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Characterization of a bicistronic knock-in reporter mouse model for investigating the role of CABLES2 *in vivo*

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Abstract: Two members of the CDK5 and ABL enzyme substrate (CABLES) family, CABLES1 and CABLES2, share a highly homologous C-terminus. They interact and associate with cyclin-dependent kinase 3 (CDK3), CDK5, and c-ABL. CABLES1 mediates tumor suppression, regulates cell proliferation, and prevents protein degradation. Although *Cables2* is ubiquitously expressed in adult mouse tissues at RNA level, the role of CABLES2 *in vivo* remains unknown. Here, we generated bicistronic *Cables2* knock-in reporter mice that expressed CABLES2 tagged with 3×FLAG and 2A-mediated fluorescent reporter tdTomato. *Cables2-3×FLAG-2A-tdTomato* (*Cables2*^{Tom}) mice confirmed the expression of *Cables2* in various mouse tissues. Interestingly, high intensity of tdTomato fluorescence was observed in the brain, testis and ovary, especially in the corpus luteum. Furthermore, immunoprecipitation analysis using the brain and testis in *Cables2*$^{Tom/Tom}$ revealed interaction of CABLES2 with CDK5. Collectively, our new *Cables2* knock-in reporter model will enable the comprehensive analysis of *in vivo* CABLES2 function.
Key words: bicistronic expression system, *Cables2*, FLAG-tag, knock-in reporter mouse, tdTomato

Introduction

The CDK5 and ABL enzyme substrate (CABLES) family comprises two members, CABLES1 and CABLES2. Both proteins have a highly homologous cyclin box-like domain at the C-terminus characterized by 78% amino acid identity. CABLES family members have been reported to interact physically and associate with cyclin-dependent kinase 3 (CDK3), CDK5, and c-ABL [1–4]. Previous studies demonstrated that *Cables1* played a key regulatory role in human intestinal tumor progression, endometrial hyperplasia, and oocyte development [5–8]. In proliferating cells, CABLES1 localizes in the nucleus and inhibits CDK2 by mediating its interaction with WEE-1 [9]. Recently, CABLES1 has been associated with protecting p63 from protein degradation and maintaining p21/Cip1 stability [10, 11]. Furthermore, *Cables1* is an essential factor for neurite outgrowth [4] and for

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Supplementary Tables: refer to J-STAGE: https://www.jstage.jst.go.jp/browse/expanim

the interaction between Robo-Abl and the N-cadherin-β-catenin complex in neural cells of the retina [12]. During development of the mouse brain, truncated *Cables1* was found to exert a dominant negative effect on the formation of the corpus callosum [13]. *In vitro*, CABLES2 induced apoptotic cell death in both a p53-dependent and a p53-independent manner [14]. *Cables2* mRNA was found to be widely expressed in adult mouse tissues by northern blot analysis [3]. Although CABLES family members share a similar protein structure, the role of CABLES2 *in vivo* remains unknown, largely due to a lack of suitable antibodies against mouse CABLES2 and absence of a *Cables2* mouse model.

Reporter mice that express fluorescent, bioluminescent or other protein tags to visualize the gene product of interest are often used to investigate *in vivo* gene function under both physiological and pathological conditions. To ensure that the reporter gene is expressed in the same way as the gene of interest, the latter is replaced by the reporter in prototypes of knock-in mice. However, the disruption of the targeted gene in homozygous knock-in reporter mice often results in perturbed cell metabolism and consequent phenotypic abnormality. Recently, we described a bicistronic knock-in strategy for production of knock-in reporter mice [15]. A bicistronic knock-in model using 2A sequences derived from porcine teschovirus-1 (P2A) enabled the reliable generation of gene products before and after P2A sequences under the targeted gene regulation. Previously, we reported that bicistronic *Ins1-2A-Cre*/R26GRR F1 mice expressed the *Cre* gene only in pancreatic insulin-positive cells and that mice homozygous for *Ins1-2A-Cre* showed normal glucose tolerance [16]. Thus, the bicistronic knock-in system is a helpful tool for generating a reliable reporter mouse strain.

To overcome existing limitations posed by lack of specific mouse CABLES2 antibodies, while allowing the investigation of CABLES2 *in vivo*, we describe here the generation of a new knock-in reporter mouse model for *Cables2*. Using CRISPR/Cas9 nickase and a targeting vector with 5' homology arm-*3×FLAG-2A-tdTomato*-3' fused to *FRT-PGK-Neo-FRT* in the opposite orientation, a bicistronic *Cables2-3×FLAG-2A-tdTomato* knock-in reporter mouse strain, C57BL/6J-*Cables2*$^{em1\ (Tomato)\ Utr}$ (abbreviated as *Cables2*^{Tom}) was successfully generated from C57BL/6J embryonic stem (ES) cells. Transcripts of the knock-in allele were detected in all tissues examined. Western blot and immunoprecipitation (IP) analyses using anti-FLAG antibody detected CABLES2 and precipitated one of its known interacting partners. A strong tdTomato signal was observed in the brain, testis, ovary, and especially in the corpus luteum. Therefore, our bicistronic *Cables2* knock-in reporter mouse is a useful model for the comprehensive analysis of *in vivo* CABLES2 function.

Materials and Methods

Animals

C57BL/6J mice (*Mus musculus*) were purchased from Charles River Laboratories (Yokohama, Japan). Mice were maintained in plastic cages under SPF conditions in a room at 23.5 ± 2.5°C and 52.5 ± 12.5% relative humidity under a 14/10-h light/dark cycle. Mice had free access to commercial chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered water. Animal experiments were carried out in a humane manner with approval from the Institutional Animal Experiment Committee of the University of Tsukuba, in accordance with the Regulations for Animal Experiments of the University of Tsukuba and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Vector construction

The CRISPR/D10A Cas9 nickase system was designed to knock-in the 3×FLAG-tag at the C-terminus of *Cables2* and allow bicistronic expression of tdTomato based on the 2A peptide [15]. Oligos were designed to target the sites flanking the stop codon of *Cables2* (Supplementary Table 1), followed by annealing, purification, and ligation into the *Bbs*I sites on the px335 plasmid. The plasmid contained Cas9 nickase and guide RNA, and was a gift from Feng Zhang (Addgene plasmid # 42335) [17]. The core targeting vector included 3×FLAG, 2A, tdTomato, and a rabbit β-globin poly (A) signal, which were ligated into the *FRT-PGK-gb2-Neo* expression cassette comprising eukaryotic and prokaryotic promoters, the neomycin resistance gene flanked by *FRT* sequences (Gene Bridges, Heidelberg, Germany), and the PGK-diphtheria toxin A (*DTA*) gene used for negative selection. The 1.8-kb 5' homology arm of *Cables2* was inserted in the targeting vector at *PmeI* and *HindIII*, whereas the 2-kb 3' homology arm of *Cables2* linked to the 3×FLAG-tag was amplified and inserted in the *AscI* site using the In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) (Fig. 1A).

Generation of *Cables2*^{Tom} knock-in mice

B6J-S1 ES cells [18], deposited in the Riken BioResource Cell bank (AES0140), were electroporated with the linearized targeting vector and px335 plasmid, and subsequently cultured in the presence of G418. G418-

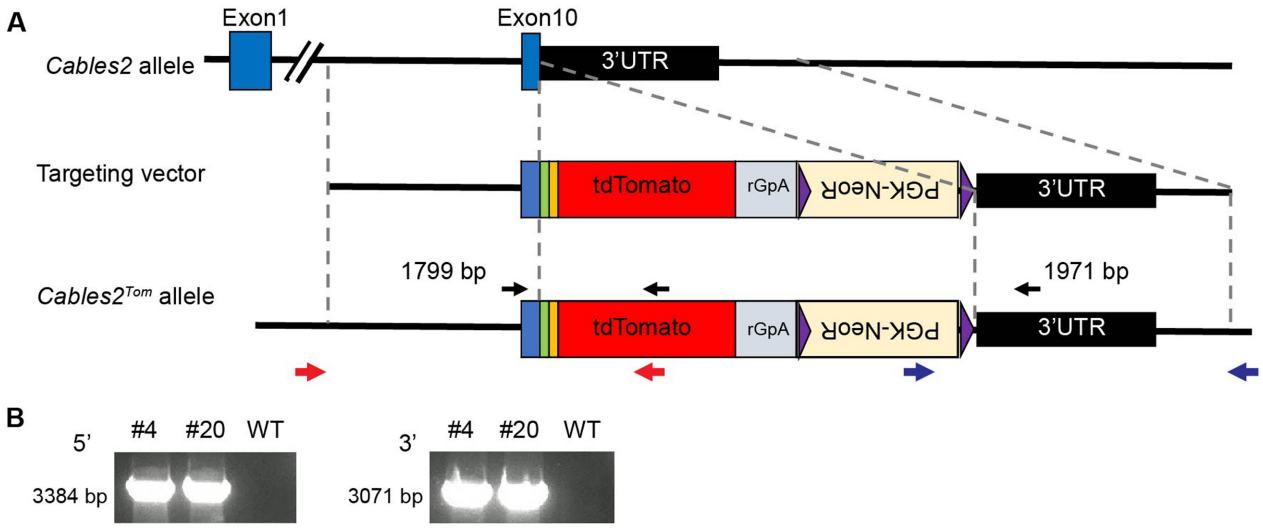

Fig. 1. Construction of the *Cables2-3×FLAG-2A-tdTomato (Cables2^{Tom})* knock-in allele. (A) Diagram showing the knock-in construction strategy, in which *Cables2* exon10 was fused with *3×FLAG* (green), *2A* (orange), *tdTomato*, rabbit β-globin poly (A) (rGpA), and *PGK-NeoR* flanked by *FRT* sequences. This cassette was inserted just before the stop codon of endogenous *Cables2*. Black arrows indicate the primers used for genotyping and detecting the tdTomato insert or wild-type alleles: Cables2-tdT F, Cables2-tdT R, and tdTomato Rv. (B) Homologous recombination of the knock-in fragment at the 5' and 3' ends (red and blue arrows in (A), respectively) was confirmed in ES cell clones. The following primers were used to detect the knock-in fragment at the 5' end: Cables2 screening 5 Fw and Cables2 screening 5 Rv (red arrows); those used for detection at the 3' end included: PGKS2 Fw and Cables2 screening 3 Rv (blue arrows).

resistant ES cells were screened and genotyped by PCR. The positive clones were cultured and aggregated with wild-type morulae, which were collected from ICR mice. The aggregated blastocysts were then transferred into the uterine horns of pseudopregnant females at 2.5 days post-coitus. Male chimeras that transmitted the knock-in allele to the germ line were propagated to generate the *Cables2^{Tom}* mouse line. Newborn mice were genotyped by extracting genomic DNA from the tail of 3-week-old mice, followed by PCR using AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and the primers listed in Supplementary Table 2.

RT-PCR and RT-qPCR

For RT-PCR, total RNA from mouse tissues was homogenized and extracted using Isogen reagent (Nippon Gene, Toyama, Japan) after heart perfusion with cold Dulbecco's PBS. Reverse transcription was performed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and the synthesized cDNAs were amplified by PCR with AmpliTaq Gold 360 Master Mix.

For RT-qPCR, total RNA was extracted from indicated tissues using the NucleoSpin RNA Plus kit (Takara Bio) and RT-qPCR was carried out using TB Green Premix Ex Taq II (Takara Bio) on a Thermal Cycler Dice Real-Time System (Takara Bio) following the manufacturer's instructions. *Cables2* gene expression was normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The primers used for RT-PCR and RT-qPCR are listed in Supplementary Table 2.

Fluorescence reporter analysis

Eight to 12-week-old mice were sacrificed and dissected tissues were fixed overnight at 4°C in 4% paraformaldehyde (Wako Industries, Osaka, Japan) in PBS. Next, tissues were embedded in frozen blocks using Tissue-Tek OCT (Sakura Finetek, Torrence, CA, USA). Blocks were sliced to 14 μm thickness using a cryostat (HM 525NX; Thermo Fisher Scientific) and placed on glass slides (Matsunami Glass, Osaka, Japan). Frozen sections were mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) for 3 min and observed immediately under a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan).

Western blotting

After heart perfusion, the indicated organs were collected, homogenized in bead tubes (Sarstedt, Nümbrecht, Germany), and proteins were extracted using T-Per Tissue Protein Extraction Reagent (Thermo Fisher Scientific). Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Billerica, MA, USA), and blocked overnight in 5% skimmed milk (DIFCO, BD Biosciences, Franklin Lakes, NJ, USA). The membranes were then incubated at room temperature for 1 h with primary anti-FLAG antibody (F1804; Sigma-Aldrich, St.

Louis, MO, USA) and anti-GAPDH antibody (sc-25778; Santa Cruz Biotechnology, Dallas, TX, USA). After washing three times in 0.05% Tween in Tris-buffered saline, the membranes were incubated for 1 h at room temperature with secondary anti-mouse IgG horseradish peroxidase (HRP)-linked whole antibody (NA931; GE Healthcare Life Sciences, Chicago, IL, USA) and anti-rabbit IgG HRP-linked whole antibody (NA934; GE Healthcare Life Sciences), and were detected using an iBright CL1000 Imaging System (Thermo Fisher Scientific).

Immunoprecipitation

After collecting samples in PBS, mouse tissues were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% deoxycholic acid, and 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and centrifuged to collect the supernatant. For each tissue, 1–2 mg protein was incubated with anti-FLAG antibody (F1804; Sigma-Aldrich) overnight, after which Dynabeads Protein G beads (Veritas, Invitrogen, Carlsbad, CA, USA) were added and the suspension was incubated with rotation overnight at 4°C. The beads were washed three times with PBS, resuspended, and boiled in Laemmli sample buffer. The precipitated proteins were analyzed by SDS-PAGE and western blotting using anti-CDK5 antibody (ab40773; Abcam, Cambridge, UK), anti-FLAG antibody (F1804; Sigma-Aldrich), and anti-GAPDH antibody (sc-25778; Santa Cruz Biotechnology).

Results

Generation of knock-in mice expressing *Cables2* tagged with 3×FLAG and bicistronic tdTomato

Bicistronic *Cables2* knock-in reporter mice were generated from knock-in C57BL/6J ES cells, in which the C-terminus of *Cables2* was tagged with three copies of *FLAG*, *tdTomato* (including the *2A* sequence), and *FRT*-flanked *PGK-Neo*. The tags were homologously integrated right before the stop codon of *Cables2*. To express the fluorescent reporter under the control of the endogenous *Cables2* promoter, *2A* sequences were placed between the *Cables2-3×FLAG*-tag and *tdTomato* (Fig. 1A). The CRISPR/D10A Cas9 nickase system was employed to generate this knock-in model. The left and right target sequences for gRNA (Supplementary Table 1) were ligated into a px335 vector and co-electroporated with the targeting vector into ES cells. To screen for targeted gene insertion, homologous recombination at the 5' and 3' end of neomycin-resistant ES cell clones was confirmed by PCR using locus- and insertion-specific primers (Supplementary Table 2, Fig. 1B). Random integration in these clones was confirmed by negativity for *Amp* resistance. Chimeric mice were generated from ES cell clone #20. The progeny was genotyped by verifying downstream insertion of tdTomato or the 3'UTR in the wild-type allele (Supplementary Table 2).

Table 1. Medelian ratio of progeny obtained by intercrossing heterozygous mice

Total	Numbers of		
	wild-type mice	heterozygous mice	homozygous mice
39	12 (30.8%)	17 (43.6%)	10 (25.6%)

By intercrossing heterozygous *Cables2*$^{Tom/+}$ mice, homozygous *Cables2*$^{Tom/Tom}$ progeny were obtained following a Mendelian pattern of inheritance. Out of 39 newborns, 12 were wild-type, 17 were heterozygous, and 10 were homozygous (Table 1). The homologous recombination of the targeting vector was re-confirmed by PCR in *Cables2* $^{Tom/Tom}$ animals. No abnormal development, growth or physical appearance was observed in *Cables2* $^{Tom/Tom}$ mice, suggesting that knock-in mice were normal and healthy.

Expression of *Cables2* RNA in *Cables2*$^{Tom/Tom}$ knock-in mice

To characterize knock-in mice, *Cables2* RNA expression in mouse tissues was examined. Total RNA from the brain, lungs, liver, kidney, spleen, colon, testis, and ovary was extracted and analyzed by RT-PCR. When comparing homozygous and wild-type mice, both males and females (more than 3) were analyzed. Two pairs of primers were designed to detect sequences between exon 6 and exon 10 of wild-type *Cables2* or the *tdTomato* insertion in the knock-in allele (Fig. 2A). Endogenous *Cables2* was detected in all examined tissues of both knock-in and wild-type mice; whereas *tdTomato* was detected only in knock-in tissues (Fig. 2B).

To quantify *Cables2* gene expression, RT-qPCR was performed using total RNA extracted from the brain, testis, and ovary. *Cables2* was highly expressed in knock-in organs compared with their wild-type counterparts. Overexpression differed between male and female tissues, with the highest levels (4.91-fold) being in the ovary (Fig. 2C). This result indicated that *Cables2* was expressed more in *Cables2*$^{Tom/Tom}$ knock-in than in wild-type mice.

Visualization of CABLES2 expression in mouse organs and tissues

To determine CABLES2 expression patterns in organs and tissues, tdTomato fluorescent signal was observed

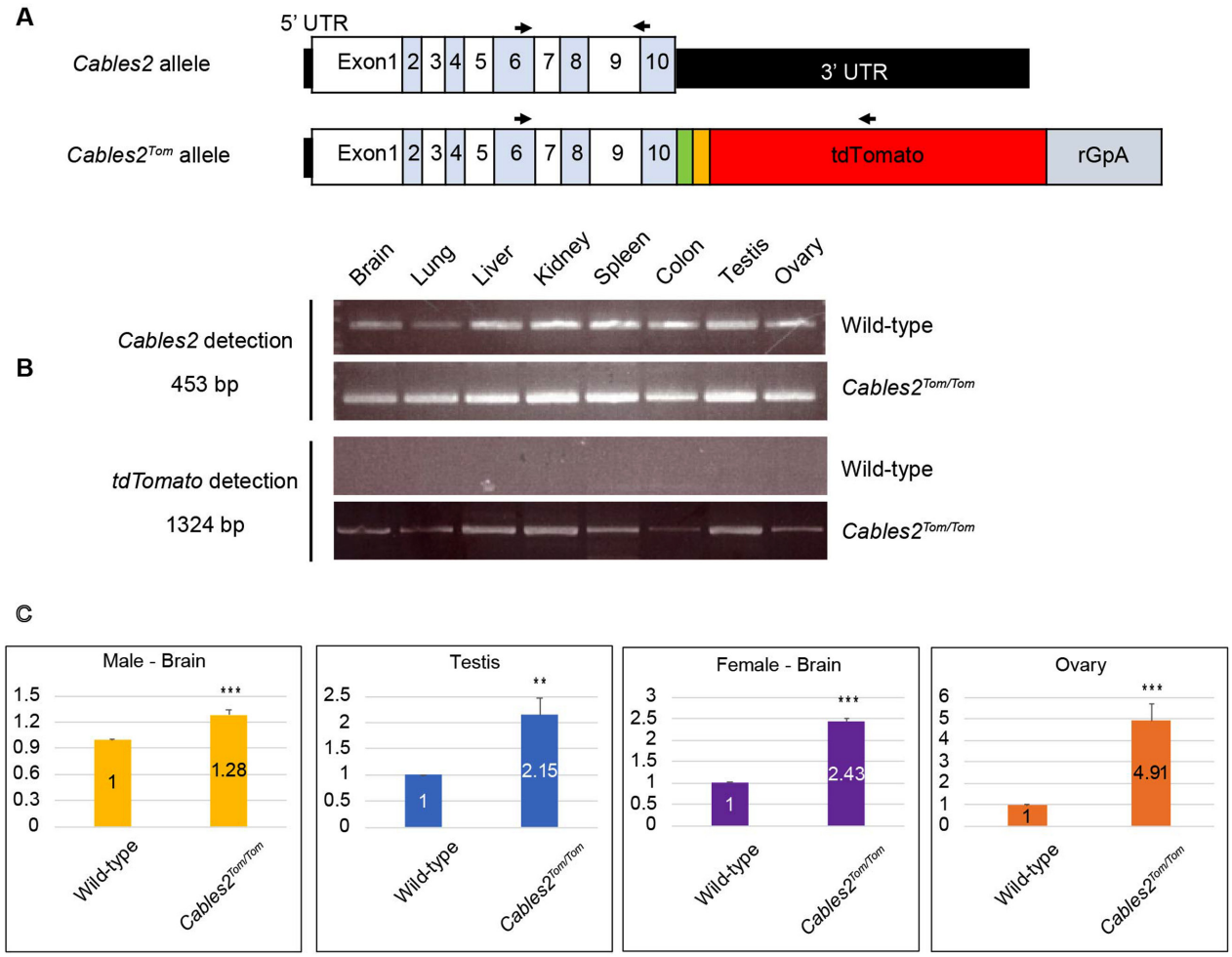

Fig. 2. RNA levels of *Cables2* in knock-in mice. (A) Diagram of mRNA transcription in wild-type and knock-in mice. The primers used in RT-PCR to detect *Cables2* or the insertion cassette in Fig. 2B were: Cables2 exon 6 Fw, Cables2 exon 9–10 Rv, and tdTomato Rv (black arrows). (B) RT-PCR results showing *Cables2* expression in several mouse tissues. (C) Quantitative real-time PCR of brain, testis, and ovary tissues collected from wild-type and knock-in mice. *Cables2* expression was detected using the primers Cables2 exon 3 qPCR Fw and Cables2 exon 4 qPCR Rv; values were normalized to *Gapdh*, which was detected by Gapdh qPCR Fw and Gapdh qPCR Rv primers. Average values of at least three independent experiments performed in duplicate are shown. Error bars denote SD. Statistical significance was determined using Student's *t*-test; ***P*<0.01; *** *P*<0.001.

in fixed samples of the brain, heart, liver, spleen, kidney, intestine, colon, testis, ovary, and uterus from both *Cables2*$^{Tom/Tom}$ and wild-type mice. As shown in Fig. 3A, the strongest tdTomato signal was detected in the brain, testis, and ovary. The liver, kidney, intestine, colon, and uterus displayed moderate tdTomato fluorescence. In contrast, fluorescent intensity of the heart and spleen did not differ between knock-in and wild-type mice.

To further investigate CABLES2 expression in mouse tissues, organs displaying high tdTomato fluorescence were analyzed histologically. Frozen sections of the brain, testis, and ovary revealed that tdTomato was uniformly expressed in the entire brain, including cortex, hippocampus and hypothalamus, as well as in almost all the cells of the testis and ovary. Interestingly, very high fluorescence intensity of tdTomato was detected in the corpus luteum, but not in the granulosa cells of the antral follicle before ovulation (Fig. 3B). These data suggest that CABLES2 is highly expressed in the brain, testis, ovary, and particularly the corpus luteum. However, we failed to confirm this observation by immunohistological staining using an anti-FLAG antibody (data not shown).

Evaluation of CABLES2 protein expression and its interaction

To detect CABLES2 directly in the brain, testis, and ovary, we performed western blot analysis using anti-FLAG antibody. Total protein was extracted from whole brain, testis, and ovary. In contrast to immunohistological analysis, here, anti-FLAG antibody detected CABLES2 fused to 3×FLAG only in *Cables2*$^{Tom/Tom}$ and not in wild-type tissues. CABLES2 expression was stronger in the brain and testis and the housekeeping control GAPDH showed similar expression in both wild-type

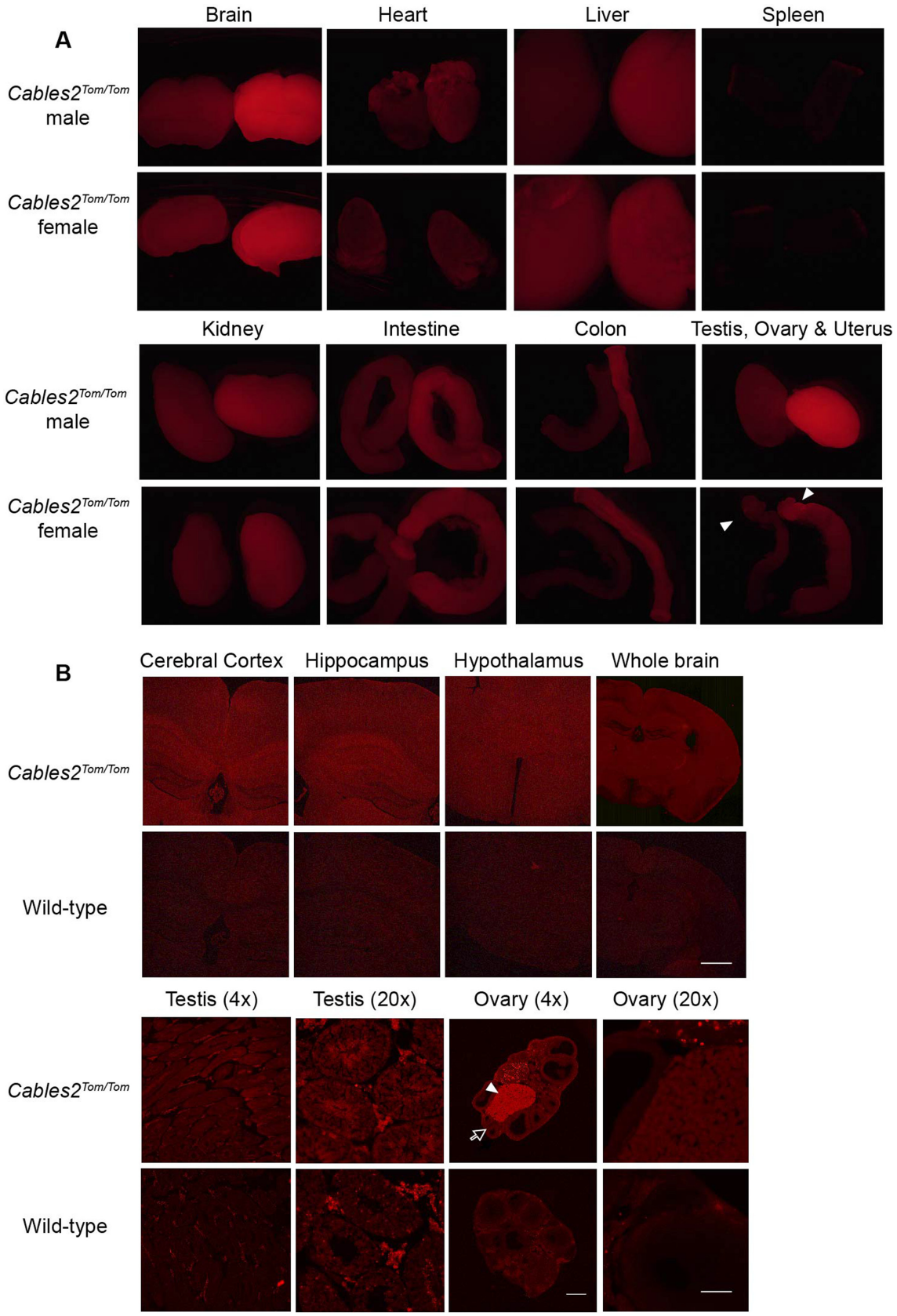

Fig. 3. Protein expression pattern of CABLES2 revealed by tdTomato reporter fluorescence. (A) The indicated mouse tissues were collected and tdTomato fluorescent signal was detected. Wild-type samples are on the left and homozygous samples are on the right in each frame. The strong tdTomato signal in the ovary is indicated by white arrows. (B) Coronal sections of the whole brain (upper panels), testis, and ovary (lower panels) are shown at 4× and 20× magnification. The white arrow indicates the corpus luteum, the black arrowhead points to the antral follicle. Scale bar, 1 mm in the whole brain, 200 μm in the ovary (4×), and 50 μm in the ovary (20×).

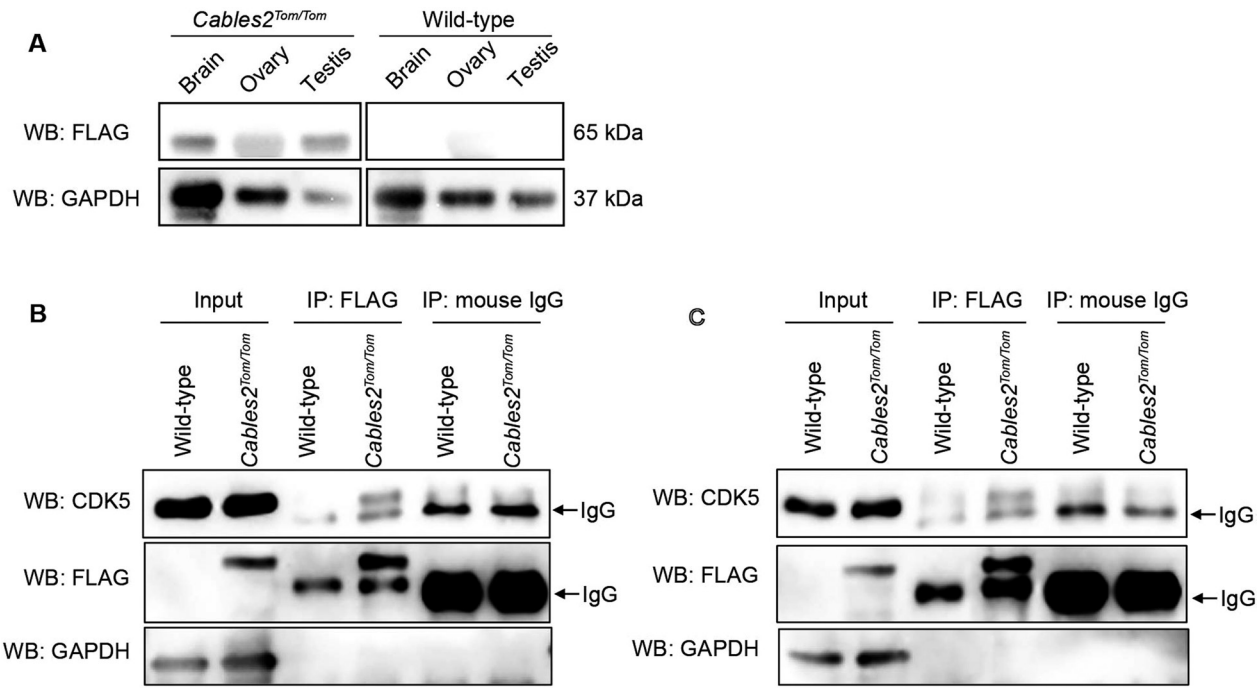

Fig. 4. Expression of CABLES2 in *Cables2*$^{Tom/Tom}$ and examination of CABLES2 interaction with CDK5. (A) Western blot (WB) results showing CABLES2 expression in the brain, testis, and ovary of knock-in mice but not in wild-type animals using an anti-FLAG antibody. GAPDH served as internal control. (B) Immunoprecipitation (IP) results showing the presence of CDK5 in mouse brain (B) and testis (C) complexes precipitated with anti-FLAG antibody but not input or negative control mouse IgG. WB with anti-FLAG antibody confirmed FLAG detection in *Cables2*$^{Tom/Tom}$ tissues.

and knock-in tissues (Fig. 4A). This result validated the use of *Cables2*$^{Tom/Tom}$ mice to detect CABLES2 expression based on the FLAG-tag.

Finally, *Cables2*$^{Tom/Tom}$ knock-in mice were employed to determine *in vivo* protein-protein interactions of CABLES2. CABLES1, the CABLES2 homolog, is a substrate and binding factor of CDK5, which is expressed and activated in the mouse brain and testis [4, 19, 20]. Therefore, to examine whether CABLES2 interacted with CDK5, IP was performed using mouse brain and testis samples from *Cables2*$^{Tom/Tom}$ mice. A high amount of protein extracted from the brain (1 mg) and testis (2 mg) was applied for IP and western blotting using anti-CDK5, anti-FLAG, and anti-GAPDH antibodies, whereas normal mouse IgG was used as negative control. IP with anti-FLAG antibody clearly showed the presence of CDK5 in the precipitated complexes with CABLES2 in both the brain (Fig. 4B) and testis (Fig. 4C), indicating physical interaction of CABLES2 with CDK5 in *Cables2*$^{Tom/Tom}$ mice. These results suggest that *Cables2*$^{Tom/Tom}$ knock-in mice can be utilized to further investigate the function of CABLES2 *in vivo*.

Discussion

In this study, we describe the first mouse model for analyzing *Cables2* function *in vivo* using a protein tag and fluorescent reporter gene. First, using CRISPR/Cas9 nickase-mediated genome editing, we generated *Cables2*$^{Tom/Tom}$ knock-in mice carrying *3×FLAG*-tag and bicistronic *tdTomato* in the *Cables2* locus. The expression of knock-in *Cables2* mRNA was confirmed in various mouse tissues. Surprisingly, the expression of *Cables2* was higher in the brain, testis, and ovary of *Cables2*$^{Tom/Tom}$ than wild-type mice. At protein level, the tdTomato signal was detected in all mouse tissues, but was stronger in the brain, testis, and particularly in the corpus luteum of the ovary. CABLES2 in *Cables2*$^{Tom/Tom}$ mice was detected also by FLAG-tag in western blots; whereas CABLES2-CDK5 interactions in the brain and testis were confirmed by IP. Taken together, these findings indicate that the novel *Cables2*$^{Tom/Tom}$ knock-in model is suitable for studying the biological function and binding network of *Cables2 in vivo*.

The bicistronic knock-in strategy employed here proved to be a reliable tool for studying location and expression of the target gene. In *Ins1-2A-Cre* knock-in mice, β-cell-specific recombination during embryonic and adult stages was confirmed by a fluorescent signal in all examined pancreatic islets in insulin-positive cells [16]. Recently, *Sox17-2A-EGFP*, *Otx2-2A-tdTomato*, and *T-2A-TagBFP* knock-in reporter mice were generated using the CRISPR/Cas9 system to easily and precisely visualize mouse endodermal, ectodermal, and mesoder-

mal tissues, respectively [21]. Here, we adopted a bicistronic strategy using *P2A* to easily detect CABLES2 expression sites by way of a fluorescent reporter in organs and tissues. The *tdTomato* gene in *Cables2*$^{Tom/Tom}$ should be regulated by the endogenous *Cables2* promoter. Expression of *Cables2* knock-in transcripts was validated at RNA level and was consistent with a previous study [3]. Given that tdTomato fluorescence was observed in a wide variety of organs and tissues, we speculated that this fluorescent reporter reflected CABLES2 expression in *Cables2*$^{Tom/Tom}$ knock-in mice.

Our characterization of *Cables2*$^{Tom/Tom}$ mice highlights the need to validate the expression level of the reporter gene upstream or downstream of the target gene when employing a knock-in strategy. The conventional internal ribosome entry site (IRES) has been widely used to co-express gene products; however, expression of IRES-dependent downstream genes has been shown to be significantly lower than that of cap-dependent upstream genes [22]. This limitation can be overcome by using a "self-cleaving" 2A peptide with elevated cleavage efficiency both *in vitro* and *in vivo* [15]. As shown in Nphs2$^{pod.T2A.ciCre.T2A.mTomato/+}$ podocyte-specific mice and *Pdx1*-2A-Dre pancreas models, use of such peptide does not perturb the expression of endogenous genes [23,24]. We previously employed the bicistronic strategy to generate *Ins1-2A-Cre* knock-in mice using *P2A* for pancreatic β-cell-specific Cre-loxP recombination and showed no influence on endogenous gene expression as homozygous mutants exhibited normal glucose tolerance [25]. In contrast, endogenous *Ddx4* levels have been recently shown to be lower in bicistronic *Ddx4-P2A-CreERT2* knock-in mice than in wild-type animals [26]. In the present study, CABLES2 displayed significantly higher mRNA expression in *Cables2*$^{Tom/Tom}$ than in wild-type tissues. A few reasons could possibly explain this phenomenon. Firstly, insertion of rabbit β-globin poly (A) just before the stop codon of endogenous *Cables2*, between CDS and endogenous poly (A), may over-protect the stability of *Cables2* transcripts and result in increased detection by RT-qPCR. Secondly, the endogenous *Cables2* 3'UTR may contain sequences important for mRNA degradation (e.g., miRNA target sequence) and their absence in the present knock-in mouse model may have led to the accumulation of *Cables2* mRNA. The third possibility is that *Cables2*^{Tom} is a hypomorphic allele. The activity of CABLES2 in *Cables2*^{Tom} animals might be attenuated by adding extra amino acids at the C-terminus, through a mechanism of transcription activation by feedback mechanism. However, the interaction of CABLES2 with CDK5, a major C-terminal partner of CABLES2, was unimpaired and the tagged CABLES2 protein would be intact. At the same time, overexpression of *Cables2* might facilitate the search for CABLES2 binding partners. Given the expression of CABLES2 across a wide variety of mouse organs and tissues, the *Cables2*$^{Tom/Tom}$ model can be used to screen and characterize *Cables2* expression *in vivo*.

Our study is the first to report CABLES2 expression pattern in the ovary, especially in the corpus luteum. The corpus luteum is an essential endocrine structure during development and maintenance of pregnancy. The formation, maintenance, and regression of the corpus luteum, defined as luteinization process, is controlled by the balance of hormones and cell cycle-regulating molecules. In general, luteinization is established and maintained by lowering the amounts of CDK2 and cyclin D1, while upregulating levels of cyclin D3, p27, and the association of p27/cyclin D3/CDK4 complexes in luteal cells [27]. Moreover, p27 and p21 cooperation is critical for the exit of differentiating granulosa cells from the cell cycle [28]. Recently, another p53 family member, p73, was characterized as a key regulator of follicle development, ovulation, and corpus luteum formation in mice [29]. Interestingly, CABLES1 was reported to associate with CDK2 [9], p21 [11], and p73 [30]. Therefore, CABLES2, whose protein structure is highly homologous to that of CABLES1 and containing the cyclin-box-like domain, may act as a cell cycle regulator to control expression in the corpus luteum, particularly with regard to the balance between cell cycle activators and inhibitors.

Using the CRISPR/Cas9 nickase system and the bicistronic strategy, a *Cables2* knock-in with 3×FLAG-tag and tdTomato reporter was established and shown to exhibit a similar expression pattern as endogenous *Cables2*. Characterization of *Cables2*$^{Tom/Tom}$ mice will provide a means for understanding the *in vivo* function of CABLES2.

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