Targeted disruption of Rab1a causes early embryonic lethality

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Abstract. Guanosine nucleotide diphosphate (GDP) dissociation inhibitor 2 (GdI2) regulates the GDP/guanosine triphosphate (GTP) exchange reaction of Rab proteins by inhibiting the dissociation of GDP and the subsequent binding of GTP. The present study aimed to determine the function of Rabla in vivo, and thus generated mice with a trapped Rabla gene. It was demonstrated that Rabla is essential for embryonic development. It was also found that one functional Rabla allele was sufficient for development in a heterozygous murine embryo, whereas a double mutant led to embryonic lethality. The dissection of uteri on embryonic day (E)10.5‑14.5 yielded no homozygous embryos, indicating that homozygotes die between E10.5 to E11.5. The gene trap construct contains a β‑galactosidase/neomycin reporter gene, allowing for heterozygotes to be stained for β‑galactosidase to determine the tissue‑specific expression of Rabla. Rabla was found to be highly expressed in the small intestine of both adult mice and embryos, although its expression levels were low in the brains of embryos. Moreover, there was no significant change in cytokine production and survival in both adult mice and embryos, although its expression levels were low in the brains of embryos. Additionally, Rabla regulates the mTOR c1 pathway in glucose homeostasis (12), colorectal cancer (13), liver cancer development (14) and breast cancer cells (15).

Introduction

Rab proteins are a subfamily of the Ras superfamily of small GTPases that are key regulators of intracellular membrane trafficking, from the formation of transport vesicles to their fusion with membranes. These proteins can be detected in an inactive or active conformation, depending on the nucleotide‑bound status, and are considered switches that cycle between an active, membrane‑associated status and an inactive cytosolic status. To date, >70 mammalian Rab proteins have been identified (1). Some Rab proteins exhibit a regulated expression, tissue‑specific expression, or developmental‑specific expression, while others are ubiquitously expressed (1). Each Rab protein exhibits a characteristic subcellular distribution (2).

Rabla regulates vesicular protein transport from the endoplasmic reticulum to the Golgi apparatus (3,4) and to the cell surface (5). It also plays a role in secretion of interleukin (IL)‑8 and growth hormones. Rabla function has been implicated in neuronal differentiation (6) and cardiac development (7). The overexpression of Rabla in transgenic mice has been shown to be associated with an increased cardiac mass and cardiac hypertrophy, leading to cardiomyopathy (7). Rabla activity is also targeted by bacterial (8‑10) and viral pathogenic (11). Additionally, Rabla regulates the mTORC1 pathway in glucose homeostasis (12), colorectal cancer (13), liver cancer development (14) and breast cancer cells (15).

The guanosine nucleotide diphosphate (GDP) dissociation inhibitor (GDI) proteins regulate the Rab family GTPase function by binding to Rab GTPase in its GDP‑bound inactive form to retrieve it from the cell membrane and to maintain a soluble pool of inactive proteins ready to be re‑used (16). The GDI family includes the GDI1 and GDI2 proteins. GDI1 is expressed primarily in neural and sensory tissues, whereas GDI2 is ubiquitously expressed (17).

In a recent study, it was demonstrated by the authors that GDI2 binds to the immunoreceptor tyrosine‑based inhibitory motif (ITIM) domain of sialic acid‑binding immunoglobulin‑type lectin G (Siglec‑G) in hematopoietic cells, such as B‑1a cells under conditions of normal homeostasis, whereas Rabla is recruited to the ITIM domain during bacterial infection (18). Therefore, it was hypothesized that GDI2 and Rabla may regulate the immune response through interaction with the ITIM domain during bacterial infection. The present study thus aimed to explore the function of Rabla in vivo by generating a Rabla null mutant model with a trapped Rabla gene. The homozygous deletion of the Rabla gene resulted in early embryonic lethality. Rabla protein was expressed from the trapped gene during early post‑implantation development, suggesting a critical role of Rabla in the transport of materials between organelles in eukaryotic cells.
Materials and methods

Reagents. Rabbit anti-mouse Rab1a antibodies (cat. no. sc-311) were obtained from Santa Cruz Biotechnology, Inc. and lipopolysaccharide (LPS; from Escherichia coli (E. coli) 053:B5 strain) were purchased from MilliporeSigma. Goat anti-mouse β-actin (cat. no. sc-1615) and horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (cat. no. sc-2004) were purchased from Santa Cruz Biotechnology, Inc. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) was obtained from Thermo Fisher Scientific, Inc.

Generation of Rabla mutant mice. A male chimeric mouse generated from the ES cell line, XB498, was obtained from Bay Genomics, LLC. The ES cell line, XB498, was generated by using a gene trap protocol with the trapping construct pGT0bps containing the intron from the engrailed 2 homeobox gene upstream of the gene encoding the β-galactosidase/neomycin-resistance fusion protein (please see Fig. 1 and https://igtg.org/cgi-bin/annotation.py?cellLine=XB498). Genotyping was determined by the polymerase chain reaction (PCR) analysis of DNA from tail biopsies, as previously described (19).

PCR-based genotyping of mice. Aliquots of 0.1 µg (10 µl) DNA were mixed with 10 µl of 2X GoTaq Green Master Mix buffer (Promega Corporation) and 10 pmol of each primer, as previously described (19). PCR amplification was carried out at 96°C for 2 min, with 35 cycles of 96°C for 10 sec, 55°C for 30 sec, and 72°C for 60 sec. To screen for the homologous recombination of DNA, the following primers were used: P1, 5’-ACTGAGTATCCCTGGCTGGC-3’ and P2, 5’-AAGAGTGAGCTAGCCAGTCA-3’. The wild-type (WT) allele was not amplified (no band was detected), while the mutant allele produced a 300-bp band corresponding to the amplification of dNA, the following primers were used: P1, 5’-ACTGAGTATCCCTGGCTGGC-3’ and P2, 5’-AAGAGTGAGCTAGCCAGTCA-3’. The PCR products were separated by agarose gel electrophoresis, stained both a WT 485-bp band and a mutant 300-bp band. The PCR products were separated by agarose gel electrophoresis, stained both a WT 485-bp band and a mutant 300-bp band. The P cR amplification was carried out at 96°C for 2 min, with 35 cycles of 96°C for 10 sec, 55°C for 30 sec, and 72°C for 60 sec. After blocking with 5% skimmed milk in PBS-T (PBS with 0.01% Tween-20) at room temperature for 1 h, the blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:1,000 dilution) at 4°C overnight. The membranes were then washed three times with X-gal wash buffer (0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂), and stained overnight at 37°C in X-gal staining buffer [0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂, 2 mM K_2Fe(CN)_6, 3H_2O, 5 mM K_2Fe(CN)_6, 1 mg/ml X-gal]. The stained sections were washed three times with X-gal wash buffer and mounted in Aquates® aqueous mounting medium (MilliporeSigma). Images were acquired using an EVOS FL Auto Imaging System (Thermo Fisher Scientific, Inc.).

Western blot analysis. Embryo lysates were prepared by incubation in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH 7.6, including protease inhibitors, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride), sonication, centrifugation was performed at 4°C and at 16,200 x g for 5 min to remove cell debris. Proteins in the lysates were determined by BCA and separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and then examined by western blotting, as previously described (21). After blocking with 5% skimmed milk in PBS-T (PBS with 0.01% Tween-20) at room temperature for 1 h, the blots were incubated with goat anti-mouse β-actin primary antibodies (1:1,000 dilution) at 4°C overnight. The membranes were then washed three times with X-gal wash buffer (0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂), and stained overnight at 37°C in X-gal staining buffer [0.1 M phosphate buffer, pH 7.3, 2 mM MgCl₂, 2 mM K_2Fe(CN)_6, 3H_2O, 5 mM K_2Fe(CN)_6, 1 mg/ml X-gal]. The stained sections were washed three times with X-gal wash buffer and mounted in Aquates® aqueous mounting medium (MilliporeSigma). Images were acquired using an EVOS FL Auto Imaging System (Thermo Fisher Scientific, Inc.).
Histological analysis and immunohistochemistry. Tissues from WT or mutant mice were fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated and embedded in paraffin according to the standard procedure and as previously described (19). Sections at a thickness of 5 µm were cut, dewaxed in xylene, dehydrated in 100% ethanol and then stained with hematoxylin and eosin [H&E; hematoxylin (cat. no. SH26‑500D, Fisher Chemical; Thermo Fisher Scientific, Inc.) for 3 min and eosin (cat. no. S176‑160Z, Poly Scientific R&D Corp.) for 30 sec] at room temperature or reacted with anti‑mouse Rab1a antibody (1:1,000; cat. no. sc‑311, Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The sections were washed in phosphate‑buffered saline (PBS) and subsequently incubated with HRP‑conjugated goat anti‑rabbit secondary antibodies (1:1,000; cat. no. sc‑2004, Santa Cruz Biotechnology, Inc.) at room temperature for 30 min. After being washed in PBS, slides were developed with 3,3’‑diaminobenzidine (DAB) and counterstained with hematoxylin for 10 sec at room temperature. For the control, immunohistochemical staining was performed by omitting the primary antibody. No significant staining was observed upon control staining (data not shown). Images were acquired using an EVOS FL Auto Imaging System (Thermo Fisher Scientific, Inc.).

Statistical analysis. The differences in cytokine concentrations were analyzed using two‑tailed t‑tests in single pairwise comparisons calculated with Excel (Microsoft). Data are presented as the mean ± SD. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Generation of Rab1a mutant mice. To determine the function of Rab1a in vivo, we obtained a Rab1a mutant mouse from MMRRC which was generated by the blastocyst injection of a Rab1a trapped embryonic stem cell clone (XB498, Bay Genomics). Gene disruption was caused by the insertion of the retroviral gene trap vector pGT0pfs containing a promoterless β‑galactosidase reporter gene. Selection for the expression of the gene requires transcription from an endogenous cellular promoter and consequently, a mutation in a cellular gene. The expression of the tagged gene can be examined by staining for β‑galactosidase. The methods used for gene trap mutagenesis have been previously reported (25‑27). In the XB498 ES cell line, the gene trap vector pGT0pfs was inserted between exons 2 and 3 of Rab1a (Fig. 1 and Data S1); the point of insertion was confirmed by PCR and DNA sequencing (Fig. 2 and Data S2). Offspring were genotyped by PCR analyses using primers P1 and P2 for the knockout (KO) PCR and primers P3 and P4 for the WT PCR (Fig. 1).
Chimeric male offspring were mated with WT C57BL/6 mice to test for the germline transmission of the disrupted Rabla allele. Heterozygous Rabla+/- mice were viable and displayed no obvious abnormality in weight or fertility during a 12-month observation period (data not shown). To remove contaminating background heterozygosity, Rabla+/- mice were backcrossed >10 generations with c57BL/6 mice.

Expression of Rabla in mice. The expression of β-galactosidase is controlled by the endogenous Rabla gene promoter. Thus, β-galactosidase expression was used in Rabla+/- mice to document the pattern of Rabla expression in mouse embryos. To visualize the expression pattern of Rabla, X-gal staining of cryosections of Rabla+/- and Rabla-/- E7 embryos was performed; sections of WT embryos served as the negative controls. Rabla was ubiquitously expressed in whole embryos (Fig. 3). To investigate endogenous Rabla protein expression, WT E7 embryos were collected, sectioned and immunostained with anti-Rabla antibodies. Similar to the Rabla gene, Rabla protein was ubiquitously expressed in the whole embryo (Fig. 3). Based on these findings, it was concluded that Rabla protein expression was consistent with Rabla β-galactosidase activity.

In addition, the expression of Rabla during embryo development was examined using X-gal staining. At E15 embryo, Rabla was mainly expressed in the intestine (Fig. 4) and a small amount of Rabla was also detected in the brain (Fig. 4), as previously reported (28,29). In adult mice, Rabla was expressed in the small intestine (Fig. 5).

Rabla deficiency causes embryonic lethality. To generate Rabla-/- mice, heterozygous Rabla+/- mice were intercrossed. The genotypes of the offspring were identified at 2 weeks after birth. None of the 250 offspring were homozygous mutants (Rabla-/-) (total, 250; Rabla+/-, 94; Rabla+-/-, 156; Rabla-/-, 0), and no increase in neonatal mortality was observed in the initial 2 weeks after birth. The ratio between the WT and heterozygote mice was 0.60, in accordance with Mendel's law. These results thus suggest that Rabla is essential for embryonic development: one functional Rabla allele is sufficient for murine embryonic development; however, a double mutant leads to embryonic lethality.

To characterize the effect of Rabla mutation on embryonic development, timed matings (breeding we set up at 5 p.m. and the following morning the presence of a copulatory plug was examined at 7 a.m. If the presence of a copulatory plug was confirmed, this day was recorded as day 0.5) were performed between mice heterozygous for Rabla. Embryos were collected at E12.5 and E14.5 from Rabla-/-breeding mice and genotyped using PCR analysis with genomic DNA. No
viable Rab1a−/− embryos were recovered (Fig. 6). The developmental retardation of Rab1a−/− embryos was apparent at E12.5, suggesting that embryonic lethality occurred prior to E12.5 (Fig. 6). Embryos at E10.5 and E11.5 were also collected and it was found that viable Rab1a−/− embryos were recovered at E10.5, whereas no viable Rab1a−/− embryos were recovered at E11.5; the data for viable embryos in different embryonic stages are summarized in Table I, the different numbers in the table indicate the viable embryos found in the different embryonic genotypes. Thus, Rab1a mutation-induced embryonic lethality occurred between E10.5 and E11.5.

Rab1a protein deficiency in Rab1a−/− KO mice. WT and mutant alleles were assessed using PCR of genomic DNA isolated from mice (Fig. 7A). Western blot analysis was also performed to test the successful disruption of the Rabla gene in Rabla−/− mice. E10.5 embryos were collected and genotyped using PCR. Rabla+/+ and Rabla−/− embryos were lysed and used for western blot analysis with anti-Rabla antibody. As shown in Fig. 7B, Rabla was completely absent in Rabla−/− embryos, indicating the functional loss of Rabla; β-actin was used as a loading control.

Table I. Effects of Rab1a mutation on the number of viable embryos.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total</th>
<th>Rab1a+/+</th>
<th>Rab1a+/−</th>
<th>Rab1a−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5</td>
<td>21</td>
<td>6</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>E11.5</td>
<td>23</td>
<td>9</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>E12.5</td>
<td>18</td>
<td>7</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>E14.5</td>
<td>15</td>
<td>5</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

E, embryonic day.

Figure 4. Expression of Rabla in embryos at embryonic day 15. X-gal staining of cryosections of Rabla−/− KO and WT embryos. Corresponding WT sections served as negative controls for specific X-gal staining in Rabla−/− KO mice. Higher magnification images are also shown. The experiments in this figure were reproduced twice and representative images are shown. Scale bar, 500 µm; scale bar in higher magnification images, 400 µm. X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; WT, wild-type; KO, knockout. The blue arrow indicates intestinal staining. The red arrow indicates brain staining.

Figure 5. Expression of Rabla in the small intestine of adult mice. X-gal staining of cryosections from the small intestine of Rabla+/+ KO and WT mice. Corresponding WT sections served as negative controls for specific X-gal staining in Rabla−/− KO mice. Higher magnification images are also shown. Experiments in this figure were reproduced twice and representative images are shown (bottom panel). Scale bar, 500 µm; scale bar in higher magnification images, 100 µm. X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; WT, wild-type; KO, knockout.

One Rabla allele is sufficient for resistance to LPS-induced sepsis. The loss of GDI2 in tumor cells alters the crosstalk between tumor cells and tumor-associated macrophages to enhance both local inflammation and tumor cell invasion and growth, resulting in inflammatory cytokine secretion by macrophages to promote metastatic growth (30). Moreover, Rabla is required for NLRP3 inflammasome activation and inflammatory lung injury (31). Recently, the authors demonstrated that Rabla bound to the ITIM domain of Siglec-G under normal homeostasis. By contrast, Rabla was recruited to the ITIM domain during bacterial infection, suggesting that GDI2 and Rabla may regulate immune response through interaction with the ITIM domain during bacterial infection (18). In the present study, to investigate whether Rabla plays a role during bacterial infection, WT and Rabla−/− mice were challenged...
with 10 mg/kg LPS and collected serum from the mice as previously described (21,22,24). As shown in Fig. 8, both WT and Rabla-/- mice produced similar levels of inflammatory cytokines following LPS stimulation. Moreover, after 120 h, 50% (8/16, Rabla-/-) and 56% (9/16, Rabla-/-) of the mice did not survive; from data pooled from two independent experiments, a similar percentage of death was observed following LPS treatment (32) (data not shown); no significant differences were observed between the WT and Rabla-/- mice as regards survival following the LPS challenge.
Discussion

The transfer of material between organelles in eukaryotic cells is predominantly mediated by vesicular transport. GTP binding proteins play key roles in the regulation of vesicular traffic at several stages of the exocytic and endocytic transport pathways. Rab GTPases are small GTP-binding proteins in the Ras superfamily. Following a vesicle fusion event, Rab is returned to its membrane of origin by GDI. GDI proteins regulate the GDP-GTP exchange reaction of Rab family members that are involved in the vesicular trafficking of molecules between cellular organelles. GDIIs decrease the rate of dissociation of GDP from Rab proteins and release GDP from membrane bound Rabs (1,33).

The authors have previously demonstrated that Rab1a may regulate the immune response through interaction with the ITIM domain during bacterial infection in vitro (18). To further investigate the function of Rab1a in vivo, the present study generated mice with a trapped Rab1a gene and uncovered a novel role for Rab1a during early embryonic development in mice. None of the 250 genotyped pups from Rab1a heterozygous mating pairs exhibited the homozygous deletion of the Rab1a allele. The present study was also unable to detect any viable Rab1a null embryos after E11.5, indicating a severe early embryonic defect caused by the complete loss of Rab1a function. However, one functional Rab1a allele is sufficient for murine embryo development, as the frequency of heterozygote offspring was as predicted.

Although Rab1a interacts with the ITIM domain during bacterial infection, there was no significant difference in cytokine production and survival between the WT and Rab1a−/− KO mice after the LPS challenge. These data suggest that one Rab1a allele is sufficient to maintain function. The conditional KO strategy is a useful method which may be used to solve the problem of embryonic lethality observed in conventional gene KOs (34). Therefore, to explore the function of Rab1a in hematopoietic cells in bacterial infection and to further investigate the role of Rab1a in embryonic development, a Rabla conditional KO mouse model is needed (34).

Rab8 is reportedly necessary for the proper localization of apical proteins and the absorption and digestion of various nutrients in the small intestine (35). Previous research has indicated that Rab11a is essential for the proper localization of apical proteins in the intestine and that the loss of Rab11 leads to the mislocalization of apical proteins in the small intestine, as demonstrated using Rab11a intestine-specific knockout (IKO) mice (36). Rab25 KO mice exhibit increased numbers of intestinal neoplasms when crossed with APCmin/+ mice (37). With the use of X-gal staining, the present study found that Rabla was mainly expressed in the small intestine in E15 embryos (Fig. 4) and in adult mice (Fig. 5). It would be of interest to determine whether Rabla also plays an important role in controlling the proper localization of apical proteins or the absorption and digestion of various nutrients in the small intestine. However, intestine specific Rabla conditional KO mice are required to further investigate the function of Rabla in the small intestine.

Although the present study demonstrates that Rabla is essential for embryonic development and homozygotes die between E10.5 and E11.5, the mechanisms underlying the regulatory effects of Rabla on embryonic development remain unclear. Moreover, while it was found that Rabla was mainly expressed in the small intestine in E15 embryos and in adult mice, it is not yet clear whether Rabla plays a critical role in the small intestine. Further experiments using Rabla conditional KO mice are thus required to provide further insight into this matter.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

GYC designed the experiments. YW, DY and GYC conducted the experiments. GC wrote the manuscript. YW and GYC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving animals were approved by the University of Tennessee Health Science Center (UTHSC) Animal Care and Use Committee (IACUC), protocol nos. 17-117 (approved January 29, 2018) and 20-0211 (approved January 26, 2021).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

3. Tisdale EJ, Bourne JR, Khosravi-Far R, der cJ and Balch WE: GYC designed the experiments. YW, dY and GYC conducted the experiments. GC wrote the manuscript. YW and GYC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.