The expression and role of Tmed2/TMED2 during the development of the murine embryo and placenta

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ABSTRACT

Normal development of the placenta is crucial for embryonic survival and growth. *Tmed2* is a gene, which has been found to be necessary for the formation of the labyrinth layer of the placenta, as well as for embryonic development. The objectives of this thesis are to: describe the transcripts of *Tmed2* using Northern Blot analysis; elucidate the expression pattern of *Tmed2* in the developing embryo and placenta using whole mount and section *in situ* hybridization; determine whether homozygous mutant embryos (*Tmed2*<sup>99J/99J</sup>) are undergoing ER stress using RT-PCR. *Tmed2* has three transcripts; however, only one of these transcripts contains exon 1 of the gene. *Tmed2* is expressed during the development of the embryo and placenta in those tissues that are affected in the *Tmed2*<sup>99J/99J</sup> mutants. Finally, *Tmed2* homozygous mutant embryos are not experiencing ER stress. *Tmed2* is probably required for the trafficking of an important protein(s) during development.
RÉSUMÉ

Le développement normal du placenta est essentiel à la survie et la croissance embryonnaire. Tmed2 est un gène qui s’est révélé nécessaire et important pour la formation de la couche labyrinthe du placenta, ainsi que pour le développement embryonnaire. Les objectifs de cette thèse sont : de décrire la transcription de Tmed2 en utilisant Northern Blot analysis; d’élucider le modèle selon lequel s’exprime Tmed2 dans le développement de l’embryon et du placenta en utilisant l’hybridation in situ complète ou en section; de déterminer si les embryons mutants homozygotes (Tmed2^{99J/99J}) subissent un stress ER en utilisant le RT-PCR. Tmed2 possède trois transcriptions; par contre, une seule de ses transcriptions contient l’exon 1 du gène. Tmed2 s’exprime lors du développement de l’embryon et du placenta dans les tissus qui sont affectés dans les Tmed2^{99J/99J} mutants. Finalement, les embryons mutants homozygotes Tmed2 ne subissent pas de stress ER. Tmed2 est probablement utilisé pour le trafic et le déplacement d’une ou de plusieurs protéines importantes lors du développement.
ACKNOWLEDGMENTS

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**LIST OF ABBREVIATIONS**

ATF6, activating transcription factor 6  
COP/II, coat protein I/II  
E, embryonic day  
ENU, N-ethyl-N-nitrosourea  
ER, endoplasmic reticulum  
ERAD, ER-associated degradation  
GAPDH, glyceraldehyde 3-phosphate dehydrogenase  
Gas1p, GPI-anchored protein  
GOLD, Golgi dynamics domain  
GRP78, glucose regulate protein 78 (heat shock 70kDa protein 5)  
GRP94, glucose regulate protein 94 (heat shock protein 90kDa beta)  
IRE1, inositol-requiring 1/ ER to nucleus signaling 1  
PCR, polymerase chain reaction  
PERK, protein kinase-like endoplasmic reticulum kinase  
PMEF, primary mouse embryonic fibroblast  
RT-PCR, reverse transcription polymerase chain reaction  
TEM, transmission electron microscopy  
TMED/Tmed, transmembrane emp24 trafficking domain  
UPR, unfolded protein response  
VCAM1, vascular adhesion molecule 1  
Xbp1, X-box binding protein 1
CONTRIBUTIONS OF AUTHORS

The candidate performed the majority of the work in this thesis; however, Li Luo provided invaluable assistance with the section *in situ* experiment, performed the Western blot analysis, and dissected the samples used for the TEM experiment.
INTRODUCTION

Development and Function of the Placenta

The placenta is an organ that is necessary for the transport of nutrients and oxygen to the fetus during gestation. It also forms the interface between the maternal and fetal circulation, and facilitates fetal waste disposal (Watson and Cross, 2005). This transport occurs either by diffusion, or by facilitated or active transport (Watson and Cross, 2005; Jones et al., 2007). The transport of nutrients is facilitated by the labyrinth layer of the placenta; it consists of branched villi which provide increased surface area for nutrient exchange. Insufficient branching or malformation of the labyrinth layer is thought to underlie some cases of miscarriage and preeclampsia associated with fetal growth restriction (Cross et al., 2003). Development of the labyrinth layer is dependent on the fusion of two extraembryonic membranes, the allantois and the chorion, in a process called chorioallantoic attachment (Downs and Gardner, 1995). The chorion originates mainly from extraembryonic ectoderm, which is a derivative of the trophectoderm layer of the blastocyst. The allantois consists of extraembryonic mesoderm and is of primitive ectodermal origin; therefore, it is entirely derived from the epiblast or inner cell mass of the blastocyst (Downs and Gardner, 1995).

Placental development begins at the blastocyst stage, embryonic day (E)3.5 in mice, at which point the separation of the first two cell lineages occurs. The outer layer of the blastocyst is the trophectoderm layer, and it is set aside from the inner cell mass. The trophectoderm layer will eventually give rise to the
mature placenta (Watson and Cross, 2005). After implantation (E4.5), the
trophectoderm layer gives rise to giant cells, as well as two diploid cell types: the
extraembryonic ectoderm and the ectoplacental cone (Watson and Cross, 2005).
The extraembryonic ectoderm gives rise to the trophoblast cells of the chorion of
the placenta, which will eventually form the labyrinth layer. The ectoplacental
cone differentiates to form the spongiotrophoblast cells, which are thought to
provide the labyrinth layer with structural support (Watson and Cross, 2005)
(Figure 1). Later, glycogen trophoblast cells differentiate from spongiotrophoblast
cells and invade the uterine wall (Watson and Cross, 2005).

**Chorioallantoic Attachment and Labyrinth Development**

The extraembryonic mesoderm, which forms the allantois, extends from
the posterior end of the embryo at E8.0 (Watson and Cross, 2005). At E8.5, the
chorion and the allantois attach in a process called chorioallantoic attachment, and
folds appear in the chorion. These folds create space for the blood vessels to grow
from the allantois. Chorioallantoic attachment is thought to be mediated by
various signaling molecules, including vascular adhesion molecule 1 (VCAM1) in
the tip of the growing allantois, and α4-integrin on the surface of the chorion
(Cross et al., 2003) (Figure 2). Fetoplacental blood vessels grow *de novo* from the
allantois to generate the fetal component of the placental vasculature while the
trophoblast undergoes villous branching to create the labyrinth layer. During
branching, the chorionic trophoblast cells differentiate into three layers of
labyrinthine trophoblast cells: two layers of syncytiotrophoblast cells and a layer
of mononuclear cells (Rossant and Tam, 2002). The syncytiotrophoblast cells line the endothelium of the fetal capillaries, while the mononuclear trophoblast cell type lines the maternal blood sinuses (Watson and Cross, 2005). The maternal blood passes through the spongiotrophoblast via sinuses and enters the spaces in the labyrinth to bathe the fetal trophoblastic villi creating a system of nutrient exchange between mother and fetus (Rossant and Tam, 2002) (Figure 3).
Figure 1: Development of the Different Cell Types of the Mouse Placenta The first differentiation event occurs at E3.5 in mice, when the trophectoderm and inner cell mass lineages are specified. Both these lineages develop into cell types which contribute to the formation of the placenta’s labyrinth layer.

(Cross et al., 2002)
Figure 2: Signaling Pathways Involved in Chorioallantoic Attachment

The schematic depicts known protein interactions and pathways during chorioallantoic attachment and subsequent development of the labyrinth layer.

(Hemberger and Cross, 2001)
Figure 3: Development of the Mouse Placenta The schematic depicts the development of the mouse placenta starting at the earliest stage E3.5. Chorioallantoic attachment begins at E8.0. The colors reflect the different lineages that can be traced throughout development.

Modified from (Rossant and Cross, 2001)
The 99J Mutation

N-Ethyl-N-Nitrosourea (ENU) is a mutagen used to introduce mutations in animal models in order to discover novel causative mutations. ENU mutagenesis targets spermatogonial stem cells, and results in random single-base pair mutations. Therefore, offspring of mice treated with ENU carry point mutations. These point mutations may lead to novel phenotypes (Cordes, 2005). The 99J mutation was found in a recessive ENU screen for developmental defects in the mouse (Garcia-Garcia et al., 2005). The 99J mutation resulted in mid-gestation embryonic lethality, which was attributed to the failure of labyrinth layer formation in homozygous mutant embryos (Figure 4). 99J homozygous mutant embryos died between E10.5 and E11.5, were smaller than their littermates, had a pointy tail bud, failed to undergo embryonic turning, and in all cases did not successfully undergo chorioallantoic attachment at E8.5 (Figure 5). At E9.5, some of the homozygous mutant embryos experienced chorioallantoic attachment. However, none of the homozygous mutant embryos developed a labyrinth layer, and had chorions which remained flat (Figure 5).

The 99J mutation was mapped using microsatellite markers, and was found to occur in Tmed2, transmembrane emp24 trafficking protein 2. The mutation was a G:C → T:A transversion in exon 1 of the gene (Jerome-Majewska et al., 2008) (Figure 6). This resulted in the nonsynonymous substitution of an alanine residue for a glutamic acid residue in the N terminus of the protein.
Figure 4: Sagittal Sections through E9.5 and E10.5 Placentas Hematoxylin and Eosin staining of normal and homozygous mutant placentas. A, D: Normal placenta showing normal attachment of the allantois (arrow) to the chorion and formation of the labyrinth layer, respectively. B, E: Homozygous mutant placenta showing an unattached allantois (n=3; arrow) and a flat chorion, respectively. C: Homozygous mutant placenta showing attachment of the allantois (n=4) but absence of a labyrinth layer (arrow). lab, labyrinth; ch, chorion; all, allantois.

(Jerome-Majewska, 2008)
**Figure 5: The 99J Embryo Phenotype**  E9.5 wildtype embryo (left) and a 99J homozygous mutant embryo (right). The mutant embryo is smaller, has a laterally restricted posterior end (arrows), and an abnormal heart.

(Jerome-Majewska, 2008)
Figure 6: The Tmed2$^{99J}$ Mutation The G:C → T:A point mutation in Tmed2 results in the substitution of an alanine residue for a glutamic acid residue. This residue is well-conserved among different species.

99J MVTLAELLALLA E LLATASGYFVSIDAHAE

Mouse MVTLAELLALLA A LLATASGYFVSIDAHAE

Human MVTLAELLVLLA A LLATVSGYFVSIDAHAE

Chimp MVTLAELLVLLA A LLATVSGYFVSIDAHAE

Rat MVTLAELLVLLA A LLATASGYFVSIDAHAE

Chick MSPVRAVLALLA A LAAPAAAYFVSIDAHAE
**TMED2**

TMED2 is part of the TMED/p24 family of proteins, which is involved in the selection and subsequent vesicular transport of proteins from the endoplasmic reticulum (ER) to the Golgi (Figure 7A) (Blum et al., 1996; Sohn et al., 1996; Dominguez et al., 1998; Fullekrug et al., 1999; Gommel et al., 1999; Emery et al., 2000). All the TMED/p24 family members have a large luminal domain and a short C-terminal membrane anchor with a highly conserved cytoplasmic tail (Blum et al., 1996). They have been found to be associated with both ER-derived coat protein I (COPI) and II (COPII) transport vesicles which mediate transport between the ER and the Golgi (Blum et al., 1996; Duden, 2003). The COPI coat surrounds the vesicle and is thought to be involved in both anterograde and retrograde transport from the ER to the Golgi, and the Golgi to the ER, respectively. The COPII coat is thought to exclusively mediate anterograde transport from the ER to the Golgi (Schimmoller et al., 1995; Stamnes et al., 1995; Sohn et al., 1996; Nickel et al., 1997; Dominguez et al., 1998; Blum et al., 1999).

TMED/p24 proteins are conserved in all eukaryotes including yeast, C. elegans, Drosophila, Xenopus, Mus musculus, and humans. TMED/p24 proteins are single pass, type I transmembrane proteins found to be associated with the cis Golgi membrane and have several conserved domains including a signal sequence at the N-terminus (Figure 7B). A signal sequence is a 20 amino acid peptide, which is necessary to target the protein to the ER where the signal sequence is recognized by signal sequence recognition particles in the ER membrane.
Subsequently the signal sequence is cleaved. The mutation in TMED2 is in the signal sequence of the protein, and therefore may affect the localization of the translated mutant protein.

In addition to the signal sequence, TMED2 as well as its family members have a Golgi dynamics domain (GOLD), which is necessary for interaction with potential cargo proteins in the ER and the Golgi, and their subsequent transport between these organelles (Anantharaman and Aravind, 2002). They also have a coiled coil domain, which is necessary for the interaction of TMED/p24 family members and the formation of heteromeric complexes from different combinations of TMED/p24 proteins, such as the TMED2/7/9/10 complex (Anantharaman and Aravind, 2002). TMED/p24 proteins form these complexes in order to perform the function of transport between the ER and the Golgi. Finally, they have cytoplasmic tails which are necessary for the protein’s interaction with COPI and COPII vesicles in the cytoplasm (Blum et al., 1996).

*Tmed2* and other family members are thought to have alternatively spliced RNA isoforms (Ensembl); however, protein isoforms have not been described. It is unknown whether two of the three RNA isoforms have functions in the cell. Only isoform 1, the largest transcript, has the signal sequence of *Tmed2*, and therefore is predicted to be the only transcript affected by the 99J mutation (Figure 9A).

All of the TMED/p24 family members have been knocked out in yeast, and the yeast were still viable (Schimmoller et al., 1995; Belden and Barlowe, 1996; Belden and Barlowe, 2001). However, when *Tmed2* was knocked out there
was an accumulation of GPI-anchored protein (Gas1p), which is a protein normally trafficked by TMED2. There was also the release of Kar2p, which is a chaperone protein. This indicates that the yeast strains lacking \textit{Tmed2} are experiencing some form of ER stress, possibly due to the accumulation of untrafficked proteins in the ER, and mounting the unfolded protein response (UPR) (Belden and Barlowe, 2001).
**Figure 7: Schematic Showing the Conserved Domains and Trafficking of TMED2/p24 Proteins**

**A.** TMED2 traffics proteins between the ER and the Golgi with the use of COPI and COPII protein coated vesicles (Duden, 2003). **B.** The signal sequence is necessary for the protein’s translocation into the ER. The GOLD domain is necessary for interaction with cargo proteins. The coiled-coil domain is necessary for the formation of heteroligomeric complexes, and the cytoplasmic domain is necessary for interaction with COPI and II proteins (Blum et al., 1996; Dominguez et al., 1998; Anantharaman and Aravind, 2002).

A.

Modified from (Duden, 2003)
B.

* Denotes 89J mutation
TMED2 and ER Stress

As mentioned previously, in yeast, deletion of \textit{Tmed2} results in the activation of the unfolded protein response (UPR). All secretory proteins enter the ER, where they fold into their native conformation. If correctly folded, the proteins are trafficked to the Golgi where they are modified by the addition of carbohydrate groups to their side-chains. However, by a process referred to as quality control, improperly folded proteins are retained in the ER (Schroder and Kaufman, 2005). When there is an accumulation of proteins in the ER, possibly due to a defect in the transportation of proteins out of the ER, this can act as a source of ER stress (Rutkowski and Kaufman, 2004).

The ability of a cell to respond to stress is essential for its homeostasis; in situations of ER stress, the UPR is activated, and three events occur: 1) ER-resident chaperones are upregulated, 2) translational attenuation occurs which reduces the number of proteins entering the ER, and 3) ER-associated degradation (ERAD) occurs which clears the ER of misfolded or accumulated proteins (Shen et al., 2002; Rutkowski and Kaufman, 2004). The UPR acts to maintain ER homeostasis in eukaryotic cells, and when it fails to do so it initiates apoptosis (Belden and Barlowe, 2001).

These three responses are achieved via signaling by three transmembrane ER proteins: ER to nucleus signaling 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase-like endoplasmic reticulum kinase (PERK). PERK is involved in the translation attenuation facet of the UPR, it homodimerizes and autophosphorylates and then acts on its substrate to inhibit protein synthesis. This
is meant to reduce the nascent protein load of the ER. On the other hand, IRE1 is involved in ERAD. IRE1 also homodimerizes and autophosphorylates, and subsequently catalyzes the splicing out of a small 26 nucleotide intron from X-box binding protein 1 (Xbp1), a transcription factor, in conditions of ER stress. The spliced form of Xbp1 is an active transcription factor, which activates transcription of genes involved in degradation. ATF6 is involved in upregulating chaperone proteins and upregulates the synthesis of Xbp1 mRNA. It performs this function by translocating to the Golgi in cases of stress where it is subsequently cleaved by proteases to become a potent transcriptional activator (Figure 8) (Rutkowski and Kaufman, 2004).
Figure 8: Schematic Showing the Pathways that are Activated in the UPR

Positive feedback loops are shown in green and negative feedback loops are shown in red. In the case of ER stress, three ER transmembrane proteins are involved in the activation of the UPR pathway: PERK, ATF6, and IRE1. PERK is autophosphorylated and acts to attenuate translation. ATF6 moves to the Golgi where it is cleaved and acts to increase the transcription of chaperone proteins such as glucose regulate protein 78 (GRP78), and glucose regulate protein 94 (GRP94), as well as to increase the transcription of Xbp1. IRE1 is also autophosphorylated and catalyzes the splicing of 26 nucleotides from Xbp1. This then permits the spliced XBPI isoform to activate the transcription of genes involved in degradation (Rutkowski and Kaufman, 2004).
Modified from (Rutkowski and Kaufman, 2004)
OBJECTIVES

The objectives of my project are to:

1) determine how many transcripts of *Tmed2* exist;

2) characterize the expression of *Tmed2* in early mouse development;

3) determine whether the 99J homozygous mutant embryos and placentas are undergoing ER stress.

HYPOTHESIS

We hypothesize that there are three *Tmed2* transcripts based on information on databases such as Ensembl and NCBI. Furthermore, we hypothesize that *Tmed2* will be widely expressed in both the embryo and placenta at various stages of development, as it is a trafficking protein. Finally, we hypothesize that 99J homozygous mutant cells are undergoing ER stress and will demonstrate signs of the UPR.
MATERIALS AND METHODS

Mice

Days on which plugs were detected before noon were considered to be E0.5. CD1 mice were used for the transcript and expression objectives (Objectives 1 and 2). The *Tmed2*99J mice were generated by ENU on a C57Bl/6J genetic background, and then backcrossed to C3H/HeJ mice. Embryos and placentas were dissected at various stages in 1X DEPC-PBS 0.2% BSA and subsequently fixed in 4% paraformaldehyde (PFA) overnight at 4°C for whole mount *in situ* hybridization and section *in situ* hybridization. Samples that were used for RNA isolation were placed in TriZol.

Probe design for *in situ* hybridization

The probe was PCR-amplified using the *Tmed2* Tran1_L and Tran1_R primers (Table 1). The expected band size is 378 base pairs. The PCR product was gel extracted (Qiagen) and subcloned into a dual promoter TOPO vector (Invitrogen). Competent cells were transformed with the plasmid and then plated on ampicillin plates with X-galactosidase. The white colonies were selected and grown in LB and ampicillin overnight. Minipreps (Qiagen) were performed, and then the plasmids were digested with EcoRI to confirm the presence of an insert. Subsequently, the clones were sequenced using M13F and R primers with an ABI POP6 sequencer.
RNA probes were transcribed using the Roche kit with a T7 probe representing the antisense strand, and an Sp6 probe representing the sense strand.

**Whole mount in situ hybridization**

Embryonic and placenta samples were dehydrated through a methanol series and stored at -20°C overnight. The samples were then rehydrated through a methanol series. The samples were bleached in 6% hydrogen peroxide in PBT for 1 hour, then washed three times in PBT. They were treated with 10µg/ml of proteinase K in PBT. The digestion was stopped with 2mg/ml of glycine in PBT and then washed twice with PBT. The samples were fixed in 0.2% glutaraldehyde + 4% PFA in PBT for 20 min, and washed twice in PBT. The samples were prehybridized at 65°C (50% formamide, 5X SSC, 1% SDS, 100µg/ml tRNA, 50µg/ml heparin) for 2 hours, and then the buffer was replaced with new hybridization buffer containing 1µg/ml dioxigenin-labeled RNA probe at 65°C overnight. The samples were washed in 50% formamide, 5X SSC, 1% SDS and in 50% formamide, 2X SSC at 65°C, followed by two 5 min washes with TBST (0.14M NaCl, 2.5mM KCl, 25mM Tris pH 7.5, 0.1% Tween-20) at room temperature. The samples were then blocked in 1% blocking reagent (Boehringer) in TBST for 1 hour. The blocking reagent was replaced with blocking reagent containing anti-dioxigenin antibody (1:1000) and incubated overnight at 4°C. Samples were washed at room temperature with TBST, then washed overnight with TBST at 4°C. The samples were washed with TBST, and then with NTMT
(0.1M Tris-HCL pH 9.5, 0.1M NaCl, 0.05M MgCl₂, 0.1% Tween-20). Then for the color reaction, they were incubated in NTMT + NBT/BCIP (Roche, 100µl/10ml of NTMT) until sufficient color was observed. After the color reaction, the samples were washed three times in PBT for 10 min, and then fixed in 4% PFA overnight. Finally, they were visualized and photographed in PBT using a Leica MZFLIII microscope and Spot Diagnostics Model 2.3.1 camera.

**Section in situ hybridization**

The sections were washed with xylene and then hydrated with an ethanol series. They were fixed in 4% PFA, and treated with proteinase K in TE, washed in PBS and fixed again in 4% PFA. They were washed in PBS, 2X SSC and then Tris/Glycine. Hybridization with DIG-labeled probe took place overnight at 65°C. Slides were washed in 5X SSC and then 0.5X SSC with formamide. They were treated with RNase A in NTE, and washed again with 0.5X SSC and 2X SSC before blocking (Boehringer). The slides where then incubated with an anti-DIG antibody overnight at 4°C. This was followed by TBS washes, and a Tris/Tween-20/levamisole wash before staining with BM purple (Roche). The sections were counterstained with Nuclear Fast Red. Photographs were taken with a Zeiss Imager.Z1 microscope and AxioCam MRc5 camera.

**Fixing and embedding tissue**

**Paraffin sections:**
After fixing overnight in 4% PFA, samples were washed for 30 min at 4°C in 1X PBS followed by 0.25M sucrose, 0.2M glycine in PBS overnight at 4°C. Tissue was washed in ethanol:saline for 30 min, and twice with 70% EtOH for 30 min at 4°C. Samples were then washed in 85% EtOH, 95% EtOH, and twice in 100% EtOH for 30 min each at room temperature. They were then washed in EtOH:xylene, and twice in xylene for 30 min each at room temperature. Finally, they were placed in fresh paraffin three times at 60°C for 30 min each, oriented, and embedded into molds. The samples were sectioned at 5µm thickness.

**Cryosections:**

Following whole mount *in situ* hybridization, samples that were cryosectioned were washed in 5% sucrose in PBS for 1 hour, followed by 30% sucrose in PBS overnight. The smaller samples, taken at E6.5 and E7.5, were first embedded in 1% agarose in PBS. Subsequently, they were washed in 30% sucrose + 50% cryomatrix (ThermoScientific) for 1 hour, and then embedded in cryomatrix. The molds were then frozen on dry ice. Samples were sectioned at 10µm thick, air-dried, washed with PBS, and coverslipped with glycerol-gelatin (Sigma).

**RNA isolation and DNase treatment**

RNA isolation was performed using the Invitrogen’s TriZol protocol (Invitrogen). 1µg of RNA was treated with 1µl of DNase in 1X buffer, and incubated for 15 minutes at room temperature. DNase was inactivated by adding 1µl of 25mM of EDTA and heating for 10 minutes at 65°C.
**Northern Blot**

Exon 4-3’ UTR probe was made by PCR-amplifying exon 4 and part of the 3’UTR of Tmed2 using the Exon4_L and Exon4_R primers (Table 1).

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon4L &amp; Exon4R</td>
<td>94°C x 3’, (94°C x 30”, 52°C x 40”, 72°C x 30”) x 35, 72°C x 5’</td>
</tr>
</tbody>
</table>

The 480 base pair product was visualized on a 0.8% agarose gel, and then was cut out with a scalpel under UV light. A gel extraction was performed using a gel extraction kit (Qiagen). 10ng of DNA was dissolved in DEPC-H₂O to a total volume of 34µl. It was denatured at 95°C and biotin-labeled according to the NEB Phototope Kit (NEB).

The exon 1 probe was made using the Tmed2L_in primer (Table 1). The oligonucleotide was 3’ end biotin-labeled using the Biotin 3’-end DNA Labeling kit (Pierce).

The nonradioactive Northern blot was carried out as indicated in the NEB Phototope kit (NEB). The following modifications were made: hybridization was done at 48°C, all washes were doubled, and the second blocking step was increased to 10 minutes.
RT-PCR

Reverse transcription was done using Invitrogen’s Superscript III following treatment with DNase. PCR was done with the following primers Xbp1 L, Xbp1 R, Tmed2 L_in, Tmed2 R_in, Tmed10 L, Tmed10 R, Gapdh L, Gapdh R, Par2 L, Par2 R, Exon2 _L, Exon 3 _L, Exon4 R (Table 1). Following the Xbp1 RT-PCR the PCR products were digested with PstI.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmed2 L_in and Tmed2 R_in</td>
<td>94°C x 3’, (94°C x 30”, 54°C x 40”, 72°C x 30”) x 35, 72°C x 5’</td>
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<tr>
<td>Xbp1 L and Xbp1 R</td>
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<td>Tmed10 L and Tmed10 R</td>
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<tr>
<td>Exon3 _L and Exon4 R</td>
<td>94°C x 3’, (94°C x 30”, 54°C x 40”, 72°C x 30”) x 35, 72°C x 5’</td>
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</table>
**Cell Culture**

Primary mouse embryonic fibroblasts were plated on gelatin plates. They were passed once, and when they reached 70% confluence, they were treated with either media-only, DMSO, tunicamycin, or thapsigargin for 3 hours. The cells were then washed twice with 1X PBS-DEPC, and then scraped and collected in TriZol for RNA isolation.

**Transmission Electron Microscopy (TEM)**

E9.5 wildtype and homozygous mutant ($Tmed2^{99J/99J}$) placentas were dissected in 0.2% BSA in PBS. Only the embryonic region of the placenta was kept and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were processed and sectioned by McGill’s Facility for Electron Microscopy Research. The sections were visualized and photographed using the Tecnai 12 120 kV microscope.
RESULTS

Objective 1: Rationale

Tmed family members have been shown to have alternatively spliced isoforms on databases such as Ensembl. Therefore, it is important to determine whether Tmed2 has more than one transcript which would be affected by the 99J point mutation in exon 1 of the gene.

Objective 1: Northern Blot

Tmed2, as well as other TMED/p24 family members, are predicted to have different RNA transcripts as a result of alternative splicing. In order to investigate whether these same transcripts exist in embryos and placentas and to confirm the information deposited in the Ensembl database, I performed Northern Blot analysis. Two probes were used, one oligo-probe which binds to exon 1 of Tmed2 and a second probe which binds from exon 4 to the 3’UTR of Tmed2. Based on Ensembl, with the first probe we expected one band with a size of 2.0kb, and with the exon 4 probe we expected three bands with the following sizes: 2.0kb, 1.6kb, and 735bp (Figure 9A).

In fact, by Northern blot using the exon 1 oligo probe I saw a single band in both an E9.5 CD1 embryo and an E9.5 CD1 placenta sample corresponding to a size of 2.0kb, as predicted (Figure 9B). However, with the exon 4 probe I saw three bands of the following sizes: 2.0kb, 1.6kb, and 1.2kb (Figure 9B). The smallest band observed (1.2kb) differs in size from the transcript predicted on Ensembl (735bp).
In addition, by RT-PCR two bands sized 920bp and 200bp were observed when using primers which bind in exon 2 and the 3’UTR. Moreover, two bands, sized 748bp and 250bp, were seen when using primers which bind in exon 3 and the 3’UTR. These bands were seen in both the wildtype and homozygous mutant embryos. This suggests that there are two splicing events occurring between exon 2 and the 3’UTR and that the mutation in Tmed2 does not affect the alternate transcripts observed.

The smallest transcript seen by Northern blot differs from the expected size and supports RT-PCR data, which suggests that there may be different splicing events occurring in the embryo than predicted by Ensembl (Figure 10). These splicing events likely occur between exon 2 and the 3’UTR, and do not seem to differ according to genotype (Figure 10B).

**Objective 1: Conclusion**

By Northern blot analysis, I have shown that Tmed2 has three transcripts. Two of these transcripts correspond in size to those predicted on Ensembl; however, one of them differs in size. Importantly, only the 2.0kb transcript possesses exon 1 of Tmed2, indicating that this would be the sole transcript to be affected by the 99J point mutation. In addition, RT-PCR data suggests that there is no difference in the splice events/transcripts generated in the wildtype and mutant embryos.
**Figure 9: Tmed2 is Alternatively Spliced** A. This schematic depicts the three predicted transcripts of Tmed2 with the colors indicating sequence similarity. All the transcripts have four exons. The first and largest transcript is 2.0kb, the second is 1.6kb, and the third is 735bp. Only the first transcript has the first exon. B. Three Northern Blots are shown, with the left one showing three transcripts of the following sizes: 2.0kb, 1.6kb, and 1.2kb in an E9.5 placental sample using the probe that binds from the fourth exon to the 3’UTR. The center lane shows a single band 2.0kb in length, with a probe which binds to exon 1 in an E9.5 embryonic sample. The right lane a single band 2.0kb in length, with a probe which binds to exon 1 in both an E9.5 embryonic and placental sample. E, embryonic day; pl, placenta.

A.

<table>
<thead>
<tr>
<th>Transcript 1</th>
<th>Transcript 2</th>
<th>Transcript 3</th>
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<td><img src="image2.png" alt="Transcript 2 Diagram" /></td>
<td><img src="image3.png" alt="Transcript 3 Diagram" /></td>
</tr>
</tbody>
</table>
B.

Exon 4 - 3'UTR

Exon 1

E9.5pl

E9.5

E9.5pl  E9.5
**Figure 10: Tmed2 Undergoes Alternate Splicing between Exon 2 and the 3’UTR**

**A.** The table shows the predicted band sizes according to the transcripts predicted on Ensembl (refer to schematic in Figure 9), as well as the bands observed from the RT-PCR. **B.** Two bands (748bp, 250bp) are observed in both mutant and wildtype embryos when primers are used between exon 3 and the 3’UTR. Lanes 1-10 are RT+ and lanes 11-20 are the RT- controls. 1 & 11, wild type E11.5; 2 & 12, wild type E10.5; 3 & 13, wild type E10.5; 4 & 14, heterozygous E10.5; 5 & 15, mutant E10.5; 6 & 16 mutant E10.5; 7 & 17 mutant E10.5; 8 & 18, mutant E10.5; 9 & 19, mutant E10.5; 10 & 20, mutant E10.5.

<table>
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<tr>
<th>Primers</th>
<th>Transcript 1</th>
<th>Transcript 2</th>
<th>Transcript 3</th>
<th>Bands Observed</th>
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<td>920 bp</td>
<td>-</td>
<td>-</td>
<td>920 bp and 200 bp bp</td>
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<tr>
<td>Exon 3-3’UTR</td>
<td>748 bp</td>
<td>748 bp</td>
<td>-</td>
<td>748 bp and 250 bp</td>
</tr>
</tbody>
</table>

A.
Objective 2: Rationale

Since our laboratory has determined that Tmed2 is required for the development of the embryo and placenta, it is necessary to determine when and where Tmed2 is expressed in wildtype tissues during development.

Objective 2: Tmed2 Expression

The expression of Tmed2 was investigated using RT-PCR, whole mount and section in situ hybridization. The data were compiled into a manuscript.

Contributions of authors

Li Luo performed the section in situ shown in this manuscript.
Tmed2, a member of the p24 family, is expressed during the development of the mouse embryo and placenta.

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ABSTRACT

TMED2, a member of the conserved p24 family of proteins, is involved in the vesicular transport of cargo proteins between the ER and the Golgi. The expression of TMED/p24 proteins in mammals is unknown; however, one member TMED10, is required for mouse development. In yeast and C. elegans, TMED2 is critical for normal movement of specific proteins between the ER and the Golgi, thus we hypothesize that it will also be required during murine development. To determine if Tmed2 is expressed in mice, we used RT-PCR, whole-mount in situ hybridization, and section in situ hybridization to examine the expression of Tmed2 between E6.5 and E10.5. Our analysis revealed that Tmed2 is expressed during these stages and that it showed specific temporal and spatial expression. This is the first study investigating the expression of a member of the TMED/p24 family during mammalian development.

INTRODUCTION

Vesicular trafficking between the endoplasmic reticulum (ER) and Golgi is mediated by coat proteins, COPI and COPII (Duden, 2003). COPI and COPII function in anterograde trafficking from the Golgi to the ER and retrograde trafficking from the ER to the Golgi respectively (Schimmoller et al., 1995; Stamnes et al., 1995; Belden and Barlowe, 1996; Sohn et al., 1996; Nickel et al., 1997; Dominguez et al., 1998). In this way, proteins are trafficked from the ER to the Golgi where they are modified and subsequently targeted to the cell surface or other destinations.
TMED family members are type I transmembrane proteins that cycle between the ER and the Golgi and are crucial to the functioning of the secretory pathway in eukaryotes. They are implicated in both the selection of properly folded cargo proteins and their trafficking between the ER and the Golgi (Blum et al., 1996; Sohn et al., 1996; Dominguez et al., 1998; Fullekrug et al., 1999; Gommel et al., 1999; Emery et al., 2000). TMED proteins have an N-terminal signal peptide, which is essential for their translocation into the ER (Blum et al., 1996). The GOLD domain, located in the N-terminal region, is thought to function in protein-protein interactions with particular cargo proteins in order to load them into vesicles (Anantharaman and Aravind, 2002). The TMED proteins contain a highly conserved repeat motif, which forms the coiled-coil region, and this is necessary for hetero-oligomerization of TMED proteins (Blum et al., 1996; Anantharaman and Aravind, 2002). Finally, at their C-termini, TMED proteins interact with COPI and COPII coat proteins (Blum et al., 1996; Dominguez et al., 1998).

The ten mammalian TMED proteins are divided into four subfamilies, α, β, δ, and γ, based on sequence homology (Dominguez et al., 1998). A member from each subfamily is required to form tetrameric complexes and TMED2 is the sole β family member in mammals.

In yeast, TMED2/Emp24p deletion mutants are viable but exhibit improper trafficking and secretion of proteins, and activation of the unfolded protein
response (Schimmoller et al., 1995; Belden and Barlowe, 1996; Marzioch et al., 1999; Belden and Barlowe, 2001). In *C. elegans*, TMED2 is important for Notch transport and quality control, such that if TMED2 and Notch are simultaneously mutated, the mutated Notch is improperly trafficked to the cell membrane, when ordinarily it would be degraded (Wen and Greenwald, 1999). In mammalian cells, TMED2 is necessary for proper trafficking, and is required for the resensitization of protease-activated receptor-2 (PAR-2) (Emery et al., 2000; Luo et al., 2007).

The specific function of the TMED family *in vivo* is still unclear. Seven *Tmed* genes have been identified in *Drosophila* and are expressed throughout development (Boltz et al., 2007). *Loj*, a *Drosophila*-specific *Tmed* gene that belongs to the γ subfamily, is required in the central nervous system for proper behavioral output, in order to have regular ovipositioning of eggs (Carney and Taylor, 2003; Boltz et al., 2007). In addition, *Tmed* genes are required for patterning of the *Drosophila* embryo (Bartoszewski et al., 2004). A family member, TMED10 is known to be required in mouse embryonic development. *Tmed10* null mice are embryonic lethal at E3.5, and heterozygous mice have Golgi apparatuses with compromised structural integrity (Denzel et al., 2000).

TMED2 and 10 are essential in mammals and *Drosophila* (Denzel et al., 2000; Springer et al., 2000; Carney and Taylor, 2003; Boltz et al., 2007). However, their expression has not been analyzed during mammalian development. Embryos homozygous for a point mutation in *Tmed2* die between E10.5 and E12.5 due to a
lack of proper placental development (unpublished results). In this paper, we report that Tmed2 is expressed as early as embryonic day (E)6.5 in the extraembryonic region of the embryo. Our data suggest that Tmed2 may be required for the normal development of the embryo and its placenta.

RESULTS AND DISCUSSION

To determine if Tmed2 is expressed during early embryonic development we performed RT-PCR analysis between E6.5 to E10.5 using primers that are complementary to exon 1 and exon 4 of Tmed2 (Figure 1A). A single 372 base pair product, which corresponds to the predicted major transcript of Tmed2 (Ensembl, ENSMUSG00000029390), was observed in the adult kidney and at all embryonic stages tested (Figure 1B).

We examined Tmed2 expression by whole-mount and section in situ hybridization to determine its localization in tissues and in specific cell types. In E6.5 embryos, Tmed2 was expressed in the ectoplacental cone (EPC) and the extraembryonic ectoderm (Figure 2A). The EPC will, ultimately, differentiate to form the spongiotrophoblast cells of the placenta (Cross, 2005), whereas the extraembryonic ectoderm, in combination with the mesoderm, eventually forms the chorion and the amnion. One day later, all E7.5 embryos expressed Tmed2 in the yolk sac, and the derivatives of the extraembryonic ectoderm, including the amnion as well as the chorion (Figure 2B, C). Tmed2 continued to be expressed in the derivatives of the ectoderm and thus at E8.5 we found specific Tmed2
expression in the chorion and giant cells (Figure 3A). These giant cells secrete factors necessary for the promotion of maternal blood flow to the implantation site (Cross, 2005). At E9.5, when the labyrinth layer, which is necessary for exchange of nutrients, of the placenta has started to form as a consequence of induced differentiation of the chorion by the allantois, Tmed2 was expressed in the cell derivatives of the chorion: the labyrinth layer, and the spongiotrophoblast layer (Figure 3B). At E10.5, as the labyrinth layer continues to develop, Tmed2 was expressed in the labyrinth layer and the spongiotrophoblast layer (Figure 3C). More specifically, it seems to be most highly expressed in the cells surrounding the maternal and fetal blood vessels (Figure 3D). Tmed2 was expressed in a subset of cells found in the maternal decidua as early as E9.5. These could be glycogen cells, which are proposed to differentiate from the EPC and spongiotrophoblast (Cross, 2005). Tmed2 was also expressed in the differentiated giant cells, which are derived from the EPC, in both E9.5 and E10.5 placentas (Figure 3B, C).

In the embryo proper, Tmed2 expression was first detected at E7.5 in late head fold stage embryos (Figure 2C). Tmed2 was expressed in the anterior mesoderm, and in the proximal end of the primitive streak (Figure 2C, D). The anterior mesoderm cells in this region are fated to form the heart and cranial structures later in development (Tam et al., 1997; Kinder et al., 1999).

In the developing head, Tmed2 was expressed in a number of structures including: the first and second pharyngeal arches, the fore-, mid- and hindbrain, the otic
placode/vesicle, and the optic placode/vesicle. As the pharyngeal arch appears, at E8.5, Tmed2 expression was highest in the anterior, distal end of the pharyngeal arch (Figure 4A, B) that is normally colonized by the neural crest cells. Tmed2 expression was detected in the first and second pharyngeal arches as well as the third pharyngeal pouch by E9.5 (Figure 4C, D). Section in situ revealed that this expression was in both paraxial and neural crest cell derived mesoderm. The pharyngeal arches will contribute to the skeletal components of the mammalian skull and jaw, and the third pouch will form the thymus. Tmed2 was expressed during the development of the eye and ear, such that at E8.5 expression was seen in the otic placode, and the optic placode (Figure 4A, B). The otic placode/vesicle will form the inner and middle ear and the optic placode/vesicle will form the lens. Additionally, Tmed2 was expressed throughout the developing brain, with expression extending dorsally from E8.5 to E10.5, such that expression was seen in the fore-, mid- and hindbrain at E10.5 (Figure 4F).

During limb bud formation, Tmed2 was expressed in both the mesoderm and ectoderm of the forming limbs. At E9.5, Tmed2 was expressed in the forelimb bud and the flank mesoderm where the hindlimb bud will emerge (Figure 4C, E, 5A). At E10.5, Tmed2 was expressed in both the fore- and hindlimb buds, with expression highest at the distal ends of the bud suggesting that it may be important during proximodistal extension of the limb buds (Figure 4F, 5B, C).
*Tmed2* was observed in the heart of late E9.5 embryos, in the distal portion of the left and right ventricle as well as the proximal part of the right atrium (Figure 4E, 5D, E). At E10.5, *Tmed2* was observed throughout the left and right ventricle, and in the periphery of the left and right atrium (Figure 5F, G). Section *in situ* at E10.5 show this expression in more detail. *Tmed2* was expressed in the endocardial cushion, the endocardium and the pericardium (Figure 5H, I). The endocardial cushion separates the heart tube into the left and right atrioventricular chambers and forms the valves of the adult heart. Thus, *Tmed2* may be important in the differentiation of the endothelial cells of the heart.

In conclusion, *Tmed2* is expressed at all stages examined and specific temporal and spatial expression in certain tissues. We observed *Tmed2* in extraembryonic tissues and it continues to be expressed in their derivatives. Our study is the first analysis of the expression pattern of a member of the TMED/p24 family during vertebrate embryogenesis. Based on our data, we propose that TMED2 has an important function in mouse development, and further study should provide insight into this specific function. A mutation in *Tmed2* in our lab is consistent with a requirement for this gene during the development of the embryo and its placenta (unpublished results). In the future it will be interesting to compare the expression patterns of other members of this family to determine if they are co-expressed during development.

**EXPERIMENTAL PROCEDURES**
Mouse lines and embryo dissection

CD1 (Charles Rivers) mice were used in this study. Days on which plugs were detected before noon, were considered to be E0.5. Pregnant females were dissected at various stages ranging between E6.5 and E10.5. E7.5 embryos were staged according to criteria described by Downs and Davies (Downs and Davies, 1993). Embryos were dissected in 0.2% BSA in PBS and were fixed overnight in 4% PFA in PBS for both whole-mount and section in situ hybridization. For RNA isolation, embryos were placed in Trizol (Invitrogen).

RT-PCR

RNA isolation was performed according to standard Trizol protocol. The RNA was treated with DNase prior to reverse transcription reaction. Reverse transcription was done using the Superscript III RT kit (Invitrogen). The following primers were designed to Tmed2 (Figure 1A) and Gapdh:

*Tmed2*F: 5’-GATGGGCTCATCTTTGAG-3’
*Tmed2*R: 5’-ACCAAAGGACCACCTGCTG-3’

*Gapdh*F: 5’-ATGACATCAAGGCTCTGTCGAG-3’
*Gapdh*R: 5’-CATACCAGGAAATGAGCTTGG-3’

RNA probe
The *Tmed2* probe was made by PCR amplification of exon 1 and 2 (Forward primer: CGAGGAGTGCTTCTTCGAG; Reverse primer: TCCCGGACTTCCATGTACTC) of the *Tmed2* gene from E8.5 decidua cDNA. The PCR amplicon size was confirmed by gel electrophoresis and then PCR purified (Qiagen) and subcloned using the dual promoter TA cloning kit (Invitrogen). Transformed bacteria were plated on ampicillin agar plates and were incubated at 37°C for 12-16 hours. Following miniprep (Qiagen) the clones were digested with *Eco*RI to confirm the presence of an insert and then sequenced (ABI POP6) using the M13 reverse primer.

The TOPO TA plasmid was linearized with *Bam*HI and using the T7 promoter the antisense probe was created and labeled with dioxigenin (Roche). For the sense probe, the plasmid was linearized with *Xba*I and the Sp6 promoter was used to generate the sense transcript. Labeling was followed with an RNA precipitation.

**Whole-mount in situ hybridization**

Embryos went through a series of methanol dehydration and rehydration steps, followed by whole-mount *in situ* hybridization according to standard protocol (Wilkinson, 1992). Embryos were examined at the following stages: E6.5 (n = 2), E7.5 (n = 5), E8.5 (n = 8), E9.5 (n = 9), E10.5 (n = 7), E8.5 decidua (n = 4).

**In situ** hybridization on sections
Embryos were embedded in paraffin and sectioned at 5µm, after which *in situ* hybridization on sections was performed according to standard protocols (Neubuser et al., 1995). Slides were counterstained with nuclear fast red (Sigma).
ACKNOWLEDGMENTS

We would like to thank Dr Aimee Ryan, Dr. Jacek Majewski, Michelle Collins, Didem Sarikaya, Nicholas Haddad, and Nathalie Magnus for their critical comments on this manuscript.
**Figure 1: Tmed2 is Expressed between E6.5 and E10.5** Expression of Tmed2 mRNA by RT-PCR between E6.5 and E10.5. **A:** Schematic showing the four exons of Tmed2 mRNA, as predicted by Ensembl. Arrowheads show where primers used in the RT-PCR bind to Tmed2. Bar shows where RNA probe used in whole-mount *in situ* hybridization and section *in situ* hybridization bind on the predicted mRNA. **B:** Expression of Tmed2 and Gapdh during embryogenesis. Lanes 1-9 are RT+ and lanes 10-18 are the RT- controls. 1 & 10, E6.5; 2 & 11, E7.5; 3 & 12, E8.5; 4 & 13, E9.5; 5 & 14 E10.5; 6 & 15 E8.5 decidua; 7 & 16 E9.5 placenta; 8 & 17, E10.5 placenta; 9 & 18, adult kidney. Gapdh, glyceraldehyde 3-phosphate dehydrogenase, a housekeeping gene was used as an internal control.
**Figure 2: Tmed2 is Expressed in the Extraembryonic Region in E6.5 and Early E7.5 Embryos**

Tmed2 mRNA at E6.5 and E7.5 was detected by whole-mount *in situ* hybridization. **A:** Lateral view of E6.5 embryo. **B:** Lateral view of late bud stage E7.5 embryo. **C, D:** Lateral and anterior view of the same late headfold stage E7.5 embryo. epc, ectoplacental cone; exe, extraembryonic ectoderm; ce, chorionic ectoderm; ys, yolk sac; am, amnion; mes, mesoderm; ps, primitive streak; nf, neural folds; ch, chorion.
Figure 3: *Tmed2* is Expressed in the Labyrinth Layer, the Spongiotrophoblast Layer, and in Giant Cells Expression of *Tmed2* in the developing placenta by whole-mount *in situ* hybridization and section *in situ* hybridization. 

**A:** E8.5 decidua. **B:** Sagittal section through E9.5 placenta. **C:** Sagittal section through E10.5 placenta. **D:** Sagittal section through E10.5 placenta (white and black arrows show fetal and maternal blood vessels respectively). gc, giant cells; ce, chorionic ectoderm; sp, spongiotrophoblast; lab, labyrinth layer; all, allantois; dc, decidua.
Figure 4: *Tmed2* is Widely Expressed in all of the Embryonic Lineages

Expression of *Tmed2* mRNA between E8.5 and E10.5 by whole-mount in situ and section in situ hybridization. **A, B:** Lateral view of E8.5 embryo. **C, D, E:** Lateral view of E9.5 embryo. The inset in C shows absence of signal with a sense probe at E9.5. **F:** Lateral view of E10.5 embryo. tb, tailbud; fb, forebrain; pa, pharyngeal arch; mb, midbrain; opp, optic placode; op, otic placode; opv, optic vesicle; ov, otic vesicle; pp, pharyngeal pouch; flb, forelimb bud; ht, heart; sm, somites; hb, hindbrain; hlb, hindlimb bud.
**Figure 5: Tmed2 Expression is Highest in the Distal Region of the Growing Limb and in a Subset of Cardiac Cells** Expression of *Tmed2* in the limb buds and hearts of E9.5 and E10.5 embryos by whole-mount *in situ* hybridization. **A:** Lateral view of E9.5 forelimb bud. **B:** Lateral view of E10.5 forelimb bud. **C:** Lateral view of E10.5 hindlimb bud. **D, E:** Lateral view of E9.5 heart. **F, G:** Lateral view of E10.5 heart. **H, I:** Section *in situ* on a sagittal section of E10.5 heart. **J:** Sagittal section showing absence of signal with a sense probe at E10.5. lv, left ventricle; la, left atrium; rv, right ventricle; ra, right atrium; oft, outflow tract; ec, endocardial cushion; en, endocardium; pc, pericardium.
REFERENCES


**Objective 3: Rationale**

Data from studies in yeast has shown that when *Tmed2*/Emp24p is knocked out, there is an accumulation of proteins indicative of the activation of the unfolded protein response due to the presence of ER stress. It is unknown whether ER stress and activation of the UPR are an aspect of the 99J phenotype in mice; therefore, we sought to determine whether there is an ER phenotype or evidence of the activation of the UPR in the 99J homozygous embryos and placentas.

**Objective 3: ER Stress**

**TEM**

In order to investigate whether there was a cellular phenotype specific to the 99J mutation associated with a changed appearance in the ER or Golgi, transmission electron microscopy (TEM) was performed on E9.5 placenta samples from both wildtype and homozygous mutant placentas. The TEM demonstrated that the ER membranes were not detected in the homozygous mutants (Figure 11).

**ER Stress**

Due to the phenotype observed in yeast when *Tmed2* is deleted, I first checked to see whether the only known cargo protein of TMED2 in mammals, Protease Activated Receptor-2 (PAR-2), is expressed during mouse development. PAR-2 is modified in the Golgi and subsequently trafficked to the plasma membrane. It is involved in cell proliferation and survival pathways (Luo et al., 2007). However, *Par-2* is not expressed at relevant stages in the mouse embryo
and placenta by RT-PCR indicating that it may not be a cargo protein of TMED2 during mouse development (Figure 12).

I next examined whether the homozygous mutant embryos were undergoing ER stress. From data previously generated in the lab, we were aware that there was no upregulation in the levels of chaperone proteins by Western Blot in the mutants compared to the wildtype (Li Luo; Figure 13). To confirm this, I performed RT-PCR using primers to a gene whose spliced isoform is only present in stressed cells and tissues, Xbp1. Xbp1 is present at low levels in unstressed cells and tissues; however, 26 nucleotides are spliced out when the UPR pathway is activated. This spliced form creates a new read-through transcript which is translated into a larger protein than the unspliced form. An established way to detect ER stress is to perform an RT-PCR to detect the smaller, spliced transcript. In order to show the different bands more clearly, I digested the PCR products with PstI, a restriction site which is not found in the spliced isoform of Xbp1 (Figure 14A). Thapsigargin is a drug which causes in the depletion of calcium from the ER thus resulting in ER stress. Primary mouse embryonic fibroblasts (PMEFs) treated with thapsigargin show a prominent upper band, which corresponds to the spliced undigested form of Xbp1. This upper band is not observed in the wildtype, heterozygous, and mutant embryos, which indicates that these samples are not experiencing ER stress and mounting the UPR (Figure 14B).

**Objective 3: Conclusion**
Although we were unable to distinguish ER membranes in the 99J homozygous placentas by TEM, activation of the UPR was not detected by RT-PCR and Western blot analysis.
**Figure 11: ER Membranes are not Easily Distinguished in the Tmed2^{99J/99J} Placentas**

Transmission electron microscopy images showing cells from both wildtype (left panels) and mutant (right panels) E9.5 placentas. There are distinguishable ER membranes in the wildtype cells (labeled N+magnification), some of which are indicated by an arrow, but no distinguishable ER membranes in the mutant samples (labeled M+magnification).
Figure 12: Par-2 is not Expressed in the Embryo or Placenta The expression of Par-2 was examined by RT-PCR and it was not expressed in the embryo or placenta at the stages examined, but was expressed in our positive controls: trophoblast stem cells (TS) and adult kidney. TS, trophoblast stem cells; Par-2, protease-activated receptor 2; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.
Figure 13: Chaperone Proteins, GRP78 and GRP94 are not Upregulated in the Tmed2^{99J/99J} Embryos A Western Blot was performed on homozygous mutant, heterozygous, and wildtype E9.5 embryos using antibodies against two chaperone proteins known to be upregulated during ER stress and activation of the UPR: GRP78 and GRP94 (Li Luo). The levels of these proteins were not increased in the homozygous mutants or heterozygotes.

(Jerome-Majewska et al., 2008)
**Figure 14: The Tmed2^{99J/99J} Embryos do not have Upregulated Levels of Spliced Xbp1**

**A.** Schematic showing that the spliced form of Xbp1, which is upregulated during ER stress lacks a PstI restriction site. **B.** 1, PMEF; 2, PMEF treated with thapsigargin; 3, wild type E11.5 embryo; 4, wild type E10.5 embryo; 5, wild type E10.5 embryo; 6, heterozygous E10.5 embryo; 7, mutant E10.5 embryo; 8, mutant E10.5 embryo; 9, mutant E10.5 embryo; 10, mutant E10.5 embryo; 11, mutant E10.5 embryo; 12, mutant E10.5 embryo. The upper band in lanes 1 and 2 of Xbp1 denote the spliced form of Xbp1 which is not digested by PstI. The lower two bands are the unspliced, digested forms of Xbp1.
B.

Unspliced $Xbp1$
Spliced $Xbp1$
Spliced $Xbp1$

(Jerome-Majewska et al., 2008)
DISCUSSION

Only the longest transcript of Tmed2 is affected by the 99J mutation

The data obtained from my Northern Blot analysis show that although there are three Tmed2 transcripts, only one of these transcripts includes the first exon of the gene. This information is consistent with ESTs published on Ensembl. However, the Northern blot analysis has also shown that only two of the transcripts are of the predicted size, suggesting that either different transcripts are present during the development of the embryo and placenta, or the information found on Ensembl is incorrect. It is important to investigate the sequences of these transcripts and to map where each splicing event is occurring in the gene. It would also be of great interest to determine the expression patterns of the other transcripts in order to be able to investigate whether the transcripts are expressed in different tissues, which together show the expression pattern I have described.

It is important to note that since only the largest transcript includes exon 1 of the gene, the 99J mutation will only directly affect this transcript.

Tmed2 is expressed during development

The expression pattern of the gene was somewhat surprising, as we were expecting a more widespread expression pattern due to the function of the gene. Instead, Tmed2 is enriched in certain tissues in comparison to others, suggesting that its function is not required in every cell of the organism. It is possible that TMED2 is involved in trafficking a necessary cell surface receptor from the ER to the Golgi, so that it subsequently localizes to the plasma membrane. In the cells
that lack TMED2, this cannot happen, and so the cell is unable to respond to signals in its extracellular environment, thereby resulting in a phenotype that is embryonic lethal. Data from cells and yeast show that TMED2 and TMED10 interact to form heteromeric complexes, which raises the question of why the \( Tmed10^{-/-} \) embryo would die so early in development, and the \( Tmed2^{99J/99J} \) embryo would die so much later. This could mean that TMED10 is able to form other complexes to compensate for the lack of TMED2. Or it could even simply be explained by their expression patterns, early in development. The expression pattern of \( Tmed10 \) has not been described, although we know that it is also expressed between E6.5 and E10.5 (Appendix II). If \( Tmed10 \) is expressed earlier than \( Tmed2 \) this would indicate that it has a function independent of \( Tmed2 \), and could result in an earlier embryonic lethality.

**TMED2 is required for the integrity of the ER membrane**

The results of the TEM suggest that TMED2 may be required for proper formation of the ER membrane; however, it would be more conclusive if this phenotype was assessed using nano-gold staining for an ER resident protein. This would confirm that there are in fact no ER membranes present in homozygous mutant cells. In addition, it is also important to determine whether the ER phenotype is present as secondary effect, rather than a primary one by performing TEM on embryos earlier in development, before an embryonic phenotype is seen, i.e. at E7.5.
"Tmed2^{99J/99J}" mutants are not undergoing ER stress and mounting the UPR

Finally, although in yeast, the \textit{Tmed2} knock-outs exhibited activation of the UPR due to ER stress, the 99J embryos in question do not seem to be activating the UPR in response to ER stress. This begs the question of whether the UPR is activated earlier, and has now resolved its source of ER stress. In this case, an accumulation of proteins in the ER due to a lack of trafficking in the mutants that do not have TMED2, can act as a source of stress to the cell. The cell then activates the UPR, and the upregulation of chaperone proteins as well as proteins involved in degradation subsequently deal with the accumulation. Alternatively the cells that are undergoing stress may undergo apoptosis. The former would resolve the source of ER stress and the latter would conclude the stress response. In both cases the UPR would no longer be active. In order to determine whether I have missed this activation, the most appropriate experiments would be to perform the \textit{Xbp1} RT-PCR at E7.5, before the 99J phenotype is apparent and to perform TUNEL assays on early embryos and placentas.
### Table 1: Primer Sequences

<table>
<thead>
<tr>
<th>PRIMERS</th>
<th>SEQUENCES</th>
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<tbody>
<tr>
<td>Tmed2in_L</td>
<td>GATGGGCCTCATCTTCGAG</td>
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<tr>
<td>Tmed2in_R</td>
<td>ACCAAAGGACCACTCTGCTG</td>
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<tr>
<td>Exon2L</td>
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<td>Exon3L</td>
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<td>Exon4L</td>
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<tr>
<td>Exon4R (3’UTR)</td>
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<tr>
<td>Tran1L</td>
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<tr>
<td>Tran1R</td>
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<td>Tmed10F</td>
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<td>Tmed10R</td>
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REFERENCES


APPENDIX I: RESTRICTION MAPPING AND SEQUENCING

Plasmid:

We purchased a Tmed2 clone in a pCMVSPORT6 plasmid from Invitrogen. First, I designed several primers to sequence the plasmid in order to determine whether the sequence is identical to that recorded on Ensembl and NCBI. Primers were designed to encompass the entire gene, and eight of these primers were used for sequencing in addition to M13 forward and reverse primers. Sequencing was done by the McGill University Genome Center. Once the sequences were generated, they were pieced together. A BLAST was performed and the Tmed2 sequence generated was 100% identical to that on Ensembl and NCBI. Additionally, we found that Tmed2 is inserted in the 3’ to 5’ direction in pCMVSPORT6, upstream of the Mlu site.
**Internal primers used for sequencing:**

<table>
<thead>
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<th>PRIMERS</th>
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<td>BEGR</td>
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<td>CH1L</td>
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<tr>
<td>ENDL</td>
<td>CCTGGTCTTCAGTGTCAGCA</td>
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</table>
**Restriction Mapping**

Following this, I performed restriction digests in order to map the restriction sites on the gene. I digested the plasmid with the following enzymes: *NcoI, SacI, XbaI, ApaI, EcoRV, BglII/SalI, SacI/KpnI, NcoI/SacI, NcoI/BglII, NcoI/XbaI, SacI/XbaI, ApaI/EcoRI, NspI, BstXI, and NcoI/SalI.*
**Restriction Map**

5'  |  NcoI  |  BglII  |  SacI  |  ApaI  |  EcoRV  |  3'

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= 100 base pairs
**APPENDIX II: Tmed10 EXPRESSION**

*Tmed10 expression coincides with Tmed2 expression:*

I investigated the expression of *Tmed10* since TMED10 is thought to form a complex with TMED2. To do this I performed RT-PCR between E6.5 and E10.5 as well as on E8.5 decidua and E9.5 and E10.5 placentas. *Tmed10* is expressed in all the stages examined, coinciding with *Tmed2* expression.

PCR conditions: 94°C x 3’, (94°C x 30’’, 54°C x 40’’, 72°C x 30’’) x 35, 72°C x 5’.

<table>
<thead>
<tr>
<th>RT+</th>
<th>RT-</th>
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<tbody>
<tr>
<td>E6.5</td>
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</tr>
<tr>
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<tr>
<td>p10.5</td>
<td>p10.5</td>
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<tr>
<td>kid</td>
<td>kid</td>
</tr>
</tbody>
</table>

[Image of RT-PCR results]
APPENDIX III: Tmed2 Expression

The whole mount *in situ* embryos were subsequently embedded and sectioned using a cryostat, in order to investigate Tmed2 expression more closely and ensure that the expression patterns observed were real and not due to trapped probe. At E6.5, expression is, as expected, not in every cell of the extraembryonic tissue, and this can be seen in the whole mount with expression most concentrated in the center of the ectoplacental cone and extraembryonic ectoderm. The black arrow shows cells with Tmed2 expression and the white arrows shows cells without Tmed2 expression. At E8.5, Tmed2 is highly expressed in the forebrain (black arrow), which is where it was seen by whole mount *in situ* hybridization. Finally, at E9.5, Tmed2 is expressed throughout the tail (black arrow), indicating that the expression seen by whole mount is real.