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Trmt112 Gene Expression in Mouse Embryonic Development

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Mouse *Trmt112*, the homologous gene of yeast *Trm112* (tRNA methyltransferase 11-2), was initially cloned from RIKEN with uncertain function. The yeast TRM112 is now known to play important roles in RNA methylation. Here, we studied the expression of *Trmt112* by *in situ* hybridization and quantitative real-time RT-PCR (QRT-PCR). A higher expression level of *Trmt112* was observed in the brain and nervous system by whole mount *in situ* hybridization from embryonic day 10.5 (E10.5) to E11.5. At later developmental stages E13.5 and E16.5, abundant expression was prominently found in various organs and tissues including developing brain, nervous system, thymus, lung, liver, intestine, kidney, and cartilage. Furthermore, *Trmt112* was persistently expressed from E9.5 to E18.5 on whole embryos and highly expressed in multiple organs at E12.5, E15.5 and E18.5 by QRT-PCR. These results showed that *Trmt112* gene was highly and ubiquitously expressed during mouse embryonic development, implying that it might be involved in the morphogenesis of diverse organs and tissues and numerous physiological functions.

Key words: TRM112, mouse development, *in situ* hybridization, QRT-PCR

I. Introduction

In transfer RNA (tRNA), base and ribose methylations are the most frequent modifications [9, 20]. The majority of methylated modifications are catalyzed by S-adenosyl-methionine-(SAM)-dependent methyltransferases (MTases) [1], whose larger family is Rossman fold MTase (RFM) containing a seven-stranded β-sheet surrounded by helices on each side [25]. Yeast tRNA MTase (Trm) has been broadly identified by now [6]. Yeast Trm112 exhibits no sequence similarity to known RFM superfamilies, implying that it lacks a methyltransferase domain and does not catalyze methylation. TRM112 is identified as a small zinc finger protein, that is required for tRNA methylation *in vivo*, but this family function is still uncertain [5, 23, 26].

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The mouse gene *Trmt112* was initially cloned from RIKEN [10]. The full-length of *Trmt112* cDNA is 1047 bp, encoding a 125-amino-acid protein that is named yeast tRNA methyltransferase 11-2 (TRM112) homolog. A BLAST search in GenBank reveals that mouse Trmt112 shares a higher similarity with archaea and eukaryotes, implying that TRM112 is an evolutionarily conserved protein. Biochemical analysis indicates that TRM112 interacts with and activates both TRM11 and TRM9, two tRNA methyltransferases necessary for the formation of 2-methylguanosine at position 10 and modification of anticodons at the wobble uridine (U34) position, respectively [3, 18, 23, 26]. In addition, TRM112 was further found to be a cofactor of eukaryotic release factor 1 (eRF1) methyltransferase [5]. Translation termination is a very essential step, which is catalyzed by protein release factors (RFs) [11]. Methylation of RFs ensures efficient translation termination and release of newly synthesized peptides from the ribosome [19, 22]. Methylation of eRF1 in yeast is performed by the heterodimeric methyltransferase (MTase)

Mtq2/Trm112 at the glutamine residue of the GGQ motift (ripeptide Gly-Gly-Gln) [4, 22], and Trm112 is necessary for the solubility and activity of the catalytic subunit of the protein methylase Mtq2p [5, 26]. More recently, the same mechanism was also found in human and mouse, the holoenzyme homologues of which are annotated as HEMK2α/hTRM112 and Pred28a (N6amt1)/mTrm112, respectively [2, 13, 14, 17, 24]. Furthermore, TRM112 has been regarded as a complex network of enzymatic activities [15], while deletion of *Trm112* gene would lead to severe growth defect in yeast [15]. In parallel, in another TRM112 homolog, arabidopsis SMO2, inactivation of SMO2 leads to a defect in progression of cell division and organ growth [7]. Besides, TRM112 might also interact with LYS9, an alcohol dehydrogenase that possesses a near Rossman fold domain that lacks the seventh β-fold [23], and other proteins with unknown function, implying that TRM112 possibly plays roles in modification or regulation of additional cellular processes [12, 15, 28].

Above all, TRM112 has been involved in multiple biological activities as an important plurifunctional factor. Nevertheless, the expression patterns of mouse *Trmt112* gene has not been reported thus far, hence we performed detailed expression analysis of *Trmt112* gene during mouse embryonic development, the results from which would offer an excellent foundation for further studies in functional analyses. The results showed that *Trmt112* was highly and ubiquitously expressed in zones of the brain, nervous system, and diverse tissues and organs, suggesting that *Trmt112* may play significant roles during embryonic development.

II. Materials and Methods

Mouse and tissue preparation

DBA/2J and C57BL/6J mice were time-mated overnight. At noon the next day the presence of a vaginal plug was considered to be embryonic day 0.5 (E0.5). All procedures were carried out following the "Rules for experiments animals" published by the Chinese Government (Beijing, China).

Whole mount in situ hybridization (WISH) and in situ hybridization (ISH)

E10.5, E11.5, E13.5 and E16.5 mouse embryos were isolated at different developmental stages and fixed in 4% paraformaldehyde in PBS at 4°C overnight. E10.5 and E11.5 were prepared for WISH. E13.5 and E16.5 embryos were paraffin embedded and sectioned at 9 μm thickness. To obtain the RNA probes, cDNA fragment of *Trmt112* (NM_026306, nt. 92-921, sense: 5'-ttcaatcgcagaaagagg-3', antisense: 5'-gtgtttctgttgtacggtct-3') were amplified by RT-PCR and inserted in pBluescript II KS$^{(+)}$ (Stratagene, La Jolla, CA, USA). Reaction for RT-PCR were performed for 32 cycles at 95°C for 2 min, 55°C for 50 s and 72°C for 45 s. RNA probe for the ISH were prepared using a DIG RNA labeling kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. WISH and ISH was performed by standard procedures as described previously [8, 16, 21, 27]. Briefly, we performed the standard procedures of the ISH experiments at least in triplicate at one time point. The experiments were pretreated by proteinase K with proper concentration and enough time to make holes on embryos prior to hybridization, and then, each parallel control was added with the sense RNA probe in contrast to the antisense probe and was hybridized at 65°C overnight. After adequate washing at 65°C by 2×SSC, 1% SDS and at room temperature by 0.2×SSC, the samples were blocked for 1 hr at room temperature and incubated with anti-digoxigenin-alkaline phosphatase conjugated antibody (Roche, 1:5000) at 4°C overnight. The slides were washed with several changes, developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP).

RNA analyses

Total RNA was isolated from E9.5 to E18.5 whole embryos and embryonic tissues (brain, tongue, heart, lung, liver and kidney) at E12.5, E15.5 and E18.5 using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and then were treated with DNaseI (Roche). cDNAs were synthesized using a SuperScript^{TM}III RNase H^{-} Reverse Transcriptase kit (Invitrogen, Eugene, OR, USA). Quantitative analysis of *Trmt112* with specific primers (NM_026306, nt. 271-492, sense: 5'-cgtaagcctttgcagtctccc-3', antisense: 5'-ttcatgttgtca cagcggagc-3') was performed using the ABI Prism 7500 Real-Time PCR System, and reactions were done using the SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions, and relative quantification was achieved following a standard curve method. Reactions for *Trmt112* were performed for 36 cycles at 95°C for 15 s, 60°C for 1 min, and then followed by a dissociation stage analysis. β-*actin* (NM_007393, nt. 520–717, sense: 5'-taccacaggcattgtgtag gact-3', antisense: 5'-ttgatgtcacgcacgatttccct-3') was used as a reference gene. All reactions, including the standards and non-template control (H_2O), were run in quartet from different embryos and organs. One-way analysis of variance (ANOVA) was used to analyze the significance of difference, followed by the Student-Newman-Keuls post-test. Students' t-test and a P value less than 0.05 was considered statistically significant.

III. Results

Gene structure and homology analysis of the mouse Trmt112

Trmt112 (gene ID: 67674) full-length cDNA has been initially identified from RIKEN [10]. Comparison with the genomic sequences showed that *Trmt112* is assembled from 4 exons spanning 1.53 kb on mouse chromosome 19E1 (Fig. 1A), the open reading frame of which starts within exon 2, ends within exon 4, and encodes a 125 amino acid protein considerably homologous with the TRM112 function domain (Fig. 1A). Amino acid sequence alignment analysis showed high similarity of mouse Trmt112 protein with other

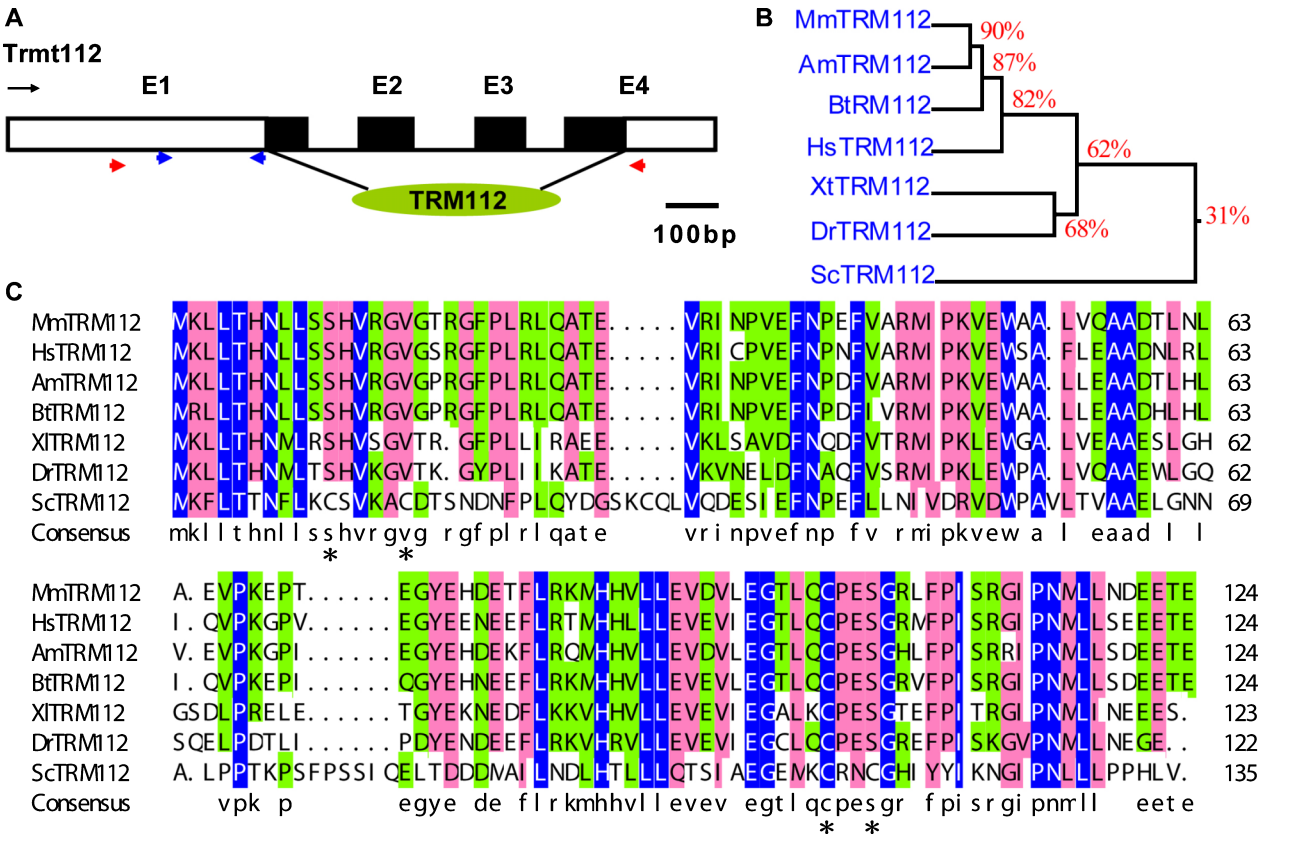
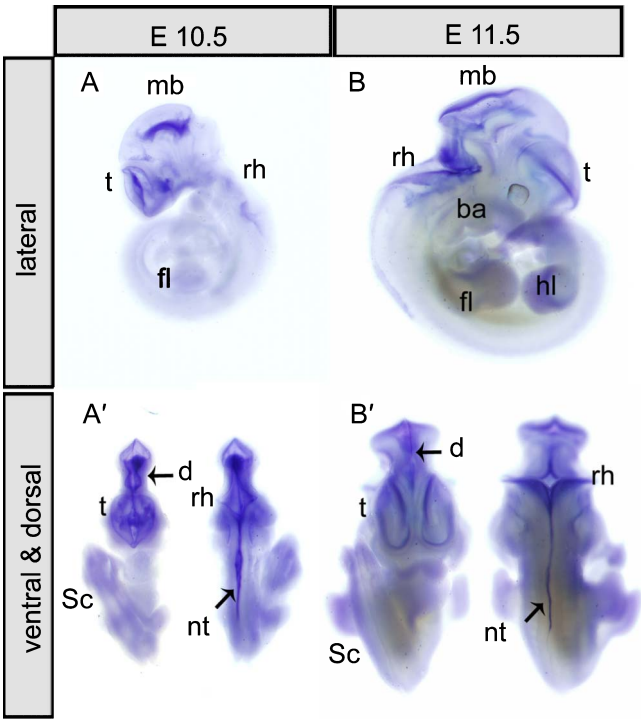


Fig. 1. Genomic organization and homology alignment of *Trmt112*. (**A**) Arrow denotes transcriptional orientations. Exons are represented as numbered boxes, introns as connecting lines, black boxes indicate coding protein, and conserved and function domain TRM112 (green oblate ellipsoid) is indicated below. Red and blue triangles were labeled the primers for *in situ* hybridization and QRT-PCR, respectively. Scale of genomic bar represents 100 bp. (**B**) Alignment of mouse TRM112 with other eukaryotes homologues. Shading colors represent highlight levels of amino acid conservation, and asterisks refer to the Cys residues for zinc binding in yeast TRM112. Mm, *Mus musculus*; Am, *Ailuropoda melanoleuca*; Hs, *Homo sapiens*; Bt, *Bos taurus*; Dr, *Danio rerio*; Xt, *Xenopus tropicalis*; Sc, *Saccharomyces cerevisiae*.



TRM112 homologues including human (*Homo sapiens*), panda (*Ailuropoda melanoleuca*), cows (*Bos taurus*), zebra fish (*Danio rerio*), clawed frog (*Xenopus tropicalis*) and yeast (*Saccharomyces cerevisiae*) (Fig. 1B), implying that TRM112 is a functionally conserved protein in the phylogenetic evolution. However, the putative zinc-binding domain of TRM112s in yeast was not present in other multicellular organisms (Fig. 1B) [5].

Expression patterns of Trmt112 in mouse embryos

The expression profile of *Trmt112* was analyzed in different tissues and at different developmental stages. *Trmt112* was firstly detected by whole mount *in situ* hybridization (WISH) at E10.5 and E11.5. At E10.5, from

Fig. 2. Expression pattern of mouse *Trmt112* at E10.5 and E11.5. (**A–B**) Lateral view of embryos, *Trmt112* was expressed in the telencephalon vesicle (t), midbrain (mb), rhombencephalon (rh), diencephalon (d) and branchial arches (ba). (**A'–B'**) Dorsal or ventral view of E10.5 and E11.5 showed *Trmt112* expression was detected through the mid-hindbrain region to rhombencephalon (rh) and spinal cord (sc).

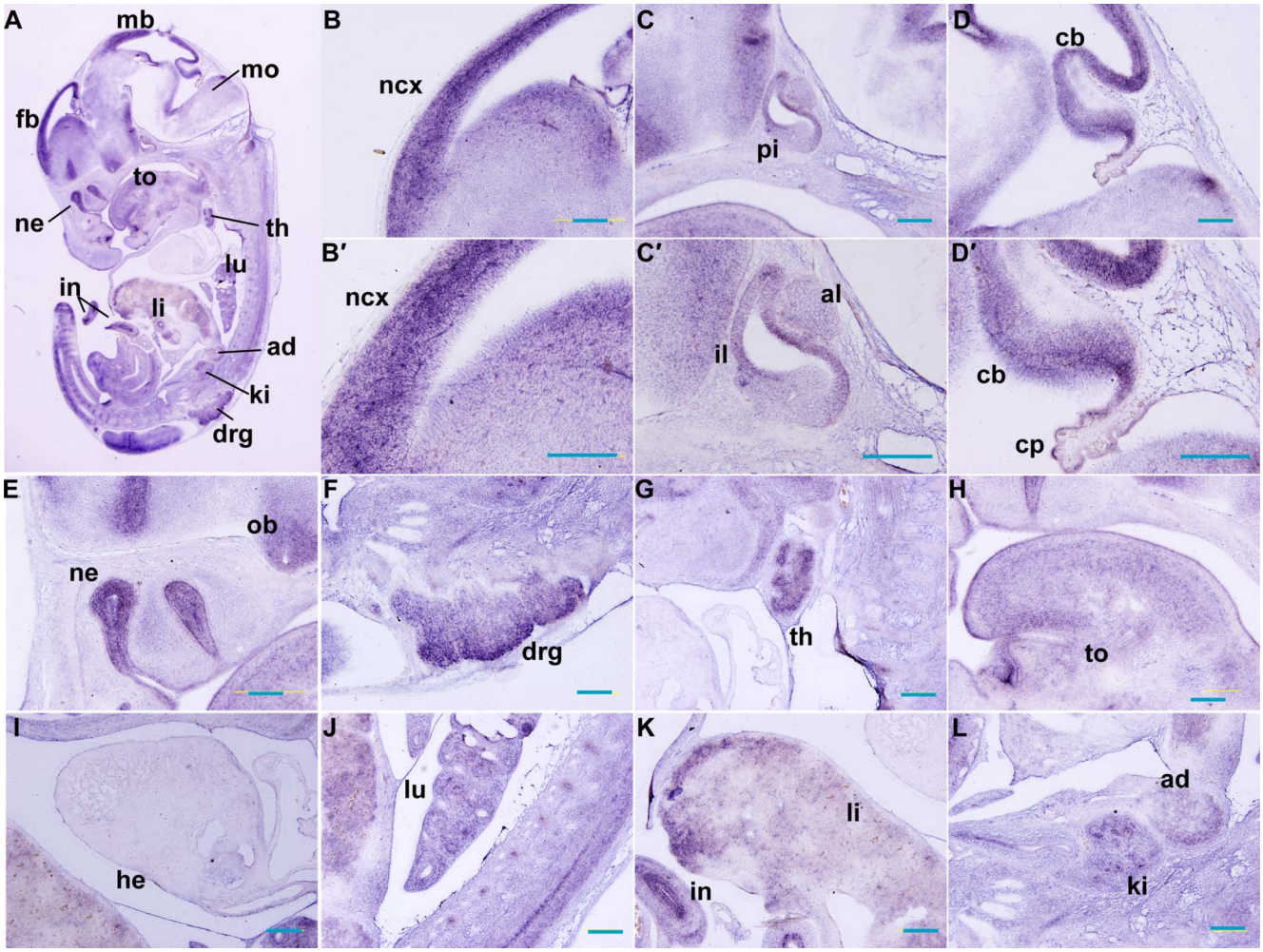


Fig. 3. Expression pattern of *Trmt112* at E13.5 by section *in situ* hybridization. (**A**) Sagittal sections of complete mouse embryos at E13.5 showed abundant expression of *Trmt112*. (**B–D & E–F**) *Trmt112* expression signals were detected in CNS. (**B'–D'**) Magnifications of observations of (**B–D**). (**G–L**) *Trmt112* prominent expression signals were detected in the main organs including thymus gland, lung, liver, intestine, kidney, and adrenal gland at E13.5. fb, forebrain; mo, medulla oblongata; ncx, neocortex; al, anterior lobe; il, intermediate lobes; pi, pituitary gland; cb, cerebellum; cp, choroid plexus; ne, nasal epithelium; ob, olfactory bulb; drg, dorsal root ganglia; th, thymus; to, tongue; h, heart; lu, lung; li, liver; in, intestine; ki, kidney. Bars=100 μm.

the lateral view, strong expressional signals of *Trmt112* were detected in the cortical anlage of the telencephalon vesicle (t), alar plate of midbrain (mb), and nervous plate of rhombencephalon (rh) (Fig. 2A). Dorsal view of the same embryos showed that the major signal was along the dorsal midline from the diencephalon (d) through the mid-hindbrain region to spinal cord (Sc) and neural tube (Nt) (Fig. 2A'). At E11.5, a similar expression pattern was maintained as E10.5, especially in brain and neural tube (Fig. 2B–B'). In addition, *Trmt112* expression was detected both in the branchial arches (ba), forelimb (fl), and hindlimb (hl) (Fig. 2A–A' & B–B') at E10.5 and E11.5. As a negative control, there were no signals detected using the *Trmt112* sense probes (data not shown).

At E13.5, the spatial expression of *Trmt112* was detected by *in situ* hybridization on histological sections. The expression was further expanded in the brain and spinal cord (Fig. 3A), and found in multiple developing organs. In the developing central nervous system (CNS), strong signals were detected in the lateral ventricular zone of the neocortex (ncx), anterior lobe (al), and intermediate lobes (il) of pituitary gland, cerebellum (cb), nasal epithelium (ne), olfactory bulb (ob), and dorsal root ganglia (drg) (Fig. 3B–F & B'–D'). In developing organs, prominent hybridization signals were observed within the thymus (th), tongue (to), epithelium of the lung (lu) and intestine (in), liver (li), kidney (ki), adrenal gland (ad) (Fig. 3G–L), but the expression in the heart (he) was low (Fig. 3I).

By E16.5, *Trmt112* was ubiquitously expressed in tissues. In the developing CNS, strong signals were detected in the regions of the neocortex (ncx), cerebellum (cb), olfactory bulb (ob), spinal cord (sc), and dorsal root ganglia (drg) (Fig. 4A–D & H–I). For instance, in the neocortex, the signals were detected in the marginal zone (mz) and

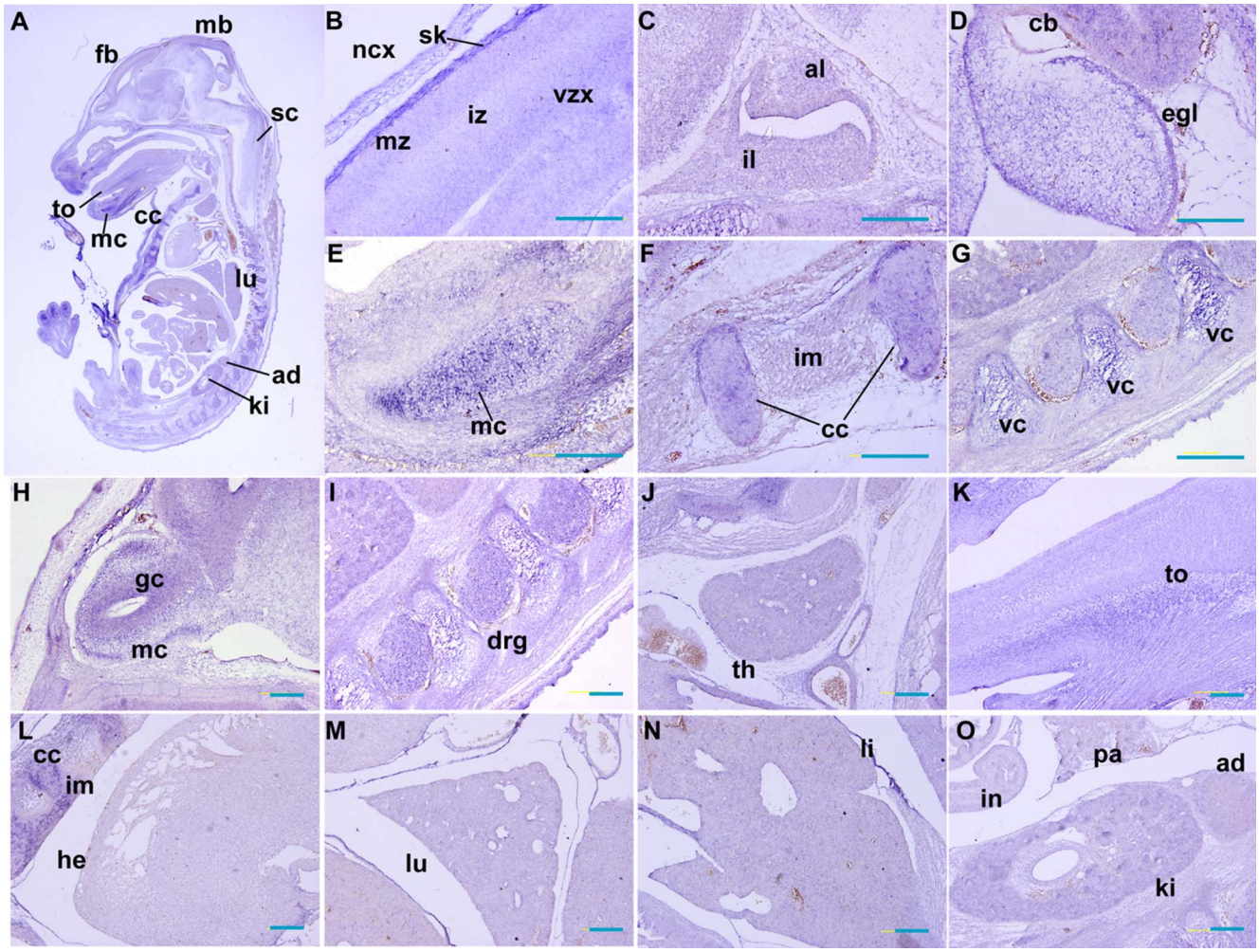


Fig. 4. Expression pattern of *Trmt112* at E16.5 by section *in situ* hybridization. (**A**) Sagittal sections of complete mouse embryos at E15.5 showed ubiquitous expression of *Trmt112*. (**B–D & H–I**) In developing CNS, strong signals were detected in the regions of the neocortex (ncx), cerebellum (cb), olfactory bulb (ob), and dorsal root ganglia (drg). (**E–G**) *Trmt112* staining was significantly present in both proliferating chondrocytes and perichondrium. (**G–O**) *Trmt112* was persistently and moderately expressed in multiple important organs at E16.5 like E13.5. fb, forebrain; sc, spinal cord; ncx, neocortex; sk, skull; mz, marginal zone; vz, ventricular zone; iz, intermedial zone; al, anterior lobe; il, intermediate lobes; cb, cerebellum; mc, Meckel's cartilage; cc, costal cartilage; vc, cartilages of vertebral column; mc, mitral cell layer; gc, granule cell layer; drg, dorsal root ganglia; th, thymus; to, tongue; h, heart; lu, lung; li, liver; in, intestine; ki, kidney. Bars=100 μm.

ventricular zone (vz), with low expression in the intermedial zone (iz) (Fig. 4B). In cerebellum, the external granular layer (egl) exhibited clear signals (Fig. 4D). In the olfactory bulb, *Trmt112* mainly distributed in the granule cell layer (gc), with weaker signals in the mitral cell layer (mc) (Fig. 4H). Alternatively, clear signals were also visible in proliferating chondrocytes and perichondrium, including skull (Fig. 4B), Meckel's cartilage (mc) (Fig. 4E), costal cartilage (cc) (Fig. 4F), and cartilages of vertebral column (vc) (Fig. 4G), which signals were not that evident at E13.5. Furthermore, *Trmt112* was moderately expressed in multiple organs like the thymus (th), tongue, heart, lung, liver, and kidney (Fig. 4G–O). As a control, sense probes were performed on adjacent tissue sections and showed no detectable labeling (data not show).

Expression analysis of Trmt112 during embryonic development

In order to further investigate the *Trmt112* expressional pattern, we utilized QRT-PCR to confirm its spatiotemporal expression during mouse development. Results showed that *Trmt112* expression was up-regulated from E9.5 to E18.5, especially up-regulated at E16.5, E17.5 and E18.5, but slightly down-regulated at E12.5 and E15.5 on whole embryos (Fig. 5A). Additionally, *Trmt112* was highly expressed in multiple tissues at different developmental stages. *Trmt112* expression was significantly up-regulated in tongue at E15.5 relative to E12.5, and in kidney at E15.5 relative to E18.5, but there no significant differences between tissues expression, expect for a litter lower expression of heart during embryonic development. (Fig. 5B).

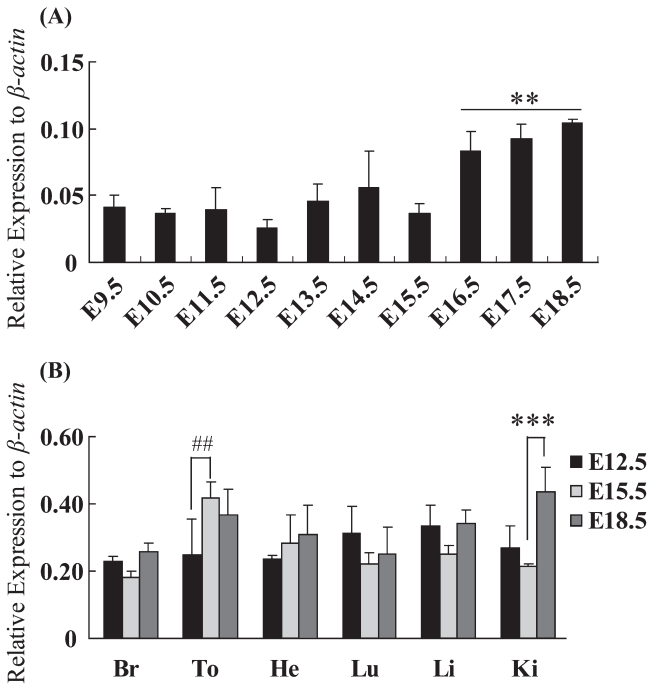


Fig. 5. *Trmt112* expression during embryonic development by QRT-PCR analysis. (**A**) Expression of *Trmt112* in whole embryos during mouse development from E9.5 to E18.5. **P<0.05 represents E16.5-E18.5 *vs* E9.5-E15.5 by one-way analysis of variance (ANOVA) followed by Tukey test. (**B**) Relative expression of *Trmt112* in diverse tissues at E12.5, E15.5 and E18.5. Values (means±SEM) represent the expression level relative to that of β-*actin* expression level from four biological replicates. (n=4). Br, brain; To, tongue; He, heart; Lu, lung; Li, liver; Ki, kidney. ##P<0.05 represents tongue expressed at E12.5 *vs* E15.5; ***P<0.01 represents kidney expressed at E15.5 *vs* E18.5 by two-way ANOVA followed by Tukey test.

IV. Discussion

In this study, *Trmt112* was prominently expressed in developing brain and neural tubes at E10.5 and E11.5, which were the proliferating zones of the nervous system, implying the possible role in the neural tube and nervous system. Section ISH results showed that *Trmt112* was ubiquitously expressed at mid-later-gestation of organogenesis stages. At E13.5, *Trmt112* was abundantly expressed in a variety of neural epithelium tissues including neocortex, cochlea, olfactory epithelium, Rathke's pouch, medulla oblongata, and dorsal root ganglia (drg). Rathke's pouch, one of the major neuroendocrine organs, which derives from the oral ectoderm and forms the anterior lobe (al) and intermediate lobes (il) of developed pituitary gland, is important for embryonic growth. At the stage of E16.5, organogenesis is largely complete, in accord with the signals detected in those tissues of brain and nervous system at E13.5, but abundant signals, that were also found in chondrocytes, where were not evident at E13.5, indicating that *Trmt112* possessed spatiotemporal specific expression patterns in development progress. QRT-PCR results showed that *Trmt112* was expressed in abundance throughout the developmental process from E9.5 to E18.5, and higher in multiple key organs. Thus, the *Trmt112* might be considered as an essential gene which persistently maintains roles in diverse organs and tissues during the embryonic stages. Alternatively, we found that *Trmt112* expression was much higher in parthenogenetic embryos than normal blastocysts of the same stage (E4.5) (data not shown). Thus we initially speculated whether *Trmt112* was associated with imprinting, but the result showed it was a biallelic gene (data not shown), possibly implying *Trmt112* expression in parthenogenetic embryos was functional at early development stage. TRM112 is an evolutionarily conserved protein, and the putative zinc-binding domain of TRM112 was present in yeast, bacteria, and some archaea but not in multicellular organisms [5]. However, disruption of the respective *Trmt112* gene in yeast and arabidopsis resulted in the slow growth of both organisms [7, 23]. Additionally, we also found mouse *Trmt112* had much higher homology with mammals than yeast, implying that *Trmt112* protein is functionally conserved in multicellular organisms.

In conclusion, we reported the characteristic expression patterns of mouse *Trmt112* during embryonic development. Our results suggested that *Trmt112* could play broad roles in the developing brain, nervous system, and morphogenesis of diverse tissues. The present study will serve as a basis for future *in vivo* genetic, functional, and mutational analyses.

V. Acknowledgments

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VI. Conflict of Interest

All authors of this paper declare no conflict of interest.

VII. References

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