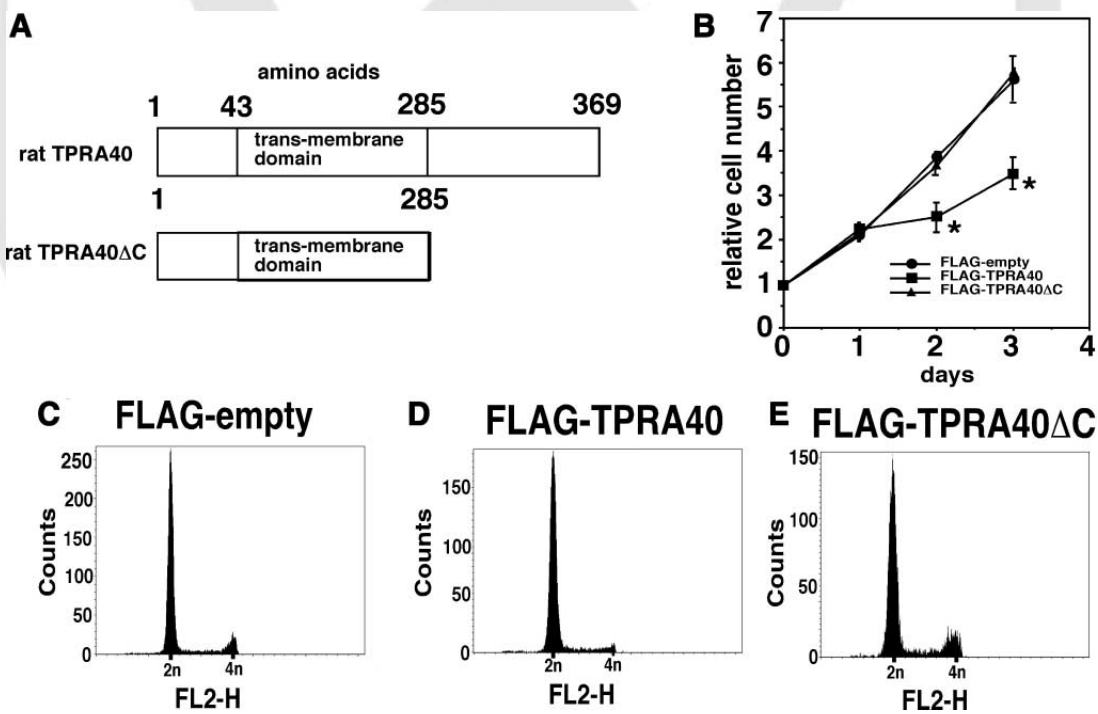

Fig. 2. Effects of TPRA40 on the proliferation of HeLa cells (A) Schematic representation of the structures of rat TPRA40 and its C-terminal deletion mutant. B: HeLa cells were transfected with the indicated vectors in addition to pMACS4.1, which encodes a truncated human CD4 surface molecule, and selected with anti-CD4 antibody and G418. Cells were counted under a bright field microscope for 4 days after selection (mean ± SE, n = 3; *$P < 0.05$ vs. control vector). C–E: HeLa cells transfected with the indicated vectors, were stimulated by serum for 20 h after the serum removal for 24 h, and subjected to FACS analysis as described under Experimental Procedures. Similar results were obtained in three separate experiments.

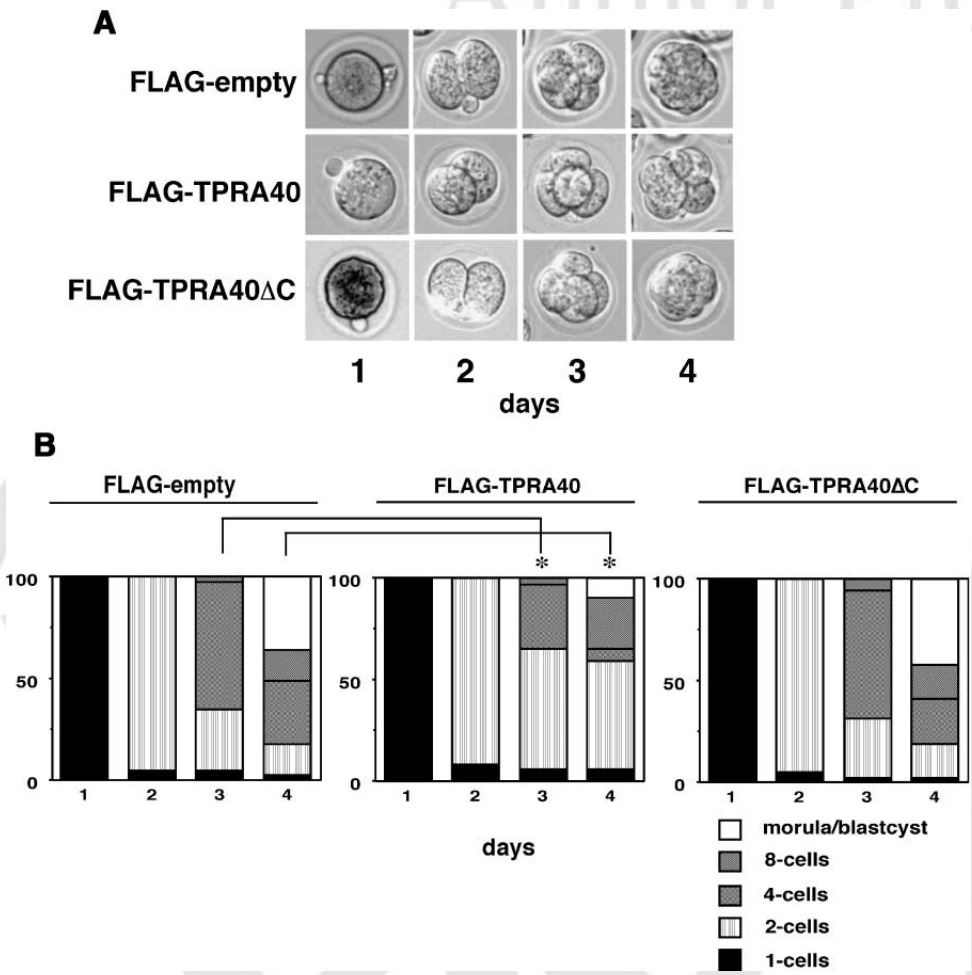

Fig. 3. Effects of TPRA40 on the cell division of mouse embryos 1 day after fertilization, embryos were injected with the indicated vectors and incubated in a drop of M16 medium. The embryos were observed at the indicated times under a bright field microscope. Thirty-nine (FLAG-empty), 34 (FLAG-TPRA40), or 38 (FLAG-TPRA40ΔC) embryos were used for each group (*$P < 0.05$ for FLAG-empty; Mann–Whitney's *U*-test). Typical images of embryos (part A) and summarized data of the developmental stages (part B) are shown.

TPRA40 interacts with NA14 in vitro and in vivo

To verify the binding of TPRA40 to NA14 in vitro, we performed GST pull-down assays with mixtures of COS7 cell lysates transfected with either FLAG-TPRA40 or GST-NA14. As shown in Figure 4A, a band with a molecular mass corresponding to approximately 40 kDa was detected only in the lysate transfected with FLAG-TPRA40 (Fig. 4A, lane 4), but not in control lysate (Fig. 4A, lane 1), indicating the expression of FLAG-TPRA40. Cell lysates transfected with FLAG-TPRA40 or GST-NA14 were mixed, pulled-down with GST-NA14, and analyzed by immunoblotting with an anti-FLAG-antibody. FLAG-TPRA40 was detected in the precipitate pulled-down with GST-NA14 (Fig. 4A, lane 6), but not in the precipitate pulled-down with GST (Fig. 4A, lane 5). FLAG-TPRA40 was undetected in precipitates not transfected with FLAG-TPRA40 (Fig. 4A, lanes 2 and 3). These results indicate that TPRA40 binds to NA14 in vitro. To test the interaction between TPRA40 and NA14 in vivo, FLAG-TPRA40 and GFP-NA14 were co-transfected into COS7 cells. Immunoblotting analyses using an anti-FLAG-antibody showed that comparable amounts of FLAG-TPRA40 were detected only in cell lysates transfected with FLAG-TPRA40 (Fig. 4B, lanes 1–4). Comparable expressions of GFP and GFP-NA14 were also confirmed using an anti-GFP antibody (Fig. 4B, lanes 5–8). Then, the cell lysates were immunoprecipitated with an anti-FLAG antibody and analyzed by immunoblotting using an anti-GFP antibody. GFP-NA14 was detected only in the precipitate transfected with FLAG-TPRA40 and GFP-NA14 (Fig. 4B, lane 12). No bands were observed in the precipitates transfected with FLAG-TPRA40 and GFP (Fig. 4B, lane 11), or in the precipitates transfected with FLAG-empty (Fig. 4B, lanes 9 and 10). These results indicate that TPRA40 interacts with NA14 in vivo. To access the specificity of the binding between NA14 and TPRA40, we examined whether FLAG-GPR30, another orphan receptor, also binds to NA14. GST-NA14 and either FLAG-TPRA40 or FLAG-GPR30 were co-transfected into COS7 cells. FLAG-TPRA40 was detected in the precipitate pulled-down with GST-NA14 while FLAG-GPR30 was not detected (Fig. 4C, lanes 5 and 6). The expressions of FLAG-TPRA40 and FLAG-GPR30 in COS7 cells were almost the same (Fig. 4C, lanes 1–3). These results indicate that TPRA40 specifically interacts with NA14.

NA14 co-localizes with TPRA40 in HeLa cells

We first examined the subcellular localizations of NA14 with distinct tag-polypeptides, FLAG-NA14 and GFP-NA14, in HeLa

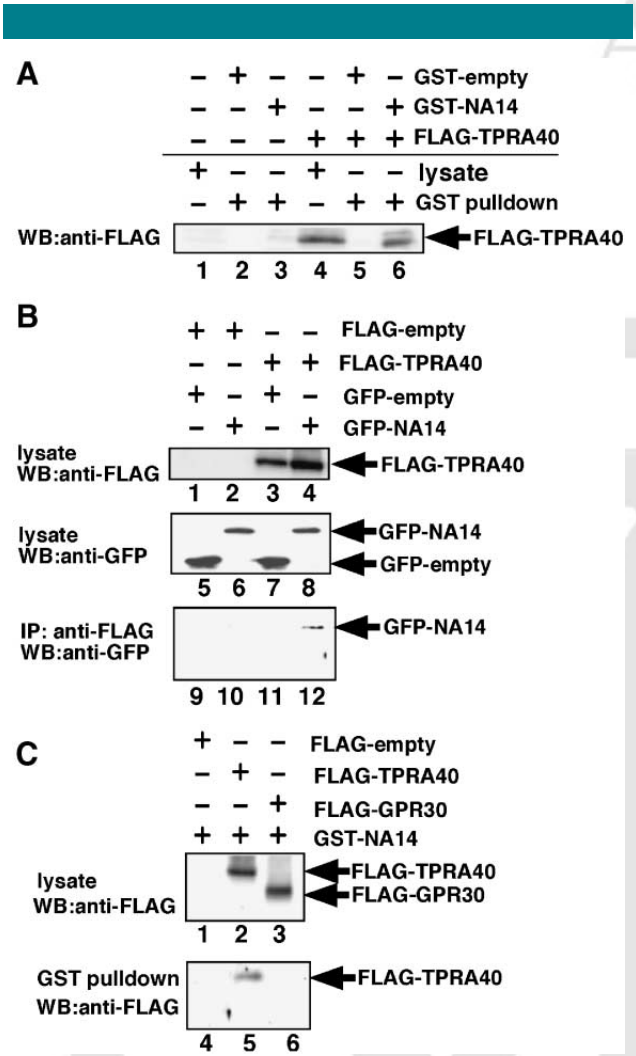

Fig. 4. Binding of TPRA40 and NA14 in vitro and in vivo (A) COS7 cells were transfected with the GST-NA14 or FLAG-TPRA40 expression vector for 48 h, and extracted as described in Experimental Procedures Section. Then the cell lysates were incubated at 4°C over night with glutathione-immobilized beads, precipitated, and subjected to immunoblotting with an anti-FLAG antibody (lanes 1–6). Similar results were obtained in three separate experiments. B: COS7 cells were co-transfected with the indicated vectors for 48 h and extracted. Cell lysates were subjected to immunoblotting with both anti-FLAG (lanes 1–4) and anti-GFP (lanes 5–8) antibodies to confirm the expressions of the proteins. Immunoprecipitation was performed with anti-FLAG antibody-immobilized beads, and the precipitated proteins were subjected to immunoblotting with an anti-GFP antibody (lanes 9–12). Similar results were obtained in three separate experiments. C: COS7 cells were co-transfected with GST-NA14 in combination with FLAG-TPRA40 or FLAG-GPR30 for 48 h and extracted. The cell lysates were subjected to immunoblotting with an anti-FLAG antibody to confirm the expressions of the proteins (lanes 1–3). The cell lysates were also incubated with glutathione-immobilized beads at 4°C overnight, and subjected to immunoblotting with an anti-FLAG antibody (lanes 4–6). Similar results were obtained in three separate experiments.

cells to select useful tag-polypeptides for immunocytochemical studies. As shown in Figure 5A, GFP-derived fluorescence (green) and anti-FLAG antibody-derived fluorescence (red) were largely merged (yellow), suggesting that both FLAG- and GFP-tags do not affect the subcellular localization of NA14. When FLAG-TPRA40 and GFP-TPRA40 were co-expressed in HeLa cells, FLAG-TPRA40 inserted into the plasma membrane and GFP-TPRA40 was distributed largely on the endoplasmic reticulum (data not shown). Therefore, FLAG and GFP as tag-polypeptides for TPRA40 and NA14, respectively, were used for further experiments. GFP-NA14 and FLAG-TPRA40 were co-transfected to HeLa cells and the localizations of these proteins were analyzed 12, 24, 36, and 48 h after transfection. Both GFP-NA14 and FLAG-TPRA40 showed punctuated localization in the cytoplasm (Fig. 5B, e–g and l–k) and partially co-localized during 12–36 h after transfection as indicated by the arrowheads (Fig. 5B, m–o). Forty-eight hours after transfection, FLAG-TPRA40 was largely transported to the plasma membrane, and small amounts of FLAG-TPRA40 were observed in the cytoplasm (Fig. 5B, h). These results indicate that FLAG-TPRA40 is transported to the plasma membrane in a time-dependent manner, and co-localizes with GFP-NA14 during the process of plasma membrane transport.

Plasma membrane transport of TPRA40 is inhibited by NA14ΔN

Since NA14 was previously reported to be associated with microtubules (MTs), we examined whether NA14 is involved in the transport of TPRA40 to the plasma membrane through MTs. NA14 contains a typical coiled-coil motif, which is speculated to be necessary for the association with MTs, at its N-terminus. The effect of N-terminal-deleted NA14 (NA14ΔN) on the plasma membrane transport of TPRA40 was examined. Immunolocalization analysis showed that GFP-NA14ΔN (Fig. 6A, o and u) was diffused widely throughout the cytoplasm in contrast to GFP-NA14 (Fig. 6A, n and t), indicating that the N-terminal coiled-coil motif of NA14 is required for its association with MTs. Co-transfection of GFP-NA14ΔN inhibited the plasma membrane transport of FLAG-TPRA40 (Fig. 6A, i and u). However, when GFP or GFP-NA14 was co-transfected, FLAG-TPRA40 was transported to the plasma membrane (Fig. 6A, g, h, s, and t). These results indicate that NA14ΔN, which cannot binds to MTs, inhibits the plasma membrane transport by dissociating FLAG-TPRA40 from MTs. FLAG-TPRA40ΔC was not transported to the plasma membrane if co-transfected with GFP, GFP-NA14, or GFP-NA14ΔN (Fig. 6A, j, k, l, v, w, and x), indicating that the interaction with NA14 is necessary for TPRA40 to be transported to the plasma membrane.

Biotinylation analysis of cell surface proteins

The involvement of NA14 in the plasma membrane transport of TPRA40 was further examined by biotinylation assays of cell surface proteins. As shown in Figure 6B, biotinylated FLAG-TPRA40 was detected by streptavidin-conjugated peroxidase, indicating the plasma membrane expression of FLAG-TPRA40. Amounts of biotinylated FLAG-TPRA40 were clearly increased when the FLAG-TPRA40 was co-transfected with GFP-NA14 than when it was con-transfected with GFP (Fig. 6B, lanes 1 and 2). In the cells transfected with GFP-NA14ΔN, no biotinylated FLAG-TPRA40 was detected under our experimental conditions (Fig. 6B, lane 3). The amounts of FLAG-TPRA40 were almost the same in all samples (Fig. 6B, lanes 4–6). These results indicate that NA14 regulates plasma membrane transport of TPRA40 in a positive manner that requires the N-terminal region of NA14.

Knockdown of NA14 by RNA interference inhibits TPRA40 transport

To confirm that NA14 is involved in the membrane transport of TPRA40, we next examined the effect of knockdown of NA14 by RNA interference. To test the effect of a small interference RNA (siRNA) mixture on NA14, a siRNA mixture was co-transfected with GFP-NA14, and GFP fluorescence in the living cells was observed 48 h after transfection. When

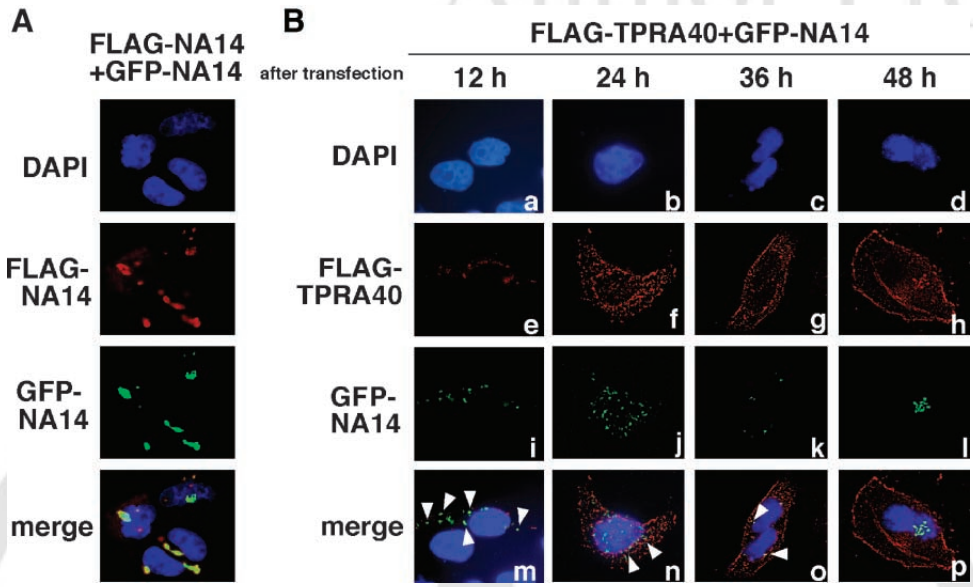

Fig. 5. Localization of TPRA40 and NA14 in HeLa cells FLAG-NA14 and GFP-NA14 expression vectors (A) or FLAG-TPRA40 and GFP-NA14 expression vectors (B) were transfected into HeLa cells, and the cells were incubated for 48 h (A) or the indicated time (B). The cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. A: Nuclei were stained with 1 μg/ml DAPI (blue). FLAG-NA14 was stained with an anti-FLAG antibody and then by incubation with a Cy3-conjugated anti-mouse IgG antibody (red). GFP-NA14 was visualized by the native fluorescence of GFP (green). A merged image is also shown. Final magnification 1,000×. Similar results were obtained in a separate experiment. B: Nuclei were stained with 1 μg/ml DAPI (DAPI; a–d, blue). FLAG-TPRA40 was stained with an anti-FLAG antibody followed by incubation with a Cy3-conjugated anti-mouse IgG antibody (TPRA40; e–h, red). GFP-NA14 was visualized by the native fluorescence of GFP (NA14; i–l, green). Merged images (merge; m–p) are also shown. The arrowheads indicate structures positive to both FLAG-TPRA40 and GFP-NA14. Final magnification 1,000×. Similar results were obtained in three separate experiments.

co-transfected with GFP-NA14, the siRNA mixture dramatically reduced the fluorescence derived from GFP-NA14 (Fig. 7A, g and h). SiRNA had no effect on GFP expression (Fig. 7A, e and f). These observations suggest that siRNA specifically down-regulates NA14. We then examined the effect of the siRNA mixture on the plasma membrane transport of FLAG-TPRA40. Although the transfection of FLAG-TPRA40 alone resulted in plasma membrane transport (Fig. 7B, c), co-transfection of the siRNA mixture and FLAG-TPRA40 suppressed plasma membrane transport (Fig. 7B, d), suggesting that NA14 is involved in the transport of TPRA40.

TPRA40/NA14 regulates cell division of early mouse embryos

To examine whether the interaction of TPRA40 with NA14 were caused in the early embryos, we determined the gene expression in the embryos using RT-PCR. NA14 and TPRA40 were abundantly expressed in the embryos as well as mouse brain (Fig. 8A), indicating that the interaction observed in HeLa cells can be present in the mouse early embryos. We examined whether NA14 is involved in the regulation of mouse embryogenesis by TPRA40. Embryos injected with GFP and FLAG-TPRA40 developed according to the time after injection, and more than half of the embryos developed to the morula/blastcyst stage by 4 days after fertilization (Fig. 8B,C). However, most embryos injected with GFP-NA14 and FLAG-TPRA40 remained at only the 2-cell stage for 4 days (Fig. 8B,C). GFP-NA14ΔN restored the development of mouse embryos to that observed in the presence of GFP (Fig. 8B,C). Immunoblotting analysis using anti-FLAG antibody indicates that the expression level of FLAG-TPRA40 in the injected embryos remained almost constant in each group (data not shown). The expression levels of GFP, GFP-NA14, and GFP-NA14ΔN in the injected embryos were confirmed by fluorescence microscopy although the GFP proteins could be undetected by westernblotting. These results show that TPRA40 regulates mouse embryogenesis through NA14.

Discussion

In this study, we have shown that an orphan receptor, TPRA40, regulates cell division of early mouse embryos. As shown in Figure 1C, shRNA-mediated knockdown of TPRA40 facilitates cell division of early mouse embryos, suggesting the involvement of TPRA40 in early mouse embryogenesis. The effect of shRNA was observed during the period 2–4 days after injection, indicating that TPRA40 is likely to be involved in embryogenesis from the first cell division. Further evidence for the involvement of TPRA40 comes from the observation that injection of the TPRA40 expression vector suppressed cell division of mouse embryos at day 4. As shown in Figure 3, approximately 10% of embryos injected with the TPRA40 expression vector developed to the morula/blastocyst stage by day 4, while approximately 40% of control embryos developed to the morula/blastocyst stage. At least 2 days after transfection are required for the functional plasma membrane transport of the TPRA40 protein in HeLa cells, and 1 day for the completion of cell division. These results indicate that the suppression of cell division by exogenous TPRA40 cDNA might be observed 3 days after injection, a supposition that is consistent with our observations as shown in Figure 3.

For many GPCRs, dimerization is required for their exit from the ER because it masks ER-retension signals or hydrophobic patches that keep the proteins in the ER (Petaja-Repo et al., 2000; Petaja-Repo et al., 2001). TPRA40ΔC has the deletion of

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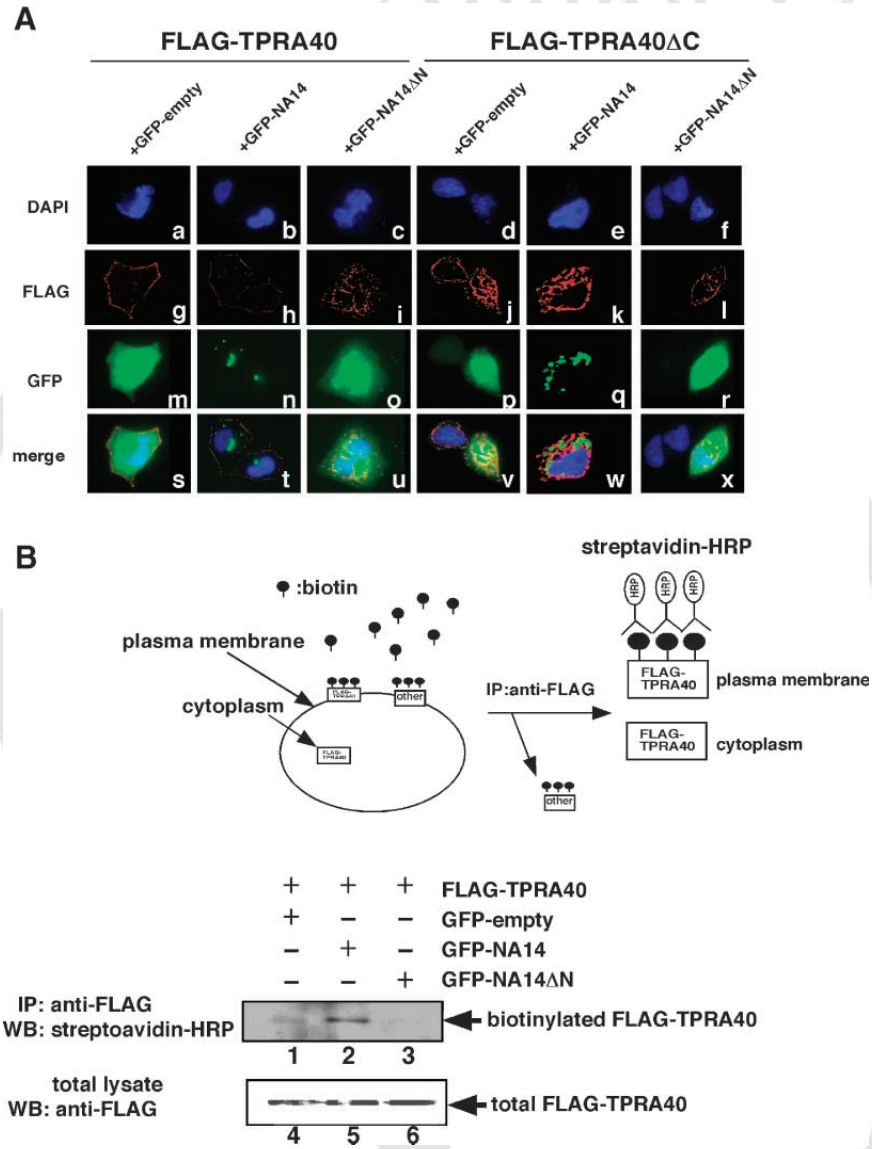

Fig. 6. Effects of NA14 and NA14ΔN on the plasma membrane transport of TPRA40 in HeLa cells (A) FLAG-TPRA40 and FLAG-TPRA40ΔC expression vectors were co-transfected with GFP, GFP-NA14, or GFP-NA14ΔN expression vectors in HeLa cells, and the cells were incubated for 48 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with an anti-FLAG antibody followed by incubation with a Cy3-conjugated anti-mouse IgG antibody. Nuclei were stained with 1 μg/ml DAPI. DAPI-derived fluorescence (DAPI; a–f, blue), Cy3-derived fluorescence (TPRA40; g–l, red), and the native fluorescence of GFP (NA14; m–r, green) were observed under a fluorescence microscope. Merged images (merge; s–x) are also shown. Final magnification 1,000×. Similar results were obtained in four separate experiments. B: Cell surface biotinylation assay. HeLa cells were transfected with FLAG-TPRA40 and GFP, GFP-NA14, or GFP-NA14ΔN for 48 h, and biotinylated on the cell surface by incubating with biotinylation reagents as shown in the upper part. Then, the cells were extracted and subjected to electrophoresis. Total FLAG-TPRA40 in the cell lysates was visualized by an anti-FLAG antibody (lanes 4–6). To detect biotinylated FLAG-TPRA40, the cell lysates were precipitated with anti-FLAG antibody-immobilized beads, and the precipitated proteins were subjected to electrophoresis. Biotinylated FLAG-TPRA40 was visualized by streptavidin-peroxidase (lanes 1–3). Similar results were obtained in a separate experiment.

the putative cytoplasmic region of the C-terminus, and is scarcely transported to the plasma membrane even 48 h after transfection in HeLa cells as shown in Figure 6A. TPRA40ΔC suppresses early embryogenesis, as shown in Figure 3, suggesting that TPRA40ΔC interferes with the function of endogenous TPRA40, and works as a dominant negative for wild-type TPRA40. Dominant negative effects of C-terminus-deleted mutants were also observed for other GPCRs. For example, the naturally occurring deletion mutant of the C-terminal cytoplasmic region of the CCR5 chemokine receptor is not transported to the plasma membrane and is retained in the ER (Benkirane et al., 1997; Schulz et al., 2000). When the mutant CCR5 and wild-type CCR5 are co-expressed, the plasma membrane transport of wild-type CCR5 is inhibited by its retention in the ER (Shioda et al., 2001). A splice variant of the dopamine D3 receptor, D3nf, has a deletion of the C-terminal portion of receptor and is localized to an intracellular compartment (Schmauss et al., 1993; Karpa et al., 2000). The D3 receptor also mislocates from the plasma membrane to the intracellular compartment in the presence of

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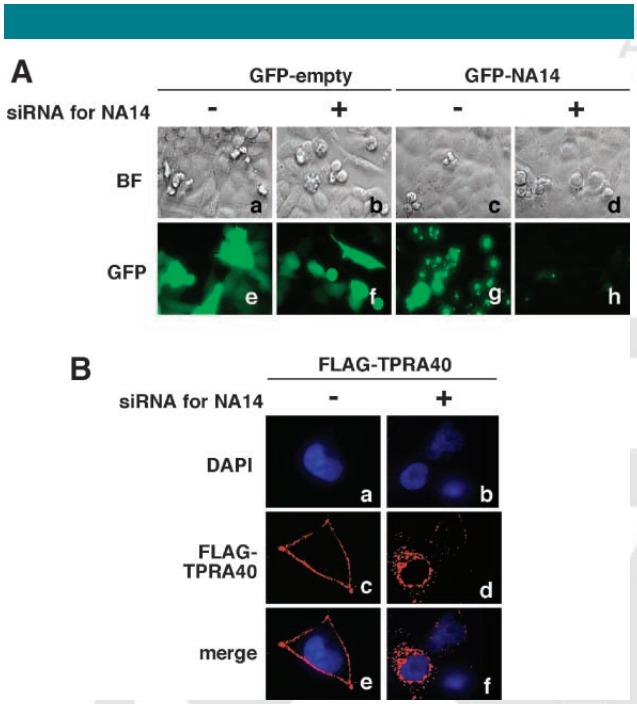

Fig. 7. Effect of siRNA-mediated knockdown of NA14 on the plasma membrane transport of TPRA40 in HeLa cells (A) GFP and GFP-NA14 expression vectors were transfected with or without siRNA mixture (4 nM) into HeLa cells. After 48 h of transfection, the GFP fluorescence in live cells was observed under a fluorescence microscope. BF indicates bright field images. Final magnification 200×. Similar results were obtained in three separate experiments. B: FLAG-TPRA40 with (+) or without (−) an siRNA mixture (4 nM) were transfected into HeLa cells, and the cells were incubated for 48 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and incubated with an anti-FLAG antibody, followed by incubation with a Cy3-conjugated anti-mouse IgG antibody. Nuclei were stained with 1 μg/ml DAPI. DAPI-derived fluorescence (DAPI; a and b), and Cy3-derived fluorescence (TPRA40; c and d) were observed under fluorescence microscope. Merged images (merge; e and f) are also shown. Final magnification 1,000×. Similar results were obtained in three separate experiments.

D3nf (Karpa et al., 2000). The results obtained using TPRA40ΔC indicate that the C-terminal cytoplasmic region of TPRA40 plays an essential role in both transport to the plasma membrane and the regulation of cell division.

We searched for molecules that bind to the C-terminal cytoplasmic region of TPRA40. Unexpectedly, Sjögren's syndrome-associated protein NA14 was identified as a binding protein for TPRA40. Sjögren's syndrome (SS) is an autoimmune disorder clinically characterized by dry mouth and eye caused by decreased function of the lacrimal and salivary glands (Thomas et al., 1998). Although NA14 was found from among the autoantigens recognized by serum from an SS patient (Ramos-Morales et al., 1998), the relationship between NA14 and the clinical expressions of SS have been poorly elucidated. NA14 is composed of 119 amino acids, and has an N-terminal acidic coiled-coil region and a C-terminal basic domain (Ramos-Morales et al., 1998). We have observed the dotted localization of NA14 in HeLa cells. Immunocytochemical analyses using an antibody against α-tubulin has suggested that these dotted structures are distributed along microtubules (data not shown). In contrast, NA14ΔN (amino acids 23–119), which is deleted from the coiled-coil region (amino acids 8–22), was diffused throughout the cytoplasm. These observations indicate that NA14 associates with MTs through its N-terminal coiled-coil region. Consistent with these data, Pfannenschmid et al. (2003) reported that NA14 is associated with microtubule (MT) structures, basal bodies in human sperm and centrosomes in HeLa cells. The binding region for NA14 to TPRA40 might be located near its C-terminal regions (amino acids 23–119) since we obtained a C-terminal fragment of NA14 (NA14ΔN) as a binding protein for TPRA40. Therefore, NA14 might bind to MTs through its N-terminal region (amino acids 1–22) and to TPRA40 at its C-terminal region (amino acids 23–119).

As demonstrated by both immunocytochemical and biochemical (cell surface biotinylation) assays, TPRA40 is transported to the plasma membrane in HeLa cells (Fig. 6A,B). NA14 is required for the transport of TPRA40 because knockdown of NA14 by siRNA inhibited the transport, as shown in Figure 7B. NA14ΔN also inhibited the plasma membrane transport of TPRA40 when the two were co-expressed in HeLa cells, suggesting that NA14ΔN functions as a dominant-negative molecule for NA14. This can be explained as follows. NA14ΔN contains binding region for TPRA40, and might compete with NA14 for binding to TPRA40. TPRA40 that binds to NA14ΔN cannot bind to MTs since NA14ΔN is deleted from the binding region for MTs. Therefore, the binding to NA14ΔN results in the retention of TPRA40 within the cytoplasm. A possible role for NA14 in MT dynamics has also been suggested by Errico et al. (2004). They showed that NA14 interacts with spastin, an AAA family ATPase that interacts with MTs, and is involved in MT dynamics (Errico et al., 2004). NA14 is required for the MT-serving activity of spastin since deletion of the NA14-binding region of spastin results in the dissociation of spastin from MTs (Errico et al., 2004). Impairment of spastin activity underlies the pathogenesis of hereditary spastic paraplegia, which is characterized by the degeneration of motor axons with the impairment of intracellular transport (Errico et al., 2002; McDermott et al., 2003; Fink, 2003a,b). These observations indicate that NA14 is involved in intracellular cargo transport as an essential adaptor molecule for interaction between the cargo and MTs.

What is the physiological significance of the suppression of early mouse embryogenesis by TPRA40? Other than TPRA40, there are a few factors that are reported to inhibit preimplantation embryogenesis (Stewart and Cullinan, 1997; Hardy and Spanos, 2002). TNF-α (Pampfer et al., 1997; Wuu et al., 1999) and IFN-γ (Haimovici et al., 1991) are factors that suppress early mouse embryogenesis; however, in contrast to TPRA40, the effects of TNF-α and IFN-γ appear only in the blastocyst stage. TNF-α mainly inhibits the proliferation of the inner cell mass (Stewart and Cullinan, 1997) while IFN-γ inhibits trophoblast outgrowth by inducing the degeneration of these cells (Haimovici et al., 1991). Therefore, TPRA40 is involved in the negative regulation of embryogenesis in a cell lineage-nonspecific manner, while TNF-α and IFN-γ are involved in the cell lineage-specific inhibition of embryogenesis. Early mouse embryogenesis is regulated not only by cytokines but by the environments in which it takes place (Fleming et al., 2004). It has been reported that maternal diabetes and hyperglycemia suppress early mouse embryogenesis during preimplantation (Moley et al., 1991, 1998a,b; Chang et al., 2005). Since TPRA40 expression is up-regulated in diabetic mice (Yang et al., 1999) and under hyperglycemic conditions in cultured cells (Fujimoto et al., 2001), TPRA40 may be a mediator of the retardation of early mouse embryogenesis under these conditions.

In conclusion, the results of the present study demonstrate one of the physiological functions of an orphan receptor, TPRA40. In addition, the present study also shows that NA14 is involved in the plasma membrane transport of TPRA40. The unexpected connection between an orphan receptor and the autoantigen of SS might shed light on the pathophysiology of SS.

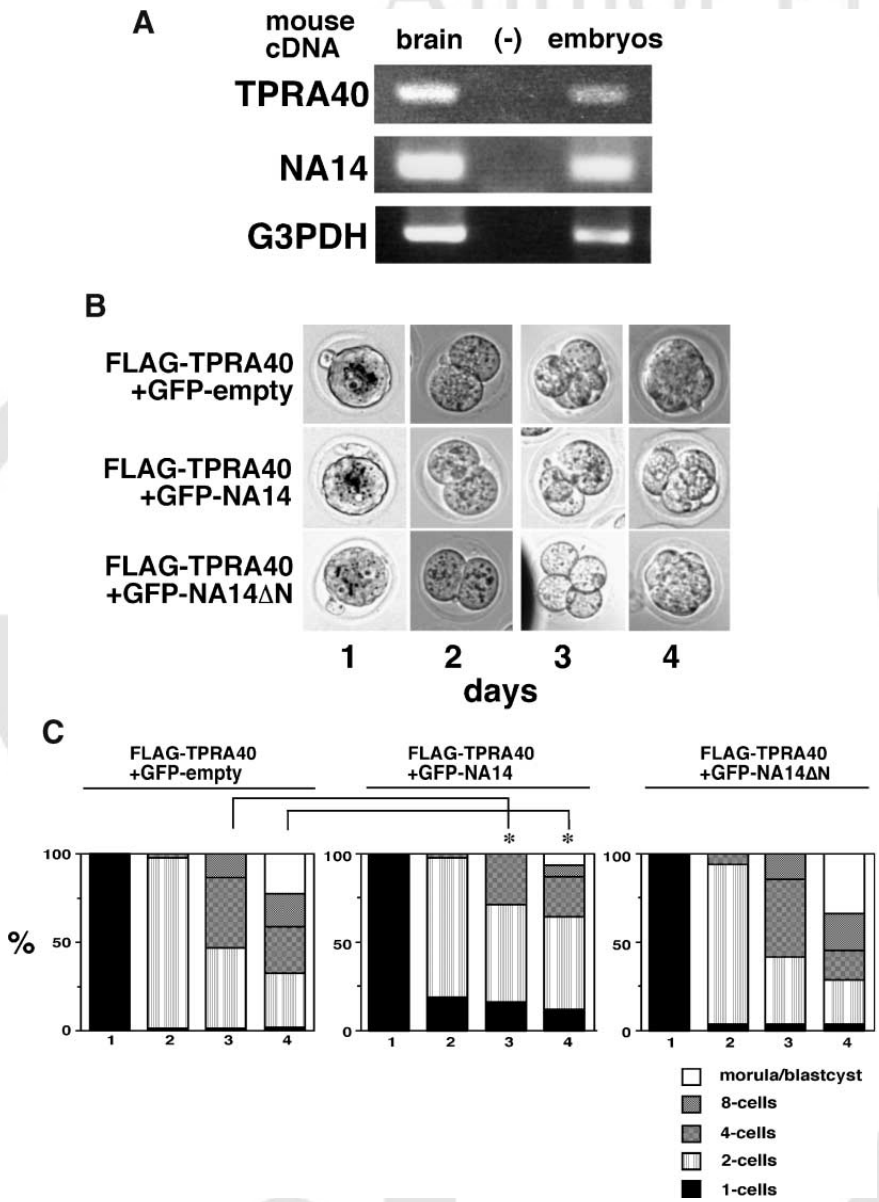

Fig. 8. Effect of TPRA40 and NA14 on cell division of mouse embryos (A) mRNA was extracted from C57/BL6 mouse brain and embryos. RT was performed, and the product of the RT reaction was amplified by PCR using the primers for NA14, TPRA40, and G3PDH. The RT-PCR products were electrophoresed in agarose gels, and stained with ethidium bromide. The figure shows representative data obtained from three independent experiments. B,C: One day after fertilization, embryos were injected with the indicated combinations of vectors, and cell divisions were observed at the indicated times under a bright field microscope. 54 (FLAG-TPRA40 + GFP-empty vector), 52 (FLAG-TPRA40 + GFP-NA14), or 51 (FLAG-TPRA40 + GFP-NA14ΔN) embryos were used in each group (*$P < 0.05$ for FLAG-TPRA40 + GFP-empty vector; Mann–Whitney's *U*-test). Typical images of embryos (upper part) and summarized data on the developmental stages (lower part) are shown.

Acknowledgments

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Literature Cited

Benkirane M, Jin DY, Chun RF, Koup RA, Jeang KT. 1997. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. J Biol Chem 272:30603–30606.

Cantrell DA. 2001. Phosphoinositide 3-kinase signalling pathways. J Cell Sci 114:1439–1445.

Chang AS, Dale AN, Moley KH. 2005. Maternal diabetes adversely affects preovulatory oocyte maturation, development, and granulosa cell apoptosis. Endocrinology 146:2445–2453.

Civelli O, Nothacker HP, Saito Y, Wang Z, Lin SH, Reinscheid RK. 2001. Novel neurotransmitters as natural ligands of orphan G-protein-coupled receptors. Trends Neurosci 24:230–237.

Drews J. 1996. Genomic sciences and the medicine of tomorrow. Nat Biotechnol 14:1516–1518.

Errico A, Ballabio A, Rugarli EI. 2002. Spastin, the protein mutated in autosomal dominant hereditary spastic paraplegia, is involved in microtubule dynamics. Hum Mol Genet 11:153–163.

Errico A, Claudiani P, D'Addio M, Rugarli EI. 2004. Spastin interacts with the centrosomal protein NA14, and is enriched in the spindle pole, the midbody and the distal axon. Hum Mol Genet 13:2121–2132.

Fink JK. 2003a Advances in the hereditary spastic paraplegias. Exp Neurol 184:S106–S110.

Fink JK. 2003b The hereditary spastic paraplegias: Nine genes and counting. Arch Neurol 60:1045–1049.
Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ. 2004. The embryo and its future. Biol Reprod 71:1046–1054.
Fujimoto K, Mizukami Y, Kimura M, Mogami K, Todoroki-Ikeda N, Kobayashi S, Matsuzaki M. 2001. Molecular cloning of rat transmembrane domain protein of 40 kDa regulated in adipocytes and its expression in H9c2 cells exposed to ischemic hypoxia and reoxygenation. Biochim Biophys Acta 1518:173–177.
Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. 1994. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 74:1141–1148.
Haimovici F, Hill JA, Anderson DJ. 1991. The effects of soluble products of activated lymphocytes and macrophages on blastocyst implantation events in vitro. Biol Reprod 44:69–75.
Hardy K, Spanos S. 2002. Growth factor expression and function in the human and mouse preimplantation embryo. J Endocrinol 172:221–236.
Hasuwa H, Kaseda K, Einarsdottir T, Okabe M. 2002. Small interfering RNA and gene silencing in transgenic mice and rats. FEBS Lett 532:227–230.
Hogan B, Beddington R, Costantini F, Lacy E. 1994. Manipulating the mouse embryo: A laboratory manual. 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Horn F, Weare J, Beukers MW, Horsch S, Bairoch A, Chen W, Edvardsen O, Campagne F, Vriend G. 1998. GPCRDB: An information system for G protein-coupled receptors. Nucleic Acids Res 26:275–279.
Horn F, Bettler E, Oliveira L, Campagne F, Cohen FE, Vriend G. 2003. GPCRDB information system for G protein-coupled receptors. Nucleic Acids Res 31:294–297.
Karpa KD, Lin R, Kabbani N, Levenson R. 2000. The dopamine D3 receptor interacts with itself and the truncated D3 splice variant d3nf: D3-D3nf interaction causes mislocalization of D3 receptors. Mol Pharmacol 58:677–683.
Kimura M, Mizukami Y, Miura T, Fujimoto K, Kobayashi S, Matsuzaki M. 2001. Orphan G protein-coupled receptor, GPR41, induces apoptosis via a p53/Bax pathway during ischemic hypoxia and reoxygenation. J Biol Chem 276:26453–26460.
Marchese A, George SR, Kolakowski LF, Jr., Lynch KR, O'Dowd BF. 1999. Novel GPCRs and their endogenous ligands: Expanding the boundaries of physiology and pharmacology. Trends Pharmacol Sci 20:370–375.
McDermott CJ, Grierson AJ, Wood JD, Bingley M, Wharton SB, Bushby KM, Shaw PJ. 2003. Hereditary spastic paraparesis: Disrupted intracellular transport associated with spastin mutation. Ann Neurol 54:748–759.
Mizukami Y, Yoshida K. 1997. Mitogen-activated protein kinase translocates to the nucleus during ischaemia and is activated during reperfusion. Biochem J 323:785–790.
Mizukami Y, Hirata T, Yoshida K. 1997. Nuclear translocation of PKC zeta during ischemia and its inhibition by wortmannin, an inhibitor of phosphatidylinositol 3-kinase. FEBS Lett 401:247–251.
Mizukami Y, Kobayashi S, Uberall F, Hellbert K, Kobayashi N, Yoshida K. 2000. Nuclear mitogen-activated protein kinase activation by protein kinase czeta during reoxygenation after ischemic hypoxia. J Biol Chem 275:19921–19927.
Mizukami Y, Iwamatsu A, Aki T, Kimura M, Nakamura K, Nao T, Okusa T, Matsuzaki M, Yoshida K, Kobayashi S. 2004. ERK1/2 regulates intracellular ATP levels through alpha-enolase expression in cardiomyocytes exposed to ischemic hypoxia and reoxygenation. J Biol Chem 279:50120–50131.
Moley KH, Vaughn WK, DeCherney AH, Diamond MP. 1991. Effect of diabetes mellitus on mouse pre-implantation embryo development. J Reprod Fert 93:325–332.
Moley KH, Chi MM, Knudson CM, Korsmeyer SJ, Mueckler MM. 1998a. Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. Nat Med 4:1421–1424.
Moley KH, Chi MM, Mueckler MM. 1998b. Maternal hyperglycemia alters glucose transport and utilization in mouse preimplantation embryos. Am J Physiol 275:E38–47.
Pampfer S, Vanderheyden I, McCracken JE, Vesela J, De Hertogh R. 1997. Increased cell death in rat blastocysts exposed to maternal diabetes in utero and to high glucose or tumor necrosis factor-alpha in vitro. Development 124:4827–4836.
Patterson C, Ruef J, Madamanchi NR, Barry-Lane P, Hu Z, Horaist C, Ballinger CA, Brasier AR, Bode C, Runge MS. 1999. Stimulation of a vascular smooth muscle cell NAD(P)H oxidase by thrombin. Evidence that p47(phox) may participate in forming this oxidase in vitro and in vivo. J Biol Chem 274:19814–19822.
Pelletier S, Duhamel F, Coulombe P, Popoff MR, Meloche S. 2003. Rho family GTPases are required for activation of Jak/STAT signaling by G protein-coupled receptors. Mol Cell Biol 23:1316–1333.
Petaja-Repo UE, Hogue M, Laperriere A, Walker P, Bouvier M. 2000. Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem 275:13727–13736.
Petaja-Repo UE, Hogue M, Laperriere A, Bhalla S, Walker P, Bouvier M. 2001. Newly synthesized human delta opioid receptors retained in the endoplasmic reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. J Biol Chem 276:4416–4423.
Pfannenschmid F, Wimmer VC, Rios RM, Geimer S, Krockel U, Leiherer A, Haller K, Nemcova Y, Mages W. 2003. Chlamydomonas DIP13 and human N A14: A new class of proteins associated with microtubule structures is involved in cell division. J Cell Sci 116:1449–1462.
Pusch O, Boden D, Silbermann R, Lee F, Tucker L, Ramratnam B. 2003. Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA. Nucleic Acids Res 31:6444–6449.
Ramos-Morales F, Infante C, Fedriani C, Bornens M, Rios RM. 1998. NA14 is a novel nuclear autoantigen with a coiled-coil domain. J Biol Chem 273:1634–1639.
Schmauss C, Haroutunian V, Davis KL, Davidson M. 1993. Selective loss of dopamine D3-type receptor mRNA expression in parietal and motor cortices of patients with chronic schizophrenia. Proc Natl Acad Sci USA 90:8942–8946.
Schulz A, Grosse R, Schultz G, Gudermann T, Schoneberg T. 2000. Structural implication for receptor oligomerization from functional reconstitution studies of mutant V2 vasopressin receptors. J Biol Chem 275:2381–2389.
Shioda T, Nakayama EE, Tanaka Y, Xin X, Liu H, Kawana-Tachikawa A, Kato A, Sakai Y, Nagai Y, Iwamoto A. 2001. Naturally occurring deletional mutation in the C-terminal cytoplasmic tail of CCR5 affects surface trafficking of CCR5. J Virol 75:3462–3468.
Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC, Hawkins PT. 1994. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. Cell 77:83–93.
Stewart CL, Cullinan EB. 1997. Preimplantation development of the mammalian embryo and its regulation by growth factors. Dev Genet 21:91–101.
Thomas E, Hay EM, Hajeer A, Silman AJ. 1998. Sjogren's syndrome: A community-based study of prevalence and impact. Br J Rheumatol 37:1069–1076.
Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT, Brown A, Rodriguez SS, Weller JR, Wright AC, Bergmann JE, Gaitanaris GA. 2003. The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci USA 100:4903–4908.
Wuu YD, Pampfer S, Becquet P, Vanderheyden I, Lee KH, De Hertogh R. 1999. Tumor necrosis factor alpha decreases the viability of mouse blastocysts in vitro and in vivo. Biol Reprod 60:479–483.
Yang H, Egan JM, Rodgers BD, Bernier M, Montrose-Rafizadeh C. 1999. Differential expression of a novel seven transmembrane domain protein in epididymal fat from aged and diabetic mice. Endocrinology 140:2859–2867.

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