Targeted disruption of the murine large nuclear KIAA1440/Ints1 protein causes growth arrest in early blastocyst stage embryos and eventual apoptotic cell death

Toshiyuki Hata \textsuperscript{a}, Manabu Nakayama \textsuperscript{a,b,*}

\textsuperscript{a} Laboratory of Pharmacogenomics, Graduate School of Pharmaceutical Sciences, Chiba University, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan
\textsuperscript{b} Department of Human Genome Research, Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan

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Abstract

The KIAA1440 protein contains no significant domains that allow for a prediction of its function, despite the fact that it is an extremely large protein comprising 2222 amino acids. In our current study, we show that the developing KIAA1440\textsuperscript{−/−} mouse embryo in a pure ICR background arrests its growth at the early blastocyst stage, whereas the majority of the KIAA1440\textsuperscript{−/−} embryos of mixed genetic backgrounds do not progress beyond the morula stage, approximately 0.5 days earlier. KIAA1440\textsuperscript{−/−} embryos exhibited no abnormal localization of E-cadherin or \(\beta\)-catenin and no obvious compaction abnormalities at the morula stage. In addition, E3.5 KIAA1440\textsuperscript{−/−} embryos are not viable even in \textit{in vitro} cultures. Both TUNEL and FAM-caspase-3/7 assays performed on these embryos consistently showed that E3.5 KIAA1440\textsuperscript{−/−} embryos had activated caspase-3/7, which then induced an apoptotic response predominantly within the inner cell mass of the blastocyst. Moreover, qRT-PCR analysis showed that KIAA1440\textsuperscript{−/−} embryos had increased levels of the unprocessed, primary U2 snRNA transcript but decreased levels of the mature U2 snRNA transcript compared to heterozygotes.

The impaired processing of U2 snRNA and the predominantly nuclear localization of KIAA1440 protein is also very consistent with recently reported data showing that it is the largest subunit of the integrator complex, which mediates U1 and U2 snRNA 3\textsuperscript{′}-end processing. Large nuclear KIAA1440/Ints1 is thus suggested to play non-redundant roles in the cell such as the formation of a scaffold for the assembly of the integrator complex.

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1. Introduction

Functional mammalian genomics still poses many challenges in spite of the accumulation of useful information and bio-resources that have resulted from various genome projects. We have identified more than 2000 human cDNAs in our laboratory that encode relatively large proteins, which we have catalogued using the nomenclature ‘KIA’ plus a unique 4-digit identifying number (KIAA0001–KIAA2038) \cite{1,2} and deposited in \textit{in silico} public databases (see HUGE database \cite{3} at http://www.kasusa.or.jp/huge). In addition, we have analyzed many of these factors using expression profiling and yeast-two hybrid screening. We have focused on large proteins as they play integral roles in the cell such as the formation of scaffolds for both the assembly of different complexes and as a centre for protein–protein interactions \cite{4}. We also propose that the inactivation of genes encoding large proteins is likely to confer definitive, observable phenotypes at a higher frequency, since at least some large proteins are likely to serve as frameworks for the intricate assembly of protein complexes. A protein complex can generally be thought as an organized hierarchy in terms of function. Some proteins are essential for the proper functioning and integrity of the complex; others are relatively less important and may have only modulator roles. Extremely large proteins...
are the key element of protein complexes, because they function as the framework around which the complex is assembled.

We have recently reported the functional characterization of the murine homologues of five human KIAA genes (KIAA1409, KIAA1440, KIAA1447, KIAA1768, KIAA1276) that encode large proteins [5]. Gene-targeting of these factors in mice causes phenotypic and developmental defects for three of these genes, which is a high success rate. Mice harbouring a targeted disruption of KIAA1409 lack the ability to drink milk, and those with a targeted disruption in the KIAA1447 gene display hind leg motor dysfunction. Disruption of KIAA1440 results in embryonic lethality at the blastocyst stage.

The mouse KIAA1440 cDNA is 7070 bp in length and encodes an extremely large protein of 2222 amino acids, which unusually contains no domains of known significance that would allow for a prediction of function. In addition, this protein shares no homology with other mouse proteins but, interestingly, is evolutionarily conserved in terms of sequence identity and large protein size among metazoans, including human, rat, mouse, globefish, zebrafish and Drosophila. Such a degree of conservation without the existence of any gene paralogues, along with the extremely large size of the protein, suggests that KIAA1440 has specialized functions that may not be compensated for by other proteins, particularly during the early embryonic stages of development.

In our present study, we extensively analyzed KIAA1440 to further elucidate its functional properties using gene-targeting in the mouse. This was followed by an assessment of the affected developmental periods and subcellular localization of the protein.

2. Materials and methods

2.1. Preparation of preimplantation embryos

KIAA1440 gene-targeted mice have been described previously [5] and were backcrossed with C57ICR mice (CLEA JAPAN, INC.) to obtain a pure genetic background. N8-10 offspring were used in the experiments of this study. Fifteen to 21 one-cell stage embryos and blastocysts obtained from wild-type females were naturally mated with KIAA1440-/- males to obtain KIAA1440+/+ , KIAA1440-/- and KIAA1440+/- preimplantation embryos. Noon of the day at which the vaginal plug was detected was designated embryonic day (E) 0.5 (E0.5). E2.75 morulae were collected by flushing the oviducts with M2 medium (Specialty Media). E3.5 blastocysts were isolated by flushing the uteri. After incubation for 5 min at 95 °C, lysates were subjected to PCR in a total volume of 50 μl with LA Taq polymerase (TaKaRa BIO INC.) using three primers: KIAA1440 upper (5’-TGTCACACTCT CCTCAGGCCA T3’); KIAA1440 lower (5’-AGTACAGCGT CAGAAGCGGT G-3’); and neomycin (5’-CATGCATACG CAGAATGCGT C-3’). Wild-type alleles of the mouse KIAA1440 gene are detectable using primers KIAA1440 upper and lower, and the targeted allele is detected by primers KIAA1440 upper and neomycin. The amplification protocol was as follows: preincubation at 95 °C for 4 min, followed by 35 cycles at 95 °C for 20 s and 66 °C for 1 min. PCR products were electrophoresed on agarose gels and visualized by ethidium bromide staining.

2.2. Immunofluorescence analysis of whole-mount embryos

E2.75 morulae were isolated and treated with acidic tyrode solution to remove the zona pellucida. The embryos were then fixed with 2% paraformaldehyde (PFA) in PBS for 20 min at room temperature, and permeabilized with 0.25% Triton X-100 in PBS for 10 min, also at room temperature. After preincubation in 3% bovine serum albumin (BSA) and 0.01% Tween 20 in PBS for 1 h, the embryos were incubated with mouse anti-E-cadherin monoclonal antibody (clone 36, BD Biosciences) at a 1:1250 dilution in 1% BSA/0.01% Tween 20/PBS, or with mouse anti-j-catenin monoclonal antibody (clone 14, BD Biosciences) at a 1:50 dilution in the same buffer for a further 1 h at room temperature. The embryos were then incubated with FluoroLink Cy3-labeled donkey anti-mouse IgG (H and L) antibody (GE Healthcare Bio-Sciences Corp.) (1:1000) for 1 h at room temperature, mounted in SlowFade Antifade medium (Invitrogen Corp.) and observed with an Olympus IX71 fluorescence microscope.

2.3. In vitro culture of blastocysts

E3.5 blastocysts were isolated and washed with M2 medium followed by ES medium (KnockOut Dulbecco’s Modified Eagle Medium, Invitrogen Corp.) containing 15% KnockOut Serum Replacement (Invitrogen Corp.), 2 mM Glutamax-I supplement (Invitrogen Corp.), 1× MEM non-essential amino acids solution (Invitrogen Corp.), 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 μM leukemia inhibitory factor (LSGRO) (Invitrogen Corp.). Individual embryos were then transferred into gelatin-coated cell culture dishes containing ES medium and incubated for 72 h at 37 °C in 5% CO2.

2.5. TUNEL assay

Detection of apoptotic cells within blastocysts was performed using an In Situ Cell Death Detection Kit, TMR-red (Roche Diagnostics Corp.), based on the terminal dUTP nick-end labeling (TUNEL) reaction. The TUNEL method labels DNA strand breaks by terminal deoxynucleotidyl transferase, which catalyzes the polymerization of labeled nucleotides to free 3′-OH DNA ends in a template-independent manner. Harvested E3.5 blastocysts were cultured in ES medium for 12 h, fixed with 4% PFA in PBS for 20 min at 4 °C and permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature. For the labeling of apoptotic cells with TMR-red fluorescence, embryos were incubated in TUNEL reaction mixture for 1 h at 37 °C. After nuclear DNA staining with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS, the embryos were transferred into mounting medium and images were captured using fluorescence microscopy.

2.6. Detection of activated caspase-3

For the detection of activated caspase-3 in blastocyst cells, a Vybrant FAM Caspase-3 and -7 Assay Kit was employed (Invitrogen Corp.). E3.5 blastocysts were cultured in ES medium for 6 h and then transferred into ES medium containing FAM-DEVd-FMK caspase-3-caspase-7 reagent, which is caspase-specific fluorescent inhibitor, and incubated for 1 h at 37 °C. Embryos were then washed three times with ES medium followed by apoptosis wash buffer. The embryos were then fixed with 4% PFA in PBS for 20 min at room temperature and permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. After nuclear DNA staining with DAPI, embryos were observed using a fluorescence microscope and genotyped.

2.7. Quantitative reverse transcriptase-polymerase chain reaction

Fifteen to 21 one-cell stage embryos and blastocysts obtained from wild-type ICR mice were used to extract total RNA. After several washes in PBS, total RNA
was extracted using 400 μl of TRizol Reagent (Invitrogen Corp.) and 100 μg of
glycogen as a carrier. Prior to extraction, 5 pg of green fluorescent protein (GFP)
RNA per embryo was added as an external control. cDNA synthesis was performed
by random priming using TaqMan Reverse Transcription Reagents (Applied
Biosystems). Reverse transcription (RT) products at levels that were equivalent to
one or two embryos were then subjected to PCR. Quantitative real-time PCR was
performed with a SYBR Green PCR Master Mix (Applied Biosystems) using the
ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The primers
used were as follows: KIAA1440 upper2 (5′-CATCATGGGC CACCTCTTCT C-
3′); KIAA1440 lower2 (5′-AGCAGAGTGA CCATGGGCGT-3′); GFP upper (5′-
GAGTGCCAT CCGAAAGGT A-3′); GFP lower (5′-CGTCTTGTAG TTTCCGCTAT C-3′). The KIAA1440 primer set was designed to overlap with
an intron to ensure specific amplification of cDNA and not genomic DNA. Serial
dilution of plasmid DNA containing mouse KIAA1440 and GFP gene inserts,
namely a native-form KIAA1440 expression vector and pcDNA-DEST35 vector
(Invitrogen corp.), were subjected to PCR to generate standard curves. The PCR
analyses of both samples and standards were all performed in triplicate at the same
time using the following protocol: denaturation at 50 °C for 2 min and 95 °C for
10 min, amplification and quantification program for 50 cycles at 95 °C for 15 s and
60 °C for 1 min, and melting at 95 °C for 15 s, 60 °C for 20 s and 60 °C
10 min; amplification and quantification program for 50 cycles at 95 °C for 15 s and
60 °C for 20 s and 60 °C

2.8. Generation and transfection of the KIAA1440 expression construct

Both C-terminal EGFP- or N-terminal EGFP-fused KIAA1440 expression vectors
were generated by insertion of mouse KIAA1440 open reading frame [5]
either upstream or downstream in frame with the EGFP insert of the pEGFP-C3
vector (Clontech Laboratories, Inc.). The empty vector control was constructed
by removal of EGFP from the pEGFP-C3 vector. The native-form KIAA1440
expression vector was then generated by the replacement of EGFP with the full-
length KIAA1440 open reading frame in pEGFP-C3. COS7 cells were
transfected with FuGENE 6 (Roche Diagnostics Corp.), according to the
manufacturer’s instruction. Forty-eight hours after incubation, cells were lysed
in 4+ SDS sample buffer (250 μM Tris–HCl (pH 6.8), 8% SDS, 28% glycerol,
0.02% bromophenol blue and 20% β-mercaptoethanol).

For the immunodetection of endogenous mouse KIAA1440 protein, whole-
cell lysates were prepared from non-transfected NIH 3T3 cells. After boiling for
3 min at 95 °C, the lysates were separated on 2–15% polyacrylamide gradient
gels (Daichi Pure Chemicals) and blotted onto PROTRAN BA85 nitrocellulose
membranes (Schleicher&Schuell Bioscience, Inc.). Western blot analysis was
then performed according to previously described standard procedures [6].
Briefly, the membranes were incubated with mouse anti-GFP monoclonal antibody (1:2000, clone GF200, Nacalai Tesque, Inc.), anti-mouse KIAA1440
polyclonal antiserum (1:10,000) or affinity-purified anti-mouse KIAA1440
antiserum. The signals were detected with ECL Plus Western Blotting Detection
kit (Amersham Biosciences) for 1 h at room temperature, the cells were incubated with either anti-mouse
KIAA1440 antiserum (1:300 in 1% BSA/PBS) or affinity-purified anti-mouse
KIAA1440 antiserum for 1 h at room temperature. Negative control cells were
incubated in buffer without primary antibodies. The cells were then incubated with
FluoroLink Cy3-labeled goat anti-rabbit IgG (H and L) antibody (1:1000, GE
Healthcare Bio-Sciences) for 1 h at room temperature. After nuclear DNA staining
with DAPI, the cells were photographed under a fluorescence microscope.

2.10. Production and purification of antibodies against the mouse
KIAA1440 protein

DNA sequences corresponding to amino acid residues 241–544 of the mouse
KIAA1440 protein was subcloned into both the pDEST17 vector (Invitrogen Corp.)
and pMAL-c2 vector (New England Biolabs). Polyclonal antibodies were raised
by immunizing rabbits with the corresponding 6× His fusion proteins that were
expressed in E. coli BL21 cells and purified using Ni-columns. The antisera were
purified with maltose-binding protein (MBP) fusion protein affinity columns. After
washing with high salt buffer (10 mM Tris–HCl (pH 7.5) and 500 mM NaCl),
purified antibodies were eluted with 100 mM glycine (pH 2.5).

2.11. Quantitative analysis of U2 small nuclear RNA (snRNA)

Blastocysts derived from KIAA1440−/− intercrosses were washed with PBS,
and each blastocyst was transferred into individual tubes. Embryos were then
lysed with CellsDirect Reagent (Invitrogen Corp.) containing a carrier GFP
RNA (final concentration: 5 ng/μl) and incubated for 10 min at 75 °C. One third
of each lysate sample was subjected to PCR genotyping; the remaining two
thirds were treated with DNase I (TaKaRa BIO INC.) and used for qRT-PCR
analysis. DNase I was inactivated by heating the samples for 10 min at 80 °C
in the presence of EDTA. qRT-PCR was performed with either a SuperScript III
Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen Corp.) or a TaqMan
One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) and an ABI
PRISM 7700 Sequence Detection System. The following primers were used: a,
U2 snRNA upper 1 (5′-CTCCGCTCTTT TGGCTAAGAT-3′); b, U2 lower 1 (5′-
CGTCTTCGTGA GGTTACTGCA-3′); c, U2 upper 2 (5′-GGAAAGTGAGG GTTGGAATAG GAGCTT-3′); d, U2 lower 2 (5′-TAAAGAAAGT GTGACT-
GCCCT ACA-3′); e, 18S ribosomal RNA (rRNA) upper (5′-GGAGAAGGCG GCACCTACCC-3′); f, 18S rRNA lower (5′-GGACACTCATG CATAGACCAG CATCG-3′). Total U2 transcripts (mature plus primary) and 18S rRNA were detected
by using the SYBR Green method and primers a and b, and primers c and f,
respectively. Primary U2 transcripts were detected by using the TaqMan Probe
method and primers c and d, and a TaqMan MGB Probe (5′-TCCACCATC GTCGATTTTAA-3′).

Next, we subcloned the target sequences of U2 snRNA and 18S rRNA. Serial
dilutions of these plasmid DNAs were subjected to PCR in triplicate and standard
curves were generated. The PCR protocol used for the detection of total U2 transcripts and 18S rRNA was as follows: reverse transcription at 55 °C for 5 min;
denaturation at 95 °C for 5 min; amplification and quantification program for 40 cycles
at 95 °C for 15 s, 57 °C for 30 s and 68 °C for 30 s; melting curve program
as described above. The protocol used for the detection of primary U2 transcripts
was as follows: reverse transcription at 48 °C for 30 min; denaturation at 95 °C
for 10 min; amplification and quantification program for 50 cycles at 95 °C for 15 s
and at 60 °C for 1 min. The quantities of each transcript were calculated from the
standard curves. We analyzed U2 snRNA and 18S rRNA using 55 embryos (wild
type: heterozygous/homozygous, 15:29:11) and 57 embryos (14:27:16),
respectively. Total and primary U2 transcripts were simultaneously analyzed using
the same total RNAs derived from individual embryos. Statistical analysis was
performed with the t-test or Welch’s test.

3. Results

3.1. The development of KIAA1440−/− embryos arrests at the early
blastocyst stage

Our previously reported preliminary results using mixed genetic background mice showed that disruption of KIAA1440
causes embryonic lethality at the blastocyst stage (E3.5) [5]. In our present study, we extensively further analyzed the function of KIAA1440 using knockout mice of a pure genetic background. Because of the advantageous numbers of eggs and pups that were obtained after natural mating, KIAA1440−/− mice were repeatedly backcrossed with ICR strain. Eight to ten backcross (N8–N10) litters of a pure ICR genetic background were then used in subsequent experiments. Embryos from heterozygous matings were isolated from oviducts or uteri, imaged, and then genotyped by PCR. The numbers of wild-type (+/+), heterozygous (+/−) and homozygous (−/−) E3.5 embryos were 51 (22%), 115 (50%) and 63 (28%), respectively. These ratios were in accordance with the rules of Mendelian inheritance and almost identical results were obtained from our analysis of E2.75 embryos (Fig. 1A). E3.5 KIAA1440−/− embryos were found to reach the early blastocyst stage, but the blastocoel of the homozygotes never expanded fully, in contrast to both the wild-type and heterozygous embryos (Fig. 1B, 1C).

Fig. 1. Genotyping of preimplantation embryos from KIAA1440+/− heterozygote intercrosses. (A) Embryos from KIAA1440+/− heterozygote intercrosses were collected at either embryonic day 2.75 or 3.5 (E2.75 or E3.5). The genotypes of each embryo were assessed by PCR. “+/+”, “+/−” and “−/−” correspond to “KIAA1440+/+”, “KIAA1440+/−” and “KIAA1440−/−”, respectively. The number of embryos and the percentage of each genotype within this total number are indicated above each bar in the graphs. (B) Morphological analysis of preimplantation embryos from KIAA1440+/− heterozygote intercrosses. The embryos were observed via differential interference microscopy. At the E3.5 blastocyst stage, KIAA1440−/− embryos (c and c’) showed abnormal morphologies compared to KIAA1440+/+ (a and a’) and KIAA1440+/− (b and b’) embryos. In contrast, at the E2.75 morula stage, KIAA1440−/− embryos (f) showed a normal morphology that was indistinguishable from KIAA1440+/+ (d) and KIAA1440+/− (e) embryos. Scale bar, 50 μm.
panels a–c and a′–c′). Because the majority of the KIAA1440−/− embryos of mixed genetic background arrested at the morula stage (E2.75) [5], it thus appeared that the genetic background impacts upon the KIAA1440−/− phenotype. To elucidate whether any earlier stage effects could be observed in the KIAA1440−/− embryos of pure genetic background at the morula stage (E.2.75), they were compared with both the wild type and heterozygotes. No morphological difference was detected among these three genotypes of embryos (Fig. 1B, panels d–f).

3.2. KIAA1440−/− embryos show no abnormal localization of E-cadherin or β-catenin and no obvious compaction abnormalities at the morula stage

Although no obvious abnormalities of compaction at the morula stage could be observed in KIAA1440−/− embryos by differential interference microscopy, we further analyzed the development of KIAA1440−/− morulae in detail by immunohistochemistry using anti-E-cadherin and anti-β-catenin antibodies. E-cadherin is a Ca2+-dependent cell adhesion molecule and is essential for the compaction of the morula [7, 8]. β-catenin binds the cytoplasmic domain of E-cadherin and mediates cell-to-cell contacts through the microfilament network [9]. Whole-mount immunofluorescent staining of morulae using anti-E-cadherin or anti-β-catenin antibodies showed that no significant differences existed among the wild-type, heterozygous or homozygous embryos, in which each case the blastomeres of the morula were strongly adherent, and their boundary lines were indistinguishable between the embryos (Fig. 2, panels a–c, g–i). The zona pellucida was dissolved during the preparative procedure in this experiment. Both the E-cadherin and β-catenin signals were found to be restricted to the sites of cell adhesion, and no abnormalities of cellular localization were detected in either the heterozygotes or homozygotes (Fig. 2, panels d–f, j–l). Our data

![Image of Immunofluorescent Staining](image_url)

Fig. 2. Whole-mount immunofluorescent staining of E-cadherin and β-catenin at the E2.75 morula stage. Embryos at E2.75 were stained with anti-E-cadherin or anti-β-catenin antibodies followed by Cy3-labeled secondary antibodies. Differential interference images (DIC) (a–c for E-cadherin, g–i for β-catenin) and Cy3 fluorescent images (d–f for E-cadherin, j–l for β-catenin) are shown. Cy3 fluorescent images were processed with the deconvolution algorithm using the SimplePCI program. There were no detectable differences in the localization patterns of E-cadherin and β-catenin among the three genotypes. Scale bar, 50 μm.
thus show that KIAA1440−/− morulae contain blastomeres that are strongly adherent, have normal cell adhesion complexes of E-cadherin and β-catenin and display normal compaction that successfully completes.

3.3. KIAA1440−/− blastocysts lack viability even in in vitro culture

KIAA1440−/− E3.5 embryos of a pure ICR background reach the early blastocyst stage, but do not form a fully expanded blastocyst with a large blastocoel. To determine whether this phenotype is due to a delay in development or is caused by an inability of this particular developmental stage to progress, we isolated wild-type, heterozygous and homozygous embryos at E3.5 and transferred them to gelatin-coated tissue culture dishes in ES medium. The explants were incubated to observe their embryonic development in vitro and genotyped by PCR. The majority of the wild-type and heterozygous samples ruptured the zona pellucida and emerged from the zona in a hatching process. In contrast, each of the homozygotes collapsed, showing neither expansion of the blastocoel nor hatching from the zona (at 72 h of incubation in vitro; Fig. 3). We analyzed a total of 42 embryos from heterozygous natural matings in these experiments; 10 (24%) of these embryos that showed a −/− genotype all appeared dead without reaching the fully expanded blastocyst stage after 72 h incubation. In the majority of the wild-type and heterozygous embryos, trophectoderm (TE) cells were observed to have spread on the surface of the dish after hatching from the zona, and an inner cell mass (ICM) propagated on these TE spreads. The early blastocyst stage phenotype of KIAA1440−/− E3.5 embryos was therefore likely to have been caused by an arrest in development and not simply a delay in this process. KIAA1440−/− E3.5 embryos were thus also found to be non-viable even in in vitro cultures. This result strongly suggests that when the development of KIAA1440−/− embryos arrests at the early blastocyst stage in vivo, this is followed by the absence of hatching and implantation to the uterus, eventually resulting in embryonic lethality.

3.4. KIAA1440−/− embryos undergo apoptotic cell death

KIAA1440−/− E3.5 embryos appeared to undergo cell death over long-term culturing in vitro. To determine whether this was due to an apoptotic response, we performed TUNEL analysis of these embryos. After natural mating between heterozygotes, E3.5 embryos were isolated and transferred into ES medium, incubated for 12 h, and subjected to the TUNEL assay. Images of the embryos were then captured to record the signals obtained, and each was genotyped by PCR. In total, 41 embryos (wild type:heterozygous:homozygous, 12:20:9) were analyzed. In the KIAA1440−/− embryos, a large number of strong TUNEL-positive signals were observed for at least 10 cells in most instances (Fig. 4, panel f). The signals were predominantly in the ICM. However, few signals were detectable in either the wild-type or heterozygous embryos (Fig. 4, panels d, e). DAPI staining also indicated that condensation of chromatin had occurred, which is a known specific feature of apoptosis, in the KIAA1440−/− blastocysts (Fig. 4, panel i). We also confirmed whether activation of caspase-3/7 had occurred in the KIAA1440−/− embryos as these are key factors in apoptosis [10]. For this purpose we utilized FAM-DEVD-FMK, a caspase-3/7-specific fluorescent inhibitor. After natural mating of heterozygotes, E3.5 embryos were isolated and cultured for 6 h. Detection of activated caspase-3/7 was performed at a timepoint that was 6 h earlier than the TUNEL analysis. A total of 43 embryos were assessed (wild type: heterozygote: homozygote, 11:23:9). In the KIAA1440−/− embryos, weak but clear fluorescent signals were typically observed in at least three cells (average 5.1 cells) of the blastocyst (Fig. 5, panel f). These fluorescent-positive cells also showed chromatin

![Fig. 3. Viability of KIAA1440−/− blastocysts in in vitro culture. Individual E3.5 embryos were cultured in gelatin-coated tissue culture plates containing ES medium, supplemented with leukemia inhibitory factor. After 72 h, the embryos were photographed with a phase-contrast microscope and genotyped by PCR. There were no differences detected in viability or development after the hatching of KIAA1440−/− (a) and KIAA1440+/− (b) explants. Moreover, the inner cell mass (ICM) was found to have propagated on the trophectoderm (TE) cell spreads in these samples. KIAA1440−/− embryos (c), however, were unable to hatch from the zona pellucida and appeared dead. Scale bar, 100 μm.](image)

![Fig. 4. Detection of apoptosis in E3.5 blastocyst cells by TUNEL analysis. Embryos isolated at E3.5 were cultured in medium for 12 h and subjected to TUNEL analysis. Differential interference images of these embryos after culturing are shown (a–c). Cells that underwent apoptosis were positively labeled with TMR-red fluorescence (d–f). Nuclear DNA was stained with DAPI (g–i). The arrows indicate condensation of chromatin. Scale bar, 50 μm.](image)

![Fig. 5. Detection of activated caspase-3/7 in E3.5 blastocyst cells. Embryos isolated at E3.5 were cultured in medium for 6 h. Differential interference images of embryos after culturing are shown (a–c). Activated caspase-3/7 was detected by caspase 3/7-specific fluorescent inhibitor (d–f). All FAM fluorescent images shown were enhanced equally because of the relatively weak signals. Nuclear DNA staining was performed using DAPI (g–i). Scale bar, 50 μm.](image)
Fig. 4.

Fig. 5.
condensation (Fig. 5, panel i). In contrast, few fluorescent signals were evident in the majority of the wild-type and heterozygous blastocysts and no chromatin condensation was detectable in these embryos (Fig. 5, panels d–e, g–h). Hence, the results of both TUNEL and FAM-caspase-3/7 assays consistently showed that KIAA1440−/− E3.5 embryos activated caspase-3/7 and induced apoptotic cell death.

3.5. Murine KIAA1440 mRNA is induced at the E3.5 blastocyst stage

We previously reported that KIAA1440−/− blastocysts fail to generate intact transcripts for KIAA1440 [5]. To confirm that the KIAA1440 mRNA in wild-type embryos is increased at the E3.5 blastocyst stage, we compared the levels of these transcripts in blastocysts and in fertilized eggs using real-time quantitative RT-PCR. Fertilized eggs and blastocysts were isolated at E0.5 and E3.5, respectively, from wild-type mice. After the addition of exogenous GFP RNA to monitor the efficiency of RNA recovery and reverse transcription, total RNAs were purified, reverse transcribed and subjected to real-time quantitative RT-PCR. The average values from three independent experiments are shown in Fig. 6. The KIAA1440 mRNA levels in the blastocyst were 5.7-fold higher than those in the fertilized egg. Because the majority of genes remain silent transcriptionally at the single-cell stage and initiate transcription at the two-cell stage [11,12], the mRNA species in fertilized eggs are maternally derived. KIAA1440 mRNA is therefore transcribed and increases in concentration during the period from E0.5 to E3.5. Our present data also indicate that KIAA1440 mRNA is essential during immediate early development, at least in the blastocyst.

3.6. The mouse KIAA1440 protein is predominantly localized in the nucleus

Disruption of KIAA1440 induces an apoptotic response at the early blastocyst stage. To further elucidate the cascades that confer the phenotypic abnormalities as a result of the lack KIAA1440 in mouse, we analyzed the subcellular localization of this protein. Mouse KIAA1440 cDNA encodes 2,222 amino acids, a ~248 kDa protein. EGFP was fused to either the N- or C-terminal of the full-length KIAA1440 cDNA and extracts from COS7 cells transiently expressing either EGFP-KIAA1440 or KIAA1440-EGFP were analyzed by Western blot using anti-GFP antibodies. Single strong signals were detected in both samples and were of the expected size of the exogenous protein product: 275 kDa (248 plus 27) (Fig. 7A). In addition, no degradation products were evident, in spite of the extremely large size of this protein product. Both EGFP-KIAA1440 and KIAA1440-EGFP localized predominantly in the nuclei of COS7 cells, but showed marginal expression in the cytoplasm when examined by fluorescence microscopy (Fig. 7D). To exclude the possibility that the EGFP tag acts as a cellular localization signal, we analyzed the native form of KIAA1440 using anti-mouse KIAA1440 antiserum. A strong signal of approximately 250 kDa was detectable by Western blotting of extracts from COS7 cells expressing the native form of mouse KIAA1440 and immunodetection with this antiserum (Fig. 7B). The native form of the KIAA1440 protein was also found to be localized in the nuclei by immunofluorescence microscopy (Fig. 7E). We also observed robust perinuclear signals, likely originating from the Golgi apparatus, when either EGFP-KIAA1440 or native KIAA1440 was overexpressed in COS7 cells (data not shown). However, we cannot presently distinguish whether these robust signals are due to overproduction of KIAA1440 or due to alternative cellular localization properties of KIAA1440. Moreover, endogenous KIAA1440 protein in non-transfected NIH 3T3 was detectable by both Western blot and immunofluorescence microscopy analyses using affinity-purified anti-mouse KIAA1440 antiserum (Fig. 7C and F). This antiserum was affinity-purified with MBP-tagged mouse KIAA1440 protein from polyclonal antiserum from rabbits that had been immunized with His-tagged mouse KIAA1440 (amino acid residues 241–544). Taken altogether, our data confirmed that mouse KIAA1440 protein is a large nuclear protein and that its disruption arrests the development of embryos at the early blastocyst stage in mice with an ICR genetic background and subsequently induces apoptosis predominantly within the ICM of the blastocyst.

3.7. KIAA1440−/− embryos have increased levels of unprocessed, primary U2 snRNA transcript but significantly decreased levels of the mature transcript

During the course of the present study, Baillat et al. reported a new biological protein complex in nucleus—the integrator complex—that is recruited to U1 and U2 snRNA genes and mediates the 3′-end processing of snRNAs in cultured human cells [13]. Mass spectrometric analysis showed that the integrator complex comprises 12 subunits: Int1–Int12 (gene symbol: Ints1–
Interestingly, the Ints1 component contains the same peptides that we reported previously for human KIAA1440 [14]. Baillat et al. also reported that siRNA-directed depletion of KIAA1440/Ints1 affects the processing of U1 and U2 snRNA. These observations prompted us to test whether snRNA processing is affected also in KIAA1440−/− embryos.

To confirm that snRNA processing activity is decreased in KIAA1440−/− embryos, we assessed snRNA processing in KIAA1440 knockout mice by qRT-PCR using total RNA prepared from individual embryos. The gene structure of U2 snRNA is shown in Fig. 8A. U2 snRNA is transcribed by RNA polymerase II (RNAPII) beyond the 3′ box [15], and the primary transcript is cleaved just before the 3′ box. The primary unprocessed transcript was detected by using primers c and d, and both mature and primary transcripts were detected by using primers a and b. Genomic DNA and total RNA were prepared from individual blastocysts derived from KIAA1440−/− intercrosses and subjected to PCR genotyping and qRT-PCR, respectively. In a control qRT-PCR experiment without reverse transcriptase, we confirmed that the purified total RNA after DNase I treatment contained an only negligible amount of genomic DNA (data not shown).

In KIAA1440−/− embryos, the amount of primary U2 transcripts was 7.1-fold higher than that in wild-type embryos (Fig. 8B), whereas the total (mature and primary) amount of U2 transcripts decreased to 35% of that in the wild type and KIAA1440+/− embryos (Fig. 8C). Note, however, that mature U2 transcripts primarily contribute to the amount of total U2 transcripts. We arrived at this conclusion through our qRT-PCR analysis using serial-diluted control plasmid DNA that showed that the absolute amount of primary U2 transcripts was 103-fold lower than that of total U2 transcripts. The ratio of primary transcripts to total transcripts in KIAA1440−/− embryos was much higher than that in the wild type (Fig. 8D). These results are consistent with the proposal of Baillat et al. that KIAA1440 is the largest subunit of the integrator complex and that it is involved in snRNA processing.

The accumulation of primary U2 transcripts was significant in KIAA1440−/− embryos. The levels of total U2 transcripts, however, were decreased (Fig. 8C). To exclude the possibility that total U2 transcript levels in KIAA1440−/− embryos might...
Fig. 8. Quantitative analysis of U2 snRNA in E3.5 blastocysts. (A) Schematic diagrams showing the structure of the U2 snRNA gene. The genomic structure of the U2 snRNA gene is indicated at the top. The primary transcript is cleaved just before the 3′ box. Mature and primary transcripts are represented by large gray arrows. The primers used are represented by small black arrows. Total (mature + primary) transcripts were amplified with primers a and b, and primary transcripts were amplified with primers c and d. (B–E) qRT-PCR of U2 snRNAs and 18S rRNAs in blastocysts. Each genomic DNA for genotyping and total RNAs for qRT-PCR were prepared from individual blastocysts derived from KIAA1440+/− intercrosses. The samples were not pooled but analyzed individually. The relative quantities of each type of transcript relative to the average of transcript from wild-type blastocysts are shown (B, U2 primary; C, U2 total; E, 18S rRNA). (D) The ratio of primary U2 transcripts to total transcripts was calculated by dividing the number of primary transcripts by the number of total transcripts, and is shown as the relative ratio to that calculated from the wild type. The means and standard deviations were obtained from 55 (U2 snRNA) and 57 (18S rRNA) embryos. The mean values are indicated below the graphs. Two and three asterisks indicate *P*<0.01 and **P**<0.001, respectively.

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have been influenced by the relatively smaller size of these embryos, we analyzed 18S rRNA, which is less likely to be directly affected by the depletion of integrator subunits because it is transcribed by RNA polymerase I and processed in a manner different from pre-mRNA splicing [16]. In KIAA1440−/− embryos, the amount of 18S rRNA was reduced to 72% of that in the wild type (Fig. 8E). This decrease, however, is smaller than the decrease we observed with total U2 transcripts. When we normalized the level of total U2 transcripts with that of 18S rRNA, the level of total (mature) transcripts in KIAA1440−/− embryos remained reduced (i.e., 48% of that in the wild type). Taken together, these qRT-PCR results confirmed that KIAA1440 is involved in snRNA 3′-end processing in vivo, strongly suggesting that embryonic lethality of KIAA1440−/− embryos is likely caused by the accumulation of primary U2 snRNA transcripts followed by the decrease of mature U2 transcripts.

4. Discussion

In our current study, we have extensively characterized the phenotype of KIAA1440−/− mice that were bred in a pure genetic background. We have also demonstrated the subcellular localization of the KIAA1440 protein and have investigated the involvement of KIAA1440 in snRNA processing. Mouse KIAA1440 is an extremely large nuclear protein composed of 2222 amino acids, but it contains no significant domains that have been previously identified in spite of its large size. Disruption of the KIAA1440 gene forces the arrest of embryonic development at the early blastocyst stage in a pure ICR genetic background, although the majority of the KIAA1440−/− mouse embryos of mixed genetic background do not progress past the earlier morula stage. It is widely known that abnormal phenotypes conferred by targeted mutation are often influenced by the genetic background. For example, a targeted mutation of the epidermal growth factor receptor results in three non-overlapping phenotypes depending on the genetic background: preimplantation lethality in a 129/Sv-CF1 background, midgestation lethality in an inbred 129/Sv background, and prenatal lethality on the 129/Sv-CD1 or C57BL/6J background [17]. Such phenotypic variations in different genetic backgrounds suggest the existence of modifier genes. The phenotype of the KIAA1440−/− mice is thus likely to be affected by an as of yet unidentified modifier gene; and it is possible that either KIAA1440 or the KIAA1440 complex interacts developmentally with this factor.

KIAA1440−/− embryos in the pure ICR background can complete the compaction process, with no abnormal localization of E-cadherin or β-catenin, at the morula stage and initiate the differentiation of both TE cells and the ICM. This is followed, however, by an apoptotic response mainly in the ICM of the blastocyst in these knockout embryos. At this developmental stage exhibiting abnormalities in the KIAA1440−/− embryos, KIAA1440 mRNA levels are normally about 5.7-fold higher than those in single-cell stage embryos, whereas KIAA1440−/− embryos obviously fail to produce intact KIAA1440 transcripts. This strongly suggests that KIAA1440 plays a positive role in early development, not only via indirect effects resulting from the depletion of maternal KIAA1440 mRNA.

U1 and U2 snRNAs are components of the spliceosome, which is involved in pre-mRNA splicing [18,19]. The snRNAs are transcribed by RNAPII to produce short nonpolyadenylated 3′-extended precursors. The formation of proper snRNA is directed by a highly conserved cis-acting sequence, named the 3′ box, located 9–19 nucleotides downstream of the mature 3′ end of an snRNA [20,21]. The C-terminal domain (CTD) of the largest subunit of RNAPII and a processing complex directly associated with it play a critical role in RNA processing of 3′ end of snRNAs [22–24]. During the course of this study, the study of Baillat et al. [13] was reported. It described the integrator complex, which is recruited to U1 and U2 snRNA genes and mediates snRNAs’ 3′-end processing. The results of Baillat and colleagues strongly suggest that KIAA1440 is the largest subunit of the integrator complex. We have focused primarily on large proteins in our laboratory, because we contend that they serve as frameworks for the intricate assembly of protein complexes, and that the inactivation of their genes is likely to confer definitive and readily detectable phenotypes. This assertion is consistent with Baillat and colleagues’ data showing that KIAA1440 indeed belongs to a large biological protein complex, the integrator complex [13]. Furthermore, in this study we have shown that in KIAA1440−/− embryos primary U2 snRNA transcripts accumulate but mature U2 snRNA transcripts do not and are in fact reduced in amount. These results confirm that KIAA1440 is involved in snRNA processing in vivo and strongly suggest that aberrant snRNA processing likely causes the phenotypes of KIAA1440 knockout mice. We speculate a straightforward scenario on why the KIAA1440−/− genotype leads to growth arrest and cell death in KIAA1440−/− embryos. First, depletion of KIAA1440 results in the loss or significant decrease of snRNA processing activity of the integrator complex. Second, this results in the reduction of mature U2 transcripts followed by the breakdown of spliceosome machinery. Third, this breakdown leads to the accumulation of pre-mRNAs and a decrease in mature mRNAs. Finally, the decrease in mature mRNAs results in decreased de novo protein synthesis, which affects various cellular functions including development and cell survival. At present, however, we do not know whether a decrease in the amount of mature U2 transcripts (i.e., half of that found in the wild type) can impair splicing of all mRNAs or splicing of specific mRNAs, e.g., fast-turnover or rare mRNAs that are more sensitive to decreased splicing activity or mRNAs that encode functionally critical proteins necessary in early developmental stages.

Although depletion of KIAA1440/Ints1 affects the 3′-end processing of U1 and U2 snRNA, the individual functions of most of the subunits contained in the integrator complex are still unknown. The Ints6 protein was previously identified as DICE1, a candidate tumor suppressor in non-small-cell lung carcinoma, and its subcellular localization is predominantly nuclear [25,26]. Moreover, Ints9/RC-74 and Ints11/RC-68 share sequence similarities with the cleavage and polyadenylation specificity factors, PCPF-100 and PCPF-73, respectively. Ints11 is likely to be the catalytic subunit of the integrator complex because of its sequence similarity to the putative catalytic β-lactamase domain (RNA-specific endonuclease) and the results of mutant analyses
within this domain [13]. Ints9 is localized in the nucleus and Ints11 is predominantly nuclear [27]. Ints6, Ints9 and Ints11, in addition to KIAA1440/Ints1, are thus localized in the nucleus, consistent with the role and localization of the integrator complex.

As KIAA1440/Ints1 is the largest subunit of the integrator complex, the question arises as to the individual functions of this protein. As we have speculated above, KIAA1440/Ints1 should serve as a framework for the intricate assembly of the integrator complex. In fact, our preliminary results from yeast two-hybrid screening indicate that KIAA1440/Ints1 binds to Ints2 directly. Interestingly, Ints2 is also a relatively large protein (KIAA1287 reported previously by our group [28]). While further extensive studies are still needed, we speculate that both KIAA1440/Ints1 and KIAA1287/Ints2 function as scaffold proteins for the assembly of the integrator complex, and that all of the other additional subunits directly interact with these factors. Disruption of these framework proteins would inevitably result in the complete loss of integrator complex.

Scaffold proteins for the assembly of the integrator complex, and that both KIAA1440/Ints1 and KIAA1287/Ints2 function as scaffold proteins for the assembly of the integrator complex, and that all of the other additional subunits directly interact with these factors. Disruption of these framework proteins would inevitably result in the complete loss of integrator complex function. It is known that during early development in mouse, U1 and U2 snRNAs are increased three- to ten-fold compared to those in the two-cell stage to the blastocyst stage [29], but the changes that occur in the 3'-end processing activity of the U1 and U2 snRNAs during this time remain unknown. In KIAA1440−/− embryos, depletion of mature U2 snRNAs from spliceosome machinery will directly or indirectly result in an apoptotic response and early blastocyst stage arrest. We cannot, however, exclude an alternative possibility that KIAA1440 contributes directly to the regulation of embryonic development via a separate function or via RNA processing activity that is independent of U1 and U2 snRNAs.

Finally, it is noteworthy that, to our knowledge, the KIAA1440/Ints1 gene-targeted mice produced by our group are the first integrator complex subunit mutants. Comparison of the phenotypes that would result from the targeting of other integrator complex subunits, followed by overall three-dimensional structural analysis of the integrator complex in addition to identification of the Ints1–12 protein interactive domains, will be of great interest for future research.

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