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Impaired expression of importin/karyopherin β1 leads to post-implantation lethality

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Abstract

Importin β1 (Impβ)/karyopherin β1 (Kpnb1) mediates the nuclear import of a large variety of substrates. This study aimed to investigate the requirement for the *Kpnb1* gene in mouse development, using a gene trap line, B6-CB-*Ayu8108*^{GtgeoIMEG} (*Ayu8108*^{geo}), in which the trap vector was inserted into the promoter region of the *Kpnb1* gene, but in reverse orientation of the *Kpnb1* gene. *Ayu8108*$^{geo/geo}$ homozygous embryos could develop to the blastocyst stage, but died before embryonic day 5.5, and expression of the *Kpnb1* gene in homozygous blastocysts was undetectable. We also replaced the *βgeo* gene with Impβ cDNA through Cre-mediated recombination to rescue Impβ expression. Homozygous mice for the rescued allele *Ayu8108*$^{Impβ/Impβ}$ were born and developed normally. These results demonstrated that the cause of post-implantation lethality of *Ayu8108*$^{geo/geo}$ homozygous embryos was impaired expression of the *Kpnb1* gene, indicating indispensable roles of Impβ1 in early development of mice.

Keywords: Importin β1; Gene trap; Cre/lox site-specific recombination; Embryonic lethal

Active nuclear import of proteins is a highly selective process involving specific recognitions between nuclear localization signals (NLS) and suitable receptors [1]. Importin/karyopherin β1 (Impβ) is a key player in nuclear protein import and mediates targeting of a canonical NLS substrate bound to an adaptor protein, importinα [2,3]. On the nuclear membrane, Impβ interacts with nuclear envelope-localized nuclear pore complexes (NPCs) and carries the importinα/cargo complex from the cytoplasm into the cell nucleus. In the nucleus, RanGTP, which exists predominantly in the nucleus, binds to Impβ and induces the release of import cargoes. Thereafter, individual importins are recycled back to the cytoplasm. Thus, Impβ is a critical component in mechanisms involved in targeting of the NLS substrate into the nucleus [4–6].

In addition to nuclear transport, Impβ has also been shown to play a role in regulation of spindle formation and of aster promoting activity (APA) during mitosis [7–10]. Two microtubule organizing components, NuMA and TPX2, which are retained in the nucleus during interphase, bind to Impβ via importinα during mitosis and are kept away from chromatin. RanGTP releases NuMA and TPX2 from importin α/β heterodimer around chromosomes and promotes spindle formation.

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Impβ is the prototype of the karyopherin family including more than 20 members in mammals [11]. Ten of these members have been identified to have a role in nuclear import. In general, such importinβ family proteins function without the need of an adaptor protein and can directly interact with their substrates. To the best of our knowledge, only Impβ requires an adaptor, importinα, to bind to its cognate cargoes.

Extensive studies using cultured cells have clarified molecular mechanisms of nuclear transport. However, roles and functional redundancy of Impβ family members in vivo have been poorly studied. In *Drosophila*, Impβ mutant strains (*Ketel* mutations) have been isolated and analyzed [12–14]. Homozygous larvae for the loss-of-function *Ketel* allele die during the second larval instar, but on the other hand, homozygous somatic clones induced by mitotic recombination are viable in the follicle epithelium in wings and tergites. Since the *Ketel* gene is not expressed in larval and adult cells that are mitotically inactive, another import pathway might substitute for the function of Impβ in *Drosophila*.

In order to identify the requirement for the *importin/karyopherinβ1* (Kpnb1) gene in mouse development, we analyzed a *Kpnb1* mutant mouse line established by gene trapping [15]. The trap vector was inserted in the promoter region of the *Kpnb1* gene in the reverse orientation of the transcription of the *Kpnb1* gene, and expression of the *Kpnb1* gene from the mutated allele was severely decreased. Homozygous embryos could develop to the blastocyst stage, but died before embryonic day (E) 5.5, indicating that Impβ1 played an indispensable role in early development of mice.

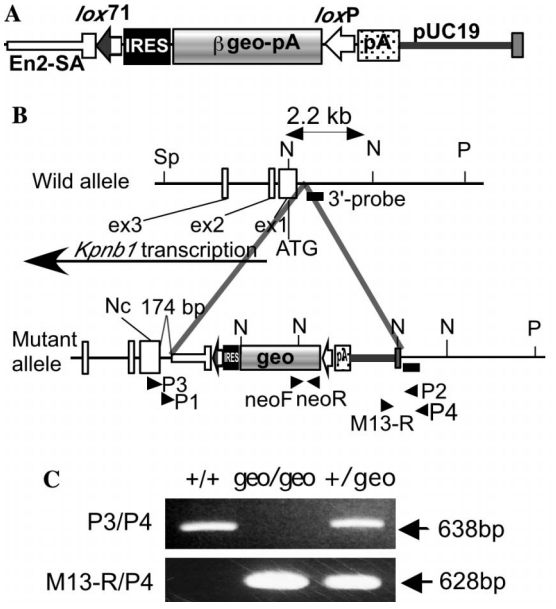

Fig. 1. Gene trap events in the Ayu8108 clone. (A) Structure of the trap vector, pU-hachi. The pU-hachi vector contains an IRES-βgeo-pA cassette flanked by *lox*71 and *lox*P to exchange the βgeo cassette into any DNA sequence through recombination by Cre. En2, the mouse *Engrailed 2* gene; SA, splice acceptor; pA, polyadenylation signal. (B) Integration pattern of the trap vector. Open boxes on the maps represent the first three exons of the *Kpnb1* gene. The trap vector was inserted 174 bp upstream from the first exon. The start codon and direction of transcription of the *Kpnb1* gene are indicated. The solid bar with "3′-probe" shows the probe used in Southern blotting for genotyping. Arrowheads indicate primers used for genotyping and RT-PCR. N, *Nco*I; Sp, *Sph*I; P, *Pst*I. (C) Genotyping by PCR. The primer pairs neo-F/neo-R and P1/P2 were used to detect the $Ayu8108^{geo}$ and wild-type alleles, respectively.

Materials and methods

Isolation of ES clones and establishment of mutant mouse lines. The gene trap vector pU-hachi and isolation of trap clones were described previously [15]. The vector contained a splice acceptor region (SA) from the mouse *En-2* gene, *lox*71, the internal ribosomal entry site (IRES) from the encephalomyocarditis virus (ECMV), the *β-galactosidase/neomysin phosphotransferase fusion* gene (*βgeo*), *lox*P, the SV40 polyadenylation sequence (pA), and pUC19 (Fig. 1A). In order to replace the *βgeo* gene with the Impβ cDNA sequence, a replacement vector carrying *lox*66-Impβ cDNA–phosphoglycerate kinase 1 (PGK)–*puromycin resistant* (*Pac*) gene–*lox*P–pSP73 was constructed. Site-specific integration in trap clones mediated by Cre-recombinase was performed as described previously [15]. Chimeric mice were produced by aggregation of ES cells with eight-cell embryos of ICR mice (Nippon Clea, Tokyo, Japan), as described previously [16]. Chimeric male mice and their heterozygous progeny were backcrossed for five to eight generations to a C57BL/6J background.

Molecular cloning of flanking genomic region by plasmid rescue. Plasmid rescue was performed as described previously [15]. Genomic DNA of Ayu8108 ES cells was digested with *Pst*I or *Sph*I, followed by self-ligation, and introduction into *Escherichia coli* cells by electroporation. The recovered plasmids were mapped by restriction enzymes and sequenced using the dideoxy chain termination method using a BigDye Terminator Cycle Sequencing (Perkin-Elmer, Foster City, CA).

Genotyping of mice. Genomic DNA was isolated by proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation from tail biopsies of newborn, E10.5 embryos, and E7.5 embryos.

For newborn mice and E10.5 embryos, Southern blot analysis was carried out. Seven micrograms of genomic DNA was digested with an appropriate enzyme, electrophoresed on 1% agarose gel, and blotted onto a nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany). Probe preparation and hybridization were performed with the DIG DNA Labeling and Detection Kit (Roche).

For E7.5 embryos, PCR analysis was carried out. To identify the mutant allele, the primers neo-F (5′-AGAGGCTATTCGGCTATGAC-3′) and neo-R (5′-CACCATGATATTCGGCAAGC-3′) were used. PCR conditions were 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s, using 0.5 U AmpliTaq DNA polymerase (Roche). To identify the wild allele, the primers P1 (5′-ATTGGCCGGACGGCTAGCGT-3′) and P2 (5′-GCTGAGCCCGAAGGCCTTTA-3′) were used. PCR conditions were 30 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s, using 0.5 U LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan).

For blastocysts and cultured embryos, individual embryos were lysed for 5 min in 2 μl of 0.005% SDS, 0.035 N NaOH, at 100 °C. After neutralization by adding 36 μl water, PCR was carried out using 5 μl of the extract. Primer pairs M13-R (5′-AGGAAACAGCTATGACCATGA-3′) and P3(5′-GGTCACCACAAGCCCATTCA-3′), P3 and P4 (5′-GAAC TCCTCGCTTCAGTTCT-3′) were used to identify mutant and wild alleles, respectively. PCR conditions were 40 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, using 0.5 U LA *Taq* polymerase (TaKaRa).

Quantitative analysis of expression of the Kpnb1 gene. Ten micrograms total RNA was reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed with a LightCycler instrument, using Faststart DNA

Master SYBR Green I Kit (Roche). PCRs were performed using B1 (5′-TAACCATCCTCGAGAAGACC-3′) and B2 (5′-ATCCCCTGGATT ATGGCAGT-3′) primers, and 40cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

Isolation and in vitro culture of blastocysts. Heterozygous female mice were superovulated and mated with heterozygous male mice, and 2-cell stage embryos were isolated and cultured in KSOM [17] medium for 2 days to the blastocyst stage. Individual blastocysts were transferred to single wells coated with gelatin in 15% FCS–DMEM. Plates were incubated and kept in a humid chamber at 37 °C, 5% CO_2 for 3 days.

Histological analysis of E5.5 embryos. Heterozygous and wild-type females were superovulated and mated with heterozygous males. Fertilized eggs were collected from the oviducts and transferred to foster mothers on the same day. Decidual swellings containing E5.5 embryos were dissected, fixed in 4% paraformaldehyde solution, and sectioned. Serial sections were stained with hematoxylin and eosin (H/E), and observed under a microscope.

RT-PCR analyses. Total RNA was isolated from individual blastocysts using the RNeasy Mini Kit (Qiagen, Valencia, CA) and suspended in 30 μl water. First-strand cDNA was synthesized using 9 μl RNA solution with oligo(dT) primers in a ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA). One-twentieth volume of the first-strand reaction was used for PCR amplification. Expressions of the *βgeo* and *Kpnb1* genes were detected using primers neo-F and neo-R, and primers B3 (5′-CTCTTCA GAATGTTCTCCGG-3′) and B4 (5′-GATCTCCGCCCTTCAGTTAA-3′), respectively. Pericentriolar material-1 (PCM1) was used as internal positive control. Primers for PCM1 were 5′-GCGTTACCCAACTT AATC-3′ and 5′-TGTGAGCGAGTAACAACC-3′. Both PCR conditions were 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s using 0.5 U LA *Taq* DNA polymerase (TaKaRa). In the case of adult mice, 10 μg of total RNA was used for first-strand cDNA synthesis using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). To detect expressions of the inserted Impβ cDNA and the endogenous *Kpnb1* gene, primers E3 (5′-GTTCGAGCTTGGAATTCATG-3′) and B5 (5′-CCGT CGAGCATTAGCATCAA-3′), and B3 and B6 (5′-CCTCTC ATTCCCAAGCATTC-3′) were used, respectively. PCR conditions were 35 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s using 0.5 U AmpliTaq polymerase (Perkin-Elmer).

5-rapid amplification of cDNA ends (RACE) and 3′RACE analyses. Total RNA was prepared using Sepazol reagent (Nacalai Tesque, Kyoto, Japan), and mRNA was purified using the Oligotex-dT30 Super mRNA Purification Kit (TaKaRa). Reverse-transcription was performed with Thermoscript (Invitrogen) using primer E1 (5′-AGCAGTGAA GGCTGTGC-3′) for 5′RACE. The 5′RACE System for Rapid Amplification of cDNA Ends Reagent Assembly Ver. 2.0 (Invitrogen) was used according to the manufacturer's instructions, using the primer E2 (5′-CTT TGTTAGGGTTCTTCTTC-3′). Products were electrophoresed and subjected to Southern blotting using digoxigenin-ddUTP-labeled oligonucleotide probes for the SA sequence in the trap vector, prepared using the DIG Oligonucleotide 3′-End Labeling Kit (Roche). Hybridization with the probes was carried out overnight at 60 °C.

The downstream cDNA fragment containing a poly(A) stretch was obtained using 3′RACE System for Rapid Amplification of cDNA Ends (Invitrogen). First-strand cDNA was synthesized with a 3′RACE Adapter Primer using the Thermoscript RT-RCR System (Invitrogen) and two nested primers P5 (5′-CATTGGCCTGCGGCTTCA-3′) and P6 (5′-CGG CTTCAAGGTCCGGTT-3′). RACE products were cloned into pGEM-T vectors (Promega, Madison, WI) and sequenced.

Results

Analysis of the insertion event in Ayu8108 trap clone

Ayu8108 ES clone cells were isolated using gene trap screening with the pU-Hachi trap vector (Fig. 1A), which was designed for the exchangeable gene trap [15]. Southern blot analysis with a probe for the pUC vector fragment revealed a single copy integration of the vector (data not shown). Genomic DNA fragments flanking both the 5′ and 3′ ends of the integrated vector were obtained by the plasmid rescue method after removal of the *βgeo* sequence by Cre-mediated recombination [18]. Sequence analysis of the flanking genomic DNA and homology search in the GenBank database revealed that the trap vector was integrated into the promoter region of the *Kpnb1* gene. Although the transcription initiation site of the *Kpnb1* gene was not yet determined, several expression sequence tag (EST) sequences covering the 5′-region of the *Kpnb1* gene show 5′-ends at 290–323 bp upstream of the start codon of the *Kpnb1* gene. Therefore, in our study, the 5′-end of the BY740663 EST sequence, which has the most extended 5′-end, was considered as the *Kpnb1* transcription initiation site. The trap vector was integrated at 225 bp upstream of the first exon, however, direction of transcription of the *βgeo* was opposite to that of the *Kpnb1* gene (Fig. 1B). Deletion of genomic DNA at the integration site was only of 46 bp, and no deletion was found in the integrated trap vector.

Chimeric mice were produced by aggregation of Ayu8108 ES cells with morulae, and the trap mouse line designated as B6-CB-$Ayu8108^{GtgeoIMEG}$ ($Ayu8108^{geo}$) was established.

Identification of the trapped transcript in the Ayu8108 line

The fact that the direction of transcription of the *βgeo* was opposite to that of the *Kpnb1* gene (Fig. 1) indicated the existence of another gene in the promoter region of the *Kpnb1* gene. In fact, ubiquitous expression of the trapped gene was observed by X-gal staining in E9.5 and E12.5 embryos (data not shown). Moreover, in adult mice, transcripts of the *βgeo* were detected in the brain, heart, lung, kidney, and testis using RT-PCR (data not shown).

To identify endogenous transcripts fused to the βgeo gene, we performed 5′RACE and obtained a clear single band (Fig. 2A). Sequence analysis revealed that transcription of the fusion transcripts started at 50 bp upstream from the insertion site, which corresponded to 124 bp upstream of the first exon of the *Kpnb1* gene, and spliced at a cryptic splice-donor site (position 52 in the pU-hachi sequence, GenBank Accession No. AB242616) within the intron of the trap vector to fuse the authentic splice acceptor (Fig. 2B). Then, 3′RACE was performed to obtain the whole transcript of the trapped gene, and a 1.3-kb product was obtained (GenBank Accession No. AB242615). As shown in Fig. 2C, the sequence was identical to the 3′-flanking genomic sequence of the trap vector. Homology search using BLAST programs [19] identified several mouse ESTs with high homology (>95%). However, in the 1.3-kb transcript, multiple stop codons appeared in all three frames, and no apparent ORF was found. Northern blot analysis was performed using both total and poly(A) RNAs from adult tissues and an RNA probe for

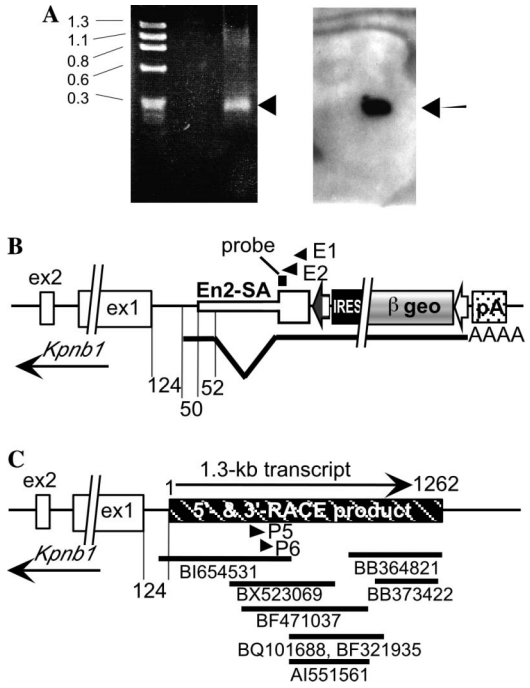

Fig. 2. Identification of the transcript trapped in the Ayu8108 clone. (A) 5′-RACE analysis of the Ayu8108 ES line. A single band (arrowhead) was obtained with E1 and E2 primers (left), and the product hybridized with an oligoprobe from the SA sequence (right, arrow), showing successful amplification of the fusion transcript with the SA sequence of the trap vector. (B) Schematic structure of transcription of the fusion transcript from the *Ayu8108*^{geo} mutant allele. Transcription started 124 bp upstream from the first exon of the *Kpnb1* gene and was spliced at a cryptic splice donor site in the intron sequence of the trap vector. (C) Transcription of the trapped gene in the wild allele. The 1.3-kb transcript identified by 5′- and 3′RACE is indicated by a thick box. The arrow on the box indicates the direction of transcription. Primers used for 3′-RACE are indicated by arrowheads. EST sequences from GenBank are shown under the map with their respective accession numbers.

the 1.3-kb product. However, only a faint signal was detected with total RNA, and no signal was obtained with poly(A) RNA (data not shown). Thus, in the Ayu8108 clones, we could not detect endogenous transcripts from the trapped gene. However, it was possible that a non-coding RNA gene was trapped.

Expression of the Kpnb1 gene is impaired in Ayu8108$^{+/geo}$ *mice*

Since the integration site of the trap vector was quite close to the first exon of the *Kpnb1* gene, it was expected that insertion would result in impaired expression of the *Kpnb1* gene, although direction of transcription was opposite. In order to examine expression levels of the *Kpnb1*gene in *Ayu8108*$^{+/geo}$ heterozygous mice, we performed real-time RT-PCR quantification in liver, kidneys, and testes of adult mice. As shown in Fig. 3, expression levels of the *Kpnb1* mRNA in *Ayu8108*$^{+/geo}$ mice were significantly lower (about 60–80%) than those of wild-type, indicating that integration of the trap vector produced a hypomorphic allele of the *Kpnb1*gene.

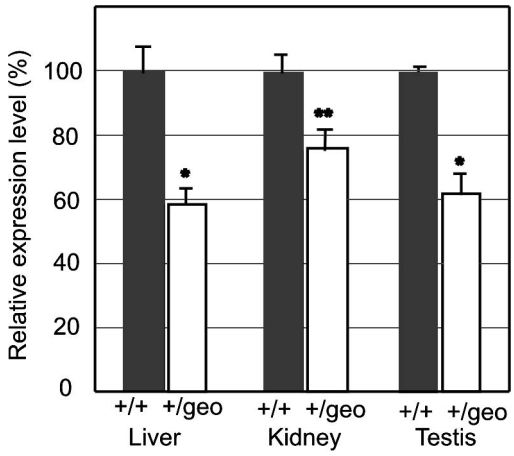

Fig. 3. Comparison of expression levels of the *Kpnb1* gene between wild-type and *Ayu8108*$^{+/geo}$ heterozygous mice by Real-time RT-PCR. Total RNAs from liver ($n = 6$), kidneys ($n = 6$), and testes ($n = 5$) of wild-type (+/+, black bars) and *Ayu8108*$^{+/geo}$ (+/geo, open bars) adult mice were used. In each tissue, relative expression level against average amount in wild-type mice is indicated with standard deviation. *$p < 0.01$; **$p < 0.05$ (Student's *t*-test).

Ayu8108$^{geo/geo}$ *homozygous embryos were lethal after implantation*

Ayu8108$^{+/geo}$ heterozygous mice were healthy in appearance and fertile. To investigate the phenotype of *Ayu8108*$^{geo/geo}$ homozygous mice, heterozygous mice were crossed, and neonates, E10.5 and E7.5 embryos, were genotyped by Southern blotting or PCR analysis (Fig. 1C). As shown in Table 1, no homozygous progeny was identified. Out of 62 deciduas obtained from E7.5, 27.4% of deciduas were empty, likely to correspond to missing homozygotes (Table 1), indicating that decreased expression of the *Kpnb1* gene caused embryonic lethality. To score rates of abortion in the uteri, heterozygous and negative littermate females were superovaluated and mated with heterozygous males. Fertilized eggs were then collected, and transferred into foster mothers on the same day, and then E5.5 deciduas were histologically analyzed. As shown in Table 2, in experiments with wild-type and heterozygous females, frequency of resorptions was 8.3% and 36.2%, respectively.

Table 1
Genotyping distribution of embryos and pups derived from heterozygous intercrosses

Age stage	+/+ (%)	+/geo, geo/+ (%)	geo/geo (%)	Resorbed (%)	Total
Blastocyst	6 (33.3)	9 (50.0)	3 (16.7)	—	18
Outgrowth	5 (20.0)	10 (40.0)	5 (20.0)	—	20
E7.5	18 (29.1)	27 (43.5)	0	17 (27.4)	62
E10.5	15 (37.5)	25 (62.5)	0	ND	40
Newborn	12 (33.3)	24 (66.7)	0	—	36

Embryos and newborn mice were genotyped by Southern blot analysis (newborn and 10.5 dpc), or by PCR analysis using P1/P2 and neo-F/R primers (7.5 dpc) or with M13-R, P3, and P4 primers (blastocysts and outgrowth blastocysts). ND, Not determined: although empty deciduas existed, their numbers were not counted.

Table 2
Frequency of resorptions of E5.5 embryos

Cross	Total No. of deciduas	No. of resorptions	Resorption frequency (%)
+/+ X +/geo	48	4	8.3
+/geo X +/geo	47	17	36.2

Fertilized eggs from the indicated crosses were transferred into oviducts. Then, at E5.5, deciduas were dissected, sectioned, and stained with hematoxylin and eosin to examine morphology of embryos. All viable embryos showed normal morphology.

The difference of 27.9% was expected to correspond to the rate of homozygotes, and no embryonal tissue was observed in the empty deciduas (data not shown). These results strongly suggested that all *Ayu8108*$^{geo/geo}$ homozygous embryos were lethal before E5.5.

To investigate whether homozygous embryos could survive to the blastocyst stage, two-cell stage embryos from heterozygous crosses were collected and cultured up to the blastocyst stage. All embryos showed normal morphology without any developmental delay. Each embryo was lysed and subjected to PCR for genotyping. At this stage, homozygous embryos were identified (Table 1). Then, growth potential of homozygous embryos was examined by in vitro culture on gelatin-coated culture slides for 3 days. All blastocysts obtained from heterozygous crosses hatched out of the zona pellucida and attached to the slides. Genotyping of explants identified 5 (20%) homozygotes (Table 1). These results suggested that *Ayu8108*$^{geo/geo}$ homozygous embryos could develop to the blastocyst stage (E3.5), and hatch out, but they died shortly after implantation by E5.5.

In order to examine expression of the *Kpnb1*gene at the blastocyst stage, RT-PCR analysis using individual blastocysts obtained from heterozygous crosses was performed. Fig. 4 depicts results of a typical experiment. In total, 70 blastocysts were examined, and 21 blastocysts (30%) showed wild-type expression pattern [βgeo(−)/Kpnb1(+)], 34 (49%) showed heterozygous expression pattern [βgeo(+)/Kpnb1(+)], and 15 (21%) showed homozygous expression pattern [βgeo(+)/Kpnb1(−)]. Results of genotyping and RT-PCR indicated that expression of the *Kpnb1* gene was undetectable by RT-PCR in *Ayu8108*$^{geo/geo}$ homozygous embryos.

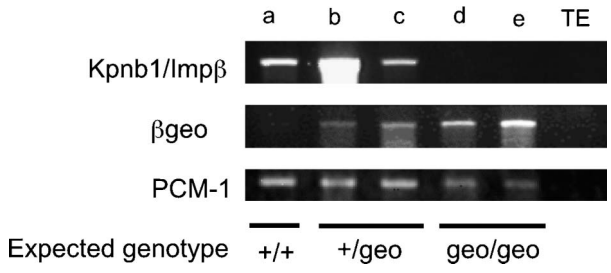

Fig. 4. RT-PCR analysis of individual blastocysts. Total RNAs from individual blastocysts (a–e) were subjected to RT-PCR to detect expression of the endogenous *Kpnb1* gene (upper panel) and the *βgeo* gene (middle panel). Detection of PCM-1 mRNA was performed as positive control (lower panel).

Expression of the Kpnb1 gene rescued post-implantation lethality

In *Ayu8108*$^{geo/geo}$ homozygous mice, expressions of the *Kpnb1* gene and of the 1.3-kb transcript were severely impaired by insertion of the trap vector. In order to determine what was responsible for lethality, we replaced the *βgeo* gene of the trap vector with Impβ cDNA, using the Cre/mutated *lox* recombination system as outlined in Fig. 5A. The inserted Impβ cDNA should be expressed under the control of the promoter for the 1.3-kb transcript, and production of the 1.3-kb transcripts should remain interrupted. After introduction of the replacement vector and of the Cre-expression vector [20] into Ayu8108 ES clones, 24 colonies were picked up, and 22 of 24 (90%) clones were shown to have the replaced alleles, *Ayu8108*$^{+/Impβ}$, by Southern blotting and PCR analyses (Fig. 5B). Then, the *Ayu8108*$^{+/Impβ}$ mouse line was established from one of the replaced clones through production of germline chimeras. Heterozygous *Ayu8108*$^{+/Impβ}$ mice were crossed, to obtain and examine phenotype of *Ayu8108*$^{Impβ/Impβ}$ homozygous mice. Genotype analysis (Figs. 1B and 5B) of 63 4-week-old offsprings identified 13 (21%) wild-type mice, 35 (56%) heterozygous mice, and 11 (17%) homozygous mice for the replaced allele, and all *Ayu8108*$^{Impβ/Impβ}$ homozygous mice appeared healthy. Expression of the endogenous *Kpnb1* gene and of the inserted Impβ cDNA was analyzed by RT-PCR using specific primer pairs for each transcript. As shown in Fig. 5C, expression of the integrated Impβ cDNA was detected in heterozygous and homozygous mice. Unexpectedly, the endogenous *Kpnb1* gene was expressed in *Ayu8108*$^{Impβ/Impβ}$ homozygous mice. This demonstrated that recovered Impβ expression from the inserted cDNA and endogenous *Kpnb1* gene rescued early embryonic lethality, and lethality was caused by impaired expression of the *Kpnb1* gene, but not of the 1.3-kb transcript.

Discussion

A hypomorphic allele of the mouse *Kpnb1* gene was generated by gene trap mutagenesis using ES cells. In heterozygous adult mice, expression level of the *Kpnb1* gene was reduced to about 60–70%, and in homozygous blastocysts, transcripts were not detected by RT-PCR. Homozygous blastocysts were able to grow on gelatin-coated slides, but homozygous implanted embryos died before E5.5 in vivo. These results indicated that Impβ protein was indispensable for the development of early stage embryos, and that any other importinβ family members could not compensate for nuclear import activity.

The developmental function of Impβ has been analyzed only in mutant *Drosophila* strains. In *Drosophila*, homozygous mutants could develop to the second larval instar

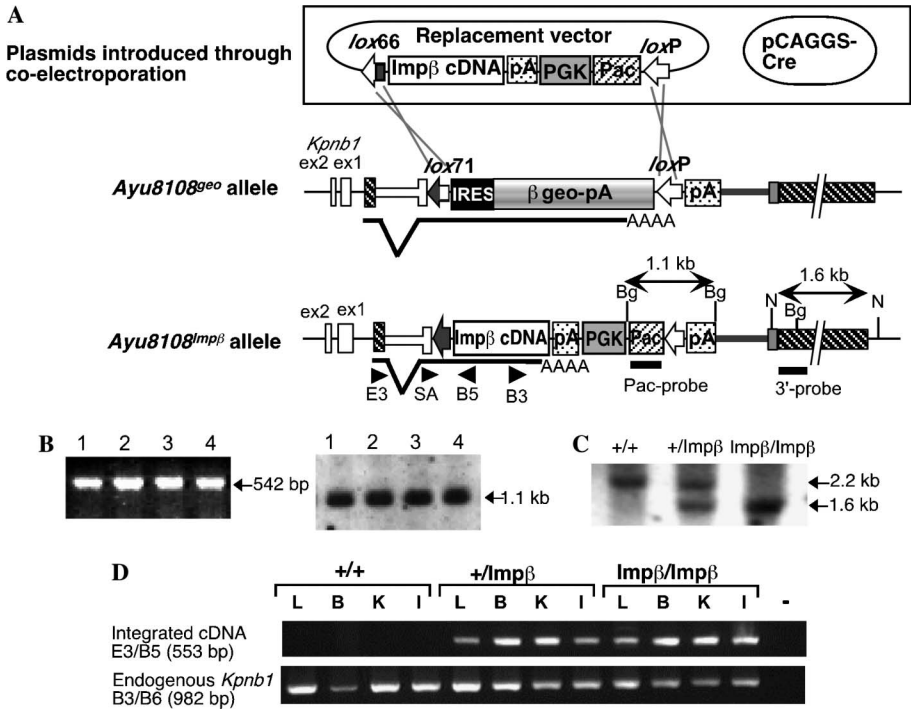

Fig. 5. Production and analysis of the *Ayu8108*^{Impβ} mouse line. (A) Site-specific replacement of the *βgeo* gene with a 2.6-kb Impβ cDNA sequence through Cre-mediated recombination. The replacement vector contains *lox*66-Impβ cDNA-pA-PGK promoter-*pac*-*lox*66 (top). Ayu8108 ES cells were co-electroporated together with the replacement vector and the Cre-expressing vector, pCAGGS-Cre. Through recombination between *lox* sites, the *βgeo* gene was replaced with the Impβ cDNA sequence (bottom). Striped boxes indicate transcribed genomic DNA regions for the 1.3-kb transcript. Replaced clones were selected in the presence of Puromycin (2 μg/ml). The integrated cDNA sequence was expressed as endogenous promoter activity which had driven the *βgeo* gene before replacement. Splicing patterns of transcripts for *βgeo* and integrated cDNA are shown under the maps. Primers used for confirmation of recombination and RT-PCR are indicated by arrowheads. Bg, *Bgl*II; N, *Nco*I. (B) Confirmation of targeted recombination at the *lox* sites. Results of representative 4 clones (1–4) are shown. Left, PCR with SA and B5 primers to detect the 5′-side junction. Right, Southern blot analysis with the Pac-probe and *Bgl*II digestion to detect the 3′-side junction. Bands with expected sizes were detected in both analyses. (C) Genotyping of the *Ayu8108*^{Impβ} mouse line. Tail DNA was digested with *Nco*I and hybridized with a 3′-probe. From the wild and *Ayu8108*^{Impβ} allele, a 2.2-kb band (Fig. 1B) and a 1.6-kb band were detected, respectively. (D) RT-PCR analysis of expression of the inserted or endogenous Impβ transcripts in each genotype. RT-PCR was performed using total RNAs from adult brain (B), kidneys (K), intestines (I), and lungs (L). To detect transcripts from the integrated Impβ cDNA and the endogenous *Kpnb1* gene, E3/B5 primer pair, and B3/B6 primer pair were used, respectively. B6 primer was in the 3′-untranslated region, therefore the B3/B6 primer pair can specifically detect endogenous *Kpnb1* expression. In homozygous *Ayu8108*$^{Impβ/Impβ}$ mice, both inserted Imp*β* cDNA and endogenous *Kpnb1* gene were expressed. −, template without RT reaction.

probably because of maternal accumulation of Impβ protein. In the case of importinβ-deficient eggs derived from germline chimeras carrying Impβ-deficient female germ cells, embryogenesis did not start at all [13]. Post-implantation lethality in our homozygous mutants for the Ayu8108 allele suggested that maternal Impβ mRNA or protein was present in mouse embryos, and that these might become depleted at the time of implantation.

In the Ayu8108 trap line, the trap vector was inserted in the promoter region of the *Kpnb1* gene in the reverse orientation. Recent genome and expression studies have revealed that many pairs of genes were driven by the same promoter sequence but in reverse direction [21,22], and that there were also non-coding RNA genes of unknown functions [23,24]. We identified a 1.3-kb product as the transcript of the trapped gene in Ayu8108 clones, but no ORF was found, and its expression was under detectable levels using northern blotting. As *Ayu8108*$^{Impβ/Impβ}$ homozygous mice were viable and healthy by expressing Impβ, but not the 1.3-kb transcript, the 1.3-kb transcript might not have a significant function in development. Yet, the 1.3-kb transcript might be a non-coding RNA of unknown function.

pU-hachi was constructed as an exchangeable gene trap vector using the Cre/mutant lox system. In this study, we used this system to insert the Impβ cDNA sequence, and we successfully identified the responsible gene for the early embryonic lethal phenotype. We clearly showed that Impβ was essential for early development in mice. Since there are many overlapping genes, insertion of a targeting vector may often result in disruptions of two genes. Our exchangeable system is expected to be a useful tool for analysis of such overlapping genes.

At present, we do not know why endogenous expression of the *Kpnb1* gene in the *Ayu8108*^{Impβ} allele recovered. We presume that there may be inhibitory element(s) for transcription in the IRES-βgeo sequence. It is known that the IRES sequence is quite GC rich and forms a complex secondary structure [25]. We have also produced the IRES-βgeo deleted allele by mating a Cre-expressing transgenic mouse line [26]. Homozygous mice for the IRES-βgeo

deleted allele were also viable and expressed the endogenous *Kpnb1* gene (data not shown). This supported the hypothesis of the existence of inhibitory elements. However, Impβ expression level from the $Ayu8108^{Impβ}$allele seemed to be lower than that of the wild-type allele, because we could not obtain compound heterozygous mice carrying both the $Ayu8108^{geo}$ and the $Ayu8108^{Impβ}$ alleles. Since we produced several alleles showing different expression levels of the *Kpnb1* gene, further analyses using these alleles would reveal the minimum requirements of Impβ expression level for development.

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