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The trafficking protein Tmed2/p24β_1 is required for morphogenesis of the mouse embryo and placenta

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ABSTRACT

During vesicular transport between the endoplasmic reticulum and the Golgi, members of the TMED/p24 protein family form hetero-oligomeric complexes that facilitate protein-cargo recognition as well as vesicle budding. In addition, they regulate each other's level of expression. Despite analyses of TMED/p24 protein distribution in mammalian cells, yeast, and *C. elegans*, little is known about the role of this family in vertebrate embryogenesis. We report the presence of a single point mutation in *Tmed2/p24*β_1 in a mutant mouse line, 99J, identified in an ENU mutagenesis screen for recessive developmental abnormalities. This mutation does not affect *Tmed2/p24*β_1 mRNA levels but results in loss of TMED2/p24β_1 protein. Prior to death at mid-gestation, 99J homozygous mutant embryos exhibit developmental delay, abnormal rostral–caudal elongation, randomized heart looping, and absence of the labyrinth layer of the placenta. We find that *Tmed2/p24*β_1 is normally expressed in tissues showing morphological defects in 99J mutant embryos and that these affected tissues lack the TMED2/p24β_1 oligomerization partners, TMED7/p24γ_3 and TMED10/p24δ_1. Our data reveal a requirement for TMED2/p24β_1 protein in the morphogenesis of the mouse embryo and placenta.

Introduction

Vesicular transport mediates molecular trafficking between the various membrane-bound compartments in a cell and thus underlies all major cellular activities. During vesicular transport members of the *Trans*membrane *e*mp24 *d*omain (TMED) or p24 protein family of trafficking proteins regulate protein-cargo selection and vesicle budding ((Carney & Bowen, 2004); (Kaiser, 2000); (Strating & Martens, 2009)). Transmembrane and secreted proteins synthesized in the endoplasmic reticulum (ER) depend on vesicular transport for proper localization to the Golgi where they are further modified before being transported to their final destinations. Properly folded proteins synthesized in the ER interact with adaptors or chaperones, such as TMED/p24 proteins, which enable them to be packaged into *co*at *p*rotein (COP) II vesicles at ER exit sites ((Bremser et al., 1999); (Gurkan et al., 2006)). After budding off the ER membrane, the vesicles move towards the Golgi via bulk flow or along microtubules (Cai et al., 2007). ER-resident proteins delivered to the Golgi along with cargo proteins are re-packaged into COPI vesicles and returned to the ER. Secretory cargo proteins deposited by COPII vesicles are transported through the Golgi stacks by COPI vesicles or by a process of cisternal maturation to the plasma membrane, endosomal/lysosomal system, or extracellular space.

Members of the TMED/p24 family are structurally related, sharing four distinct functional domains. A short signal sequence targets them to the ER membrane during translation; an N-terminal Golgi dynamics (GOLD) domain carries out cargo recognition; a coiled-coil domain mediates interactions between family members; and a short cytoplasmic tail contains conserved motifs for binding to coat complexes in COPI and COPII vesicles ((Anantharaman & Aravind, 2002); (Bethune et al., 2006); (Blum et al., 1999); (Bremser et al., 1999); (Carney & Bowen, 2004); (Dominguez et al., 1998); (Goldberg, 2000); (Lavoie et al., 1999); (Sohn et al., 1996)). Members of the TMED/p24 family fall into four subfamilies based on shared protein identity: α, β, δ and γ. Although these subfamilies are conserved in all animals and fungi, species-specific duplications and/or losses have resulted in varying numbers of genes in each TMED/p24 subfamily ((Bouw et al., 2004); (Carney & Bowen, 2004); (Dominguez et al., 1998); (Strating & Martens, 2009); (Strating et al., 2009)). Ten *Tmed/p24* genes are present in mammals: five in the γ subfamily, *Tmed1/p24*γ, *Tmed3/p24*γ_4, *Tmed5/p24*γ_2, *Tmed6/p24*γ_5, and *Tmed7/p24*γ_3; three in the α subfamily, *Tmed4/p24*α_3, *Tmed9p24*α_2 and *Tmed11/p24*α_1; one in the δ subfamily, *Tmed10/p24*δ; and one in the β subfamily, *Tmed2/p24*β_1 (Strating et al., 2009). TMED proteins, which are herein referred to by their assigned MGI nomenclature, are

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reported to exist as monomers, dimers, oligomers or hetero-oligomers ((Barr et al., 2001); (Carney & Bowen, 2004); (Jenne et al., 2002); (Luo et al., 2007); (Marzioch et al., 1999)). According to other experimental evidence, hetero-oligomers comprising one member of each of the four subfamilies form the functional units required for vesicular transport ((Blum et al., 1999); (Marzioch et al., 1999)).

Genetic and biochemical experiments reveal that interactions between TMED proteins regulate their stability: knockdown or deletion of one TMED protein led to decrease or loss of expression of TMED proteins from different subfamilies ((Blum et al., 1999); (Carney & Bowen, 2004); (Denzel et al., 2000); (Fullekrug et al., 1999); (Marzioch et al., 1999); (Takida et al., 2008); (Wen & Greenwald, 1999)). A null mutation in *Tmed10* resulted in developmental arrest before blastocyst formation and decreased expression of two interacting TMED proteins, TMED9 and TMED3, in livers of heterozygous mice (Denzel et al., 2000). In both yeast and mammalian cell lines, TMED2, the sole member of the β subfamily, is found in a complex containing TMED10 and/or TMED7, as well as TMED9 and it is required for their stability ((Barr et al., 2001); (Fullekrug et al., 1999); (Jenne et al., 2002); (Marzioch et al., 1999)).

Members of the TMED family localize to membranes of the ER, ERGIC (endoplasmic reticulum-Golgi intermediate compartment) and *cis*-Golgi as well as to COPI and COPII vesicles. Biochemical and genetic experiments demonstrate that TMED proteins bind to both COPI and COPII proteins and likely function in anterograde and retrograde transport between the ER and the Golgi ((Bethune et al., 2006); (Bremser et al., 1999); (Dominguez et al., 1998); (Goldberg, 2000)). In yeast, mutations of the *Tmed2* homolog, emp24, result in delayed maturation of Gas1p, a GPI-anchored protein, and defective transport of invertase, a soluble secreted protein ((Marzioch et al., 1999); (Muniz et al., 2000)). In mammalian cells, reduction of TMED10 levels by RNAi delayed trafficking of GPI-anchored proteins to the plasma membrane (Takida et al., 2008). Thus, TMED proteins appear to be required in both yeast and mammalian cells specifically for movement of GPI-anchored proteins to the plasma membrane.

In addition to impairing anterograde protein trafficking, mutations in TMED proteins trigger the ER stress-associated unfolded protein response. Loss of TMED proteins in yeast activates splicing of XBP1 pre-mRNA, leading to synthesis of the transcription factor that regulates the unfolded protein response. Loss of TMED also resulted in increased expression and abnormal secretion of Kar2, the yeast orthologue of *Hspa5*/*Grp78*, an ER-resident chaperone belonging to the heat shock protein 70 (HSP 70) family (Belden & Barlowe, 2001). Similarly, in mammalian cells knockdown of TMED4 led to upregulation of HSP 70 and to decreased apoptosis (Hwang et al., 2008). In *Drosophila*, mutation of Logjam, the *Tmed3* homolog, caused activation of the NF-κB pathway without the associated splicing of *Xbp1* (Boltz & Carney, 2008). Thus, TMED proteins may modulate multiple cellular stress pathways.

In *C. elegans*, a screen for suppressors of *lin-12*/*Glp* alleles encoding Notch receptors with reduced activity, identified mutations in the *C. elegans Tmed2* homolog, *sel-9* (Wen & Greenwald, 1999). All of the *lin-12*/*Glp* alleles suppressed by *sel-9* mutations carried missense mutations in their extracellular domain. Whereas in wild type strains altered Notch proteins accumulate within the cell, in the *sel-9* mutants altered Notch assembled at the cell membrane where it was functional (Wen & Greenwald, 1999). Trafficking of wild type Notch/Glp did not require *sel-9*; thus Tmed2 likely functions in quality control, specifically selecting defective Notch/Glp as cargo. Studies in primary rat astrocytes and human endothelial kidney cells found that TMED2 interacts with PAR-2, a G protein-coupled receptor that is activated at the plasma membrane by protease cleavage (Luo et al., 2007). Downstream signaling triggered by PAR-2 activation released TMED2-bound PAR-2, freeing it to traffic from the Golgi to the plasma membrane. In this case, TMED2-cargo selectivity appears to regulate post-Golgi protein trafficking.

Here we report the identification of a null allele of *Tmed2* in an N-ethyl N-nitrosourea (ENU) screen for recessive mutations perturbing the morphology of the developing mouse embryo. The mutation was discovered in a mouse line designated 99J and the mutant allele named *Tmed2*^{99J} (herein referred to as 99J). 99J homozygous mutant embryos exhibit developmental delay by E8.5, fail to undergo embryonic turning, and display posterior truncations, abnormal heart looping, and absence of the labyrinth layer of the placenta. The mutant embryos are reabsorbed by mid-gestation. Using positional cloning and sequencing we determined that the *Tmed2*^{99J} allele carries a G:C to T:A transversion in the first exon, generating an alanine to glutamic acid substitution in the TMED2 signal sequence, blocking the production of any TMED2 protein. Failure to complement a gene trap allele of *Tmed2* confirmed that the mutation in *Tmed2*^{99J} underlies the phenotypic defects. The levels of TMED7 and TMED10 protein were decreased in 99J heterozygous embryos and absent in the homozygous mutants embryo, revealing that TMED2 regulates the stability of these γ and δ subfamily members. The mid-gestation defects of *Tmed2*^{99J} suggest that the cargos selected and trafficked by TMED2 participate in the morphogenetic processes of early development.

Materials and methods

Mouse strains

The 99J line was generated by ENU mutagenesis of C57BL/6J mice ((Anderson, 2000); (Garcia-Garcia & Anderson, 2003); (Kasarskis et al., 1998)) and backcrossed to C3HeB/FeJ females. The PST809 cell line, with insertion of a gene trap cassette in the 3rd intron of *Tmed2*, *Tmed2*^{GT}, was identified on ENSEMBL (http://baygenomics.ucsf.edu/). The insertion was sequenced and verified before injection into C57 blastocysts. The chimeric offspring were bred to C57BL/Crc females and heterozygous F1 (C57/129) mice were bred to 99J heterozygous carriers for complementation analysis. CD1 (Charles Rivers, Canada) females mated to CD1 male were used to generate embryos for wholemount in situ hybridization. All mouse breedings and manipulations were performed in accordance with the Canadian Council on Animal Research. To generate embryos, females were placed with a male overnight and checked for the presence of a vaginal plug in the morning. The day that a plug was detected was considered embryonic day (E) 0.5. Mice and embryos from the 99J ENU line were genotyped by PCR with primers to D5MIT65 and D5MIT213. Mice and embryos from the Pst809 gene trap line were genotyped with primers from JaxMice that recognize the LacZ gene or with primers which were designed to distinguish the wild type *Tmed2* allele from the gene trapped allele. Primers *Tmed2*In4F and *Tmed2*In4R amplified the wild type *Tmed2* allele, and primers *Tmed2*In4F and Genetrap1R amplified the gene trap allele.

*Tmed2*In4F: *AAGTGCACAGCTGAGTGGT*
*Tmed2*In4R: *CACAGTGTCTGACCCCCTTT*
Genetrap1R: *AAGGGTCTTTGAGCACCAGA.*

Mapping of the 99J mutation

The mutation was mapped using linkage to flanking simple sequence length polymorphism (SSLP) markers from the MIT database or new markers that we generated (http://mouse.ski.mskcc.org/). Exons, splice site acceptors and splice site donors of 35 genes in the 99J minimal region were sequenced at the McGill Genome Center. Genomic DNA from 99J homozygous mutant embryos ($n=6$), carrier mice ($n=2$), and two wild type strains C3H/HeJ ($n=1$) and C57bL/6J mice ($n=1$) were analyzed with SeqMan II (DNASTAR).