O-fucosylation of thrombospondin type 1 repeats restricts epithelial to mesenchymal transition (EMT) and maintains epiblast pluripotency during mouse gastrulation

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Thrombospondin type 1 repeat (TSR) superfamilies members regulate diverse biological activities ranging from cell motility to inhibition of angiogenesis. In this study, we verified that mouse protein O-fucosyltransferase-2 (POFUT2) specifically adds O-fucose to TSRs. Using two Pofut2 gene-trap lines, we demonstrated that O-fucosylation of TSRs was essential for restricting epithelial to mesenchymal transition in the primitive streak, correct patterning of mesoderm, and localization of the definitive endoderm. Although Pofut2 mutant embryos established anterior/posterior polarity, they underwent extensive mesoderm differentiation at the expense of maintaining epiblast pluripotency. Moreover, mesoderm differentiation was biased towards the vascular endothelial cell lineage. Localization of Foxa2 and Cer1 expressing cells within the interior of Pofut2 mutant embryos suggested that POFUT2 activity was also required for the displacement of the primitive endoderm by definitive endoderm. Notably, Nodal, BMP4, Fg8, and Wnt3 expression were markedly elevated and expanded in Pofut2 mutants, providing evidence that O-fucose modification of TSRs was essential for modulation of growth factor signaling during gastrulation. The ability of Pofut2 mutant embryos to form teratomas comprised of tissues from all three germ layer origins suggested that defects in Pofut2 mutant embryos resulted from abnormalities in the extracellular environment. This prediction is consistent with the observation that POFUT2 targets are constitutive components of the extracellular matrix (ECM) or associate with the ECM. For this reason, the Pofut2 mutants represent a valuable tool for studying the role of O-fucosylation in ECM synthesis and remodeling, and will be a valuable model to study how post-translational modification of ECM components regulates the formation of tissue boundaries, cell movements, and signaling.

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Introduction

Epidermal growth factor-like (EGF) and thrombospondin type 1 repeats (TSRs) can be modiﬁed by an unusual form of O-linked glycosylation called O-fucose (Hofsteenge et al., 2001; Luther and Haltiwanger, 2009; Moloney et al., 2000a). EGF repeats and TSRs are 40–60 aa cysteine-rich motifs found in a wide variety of cell surface and secreted proteins. TSRs are somewhat larger than EGF repeats, but both contain six conserved cysteines forming three disulﬁde bonds, although the disulﬁde bonding patterns for each are distinct (Luther and Haltiwanger, 2009). O-Fucose is added to EGF repeats by protein O-fucosyltransferase 1 (POFUT1) (Wang et al., 2001), while POFUT2 O-fucosylates TSRs (Luo et al., 2006a,b). Both enzymes are highly selective, modifying only properly folded repeats containing the appropriate consensus sequences (Luther and Haltiwanger, 2009). The similarity between Notch and Pofut1 mutant phenotypes in mice and Drosophila provides evidence that the O-fucose residues on the Notch receptor EGF repeats are essential for its function (Okaajiama and Irvine, 2002; Shi and Stanley, 2003). Cell biological and biochemical studies investigating the role of O-fucose on Notch also support this view (Okajima et al., 2003; Rampal et al., 2005; Sasamura et al., 2003; Stahl et al., 2008). Further evidence for the importance of O-fucose glycans in Notch function comes from the observation that Fringe, a known modulator of Notch function, functions by adding a [3-linked N-acetylgalcosamine to O-fucose on the EGF repeats of Notch (Luther and Haltiwanger, 2009; Bruckner et al., 2000; Moloney et al., 2000a,b).

POFUT2 is closely related to POFUT1, but modiﬁes TSRs that contain the consensus sequence: C-X2–3–[(S/T)]–C-X2–G, where the cysteines are the ﬁrst and second conserved cysteines in group 1 TSRs, or the second and third cysteines in group 2 TSRs (Table 1) (Hofsteenge et al., 2001; Luo et al., 2006a,b; Gonzalez de Peredo et al., 2002; Ricketts et al., 2007; Tan et al., 2002; Wang et al., 2007). O-Fucose on TSRs can be
Table 1

<table>
<thead>
<tr>
<th>TSR class</th>
<th>Mouse protein</th>
<th># Consensus sequences</th>
<th>Family</th>
</tr>
</thead>
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<td>Group 1</td>
<td>ADAMTS1–4, ADAMTS2 (Wang et al., 2009), ADAMTS6–10, ADAMTS12, ADAMTS13 (Duncan et al., 2007), ADAMTS14–20</td>
<td>1–12</td>
<td>A disintegrin and metalloproteinase with thrombospondin type I repeats (ADAMTS)</td>
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<td></td>
<td>ADAMTS15L1 (Wang et al., 2007, 2009), ADAMTS12–6</td>
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<td>ADAMTS-like</td>
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<td></td>
<td>BAIII-3</td>
<td>4</td>
<td>Brain-specific angiogenin inhibitor</td>
</tr>
<tr>
<td></td>
<td>Clp (Propedin) (Gonzalez de Peredo et al., 2002)</td>
<td>4</td>
<td>Complement Component</td>
</tr>
<tr>
<td></td>
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<td>1</td>
<td>Cartilage intermediate layer protein/nucleotide pyrophosphohydrolase</td>
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<td>1</td>
<td>Hemicentin</td>
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<td>HMCN1</td>
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<td>PAPLN (Paplin)</td>
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<td>THBS1 (TSP1) (Hofsteenge et al., 2001; Gonzalez de Peredo et al., 2002), THBS2 (TSP2)</td>
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<td>SSPO (Scop-spondin)</td>
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<td>THSD7a, THSD7b</td>
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<td>Thrombospondin, type I, domain-containing</td>
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<td>Group 2</td>
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<td>CCN (Cryn1, Ctgf, Nov)</td>
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<tr>
<td></td>
<td>SPON1 (S-spondin) (Gonzalez de Peredo et al., 2002)</td>
<td>4</td>
<td>Spondin</td>
</tr>
</tbody>
</table>

Underlined proteins indicate Human, mouse, or rat homologues verified to contain O-fucosylated TSRs. Mouse proteins containing TSRs were identified by searching SMART database (http://www.smart.embl-heidelberg.de/) with keyword TSP1 in both normal and genomic modes. Alternative protein names are listed in parentheses.

* Fifty-one proteins that contain the consensus sequence CX(2–5)S/TCX(2)G within TSRs are listed above as putative POFUT2 targets and are divided into groups 1 or 2 based on their TSR structures (Tan et al., 2002).

All animal work was conducted according to relevant national and international guidelines. Stony Brook University operates under Assurance #A3011-01, approved by the NIH Office of Laboratory Animal Welfare (OLAW). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) which follow all the guidance set forth in: Public Health Service Policy on Humane Care and Use of Laboratory Animals distributed by Office of Laboratory Animal Welfare, NIH; Animal Welfare Act and Animal Welfare Regulations distributed by United States Department of Agriculture; and Guide for the Care and Use of Laboratory Animals distributed by the National Research Council. Stony Brook University animal facilities are accredited with AAALAC International (Association for the Assessment and Accreditation of Laboratory Animal Care International).

Generation of Pofut2 expression constructs

Mouse POFUT2 cDNA was amplified by PCR from a mouse testis cDNA library (a kind gift from Dr. William J. Lennarz) with the primers. Forward: 5′-CATATCTAAAGCTTGGCCGCTCCTGCGTGC-3′; and Reverse: 5′-CATATATCTCGAGGGTAGTACCTCATTCTCCAGTGTCTGGTTGG-3′, and digested with Hind III and Xho I, and cloned into pcDNA4/TO/myc-His vector (Invitrogen). Since this construct does not contain the Pofut2 stop codon, recombinant proteins will be expressed as myc and His tagged proteins (POFUT2-Myc-His). For the expression of untagged mouse POFUT2, the Pofut2 cDNA (including stop codon) was inserted between Hind III and Xba I sites of pcDNA4 (Invitrogen). To mutate the ERE motif (POFUT2/
E396A-myc-His), site-directed mutagenesis was carried out to replace dA at 1187 (nt) with dC.

**Transient transfection and purification of the myc- and 6× His-tagged POFT2 protein by Ni-NTA chromatography**

HEK293T cells were transiently transfected with the expression plasmids encoding full-length mouse Pofut2 with or without myc- and hexa-His-tags at its C-terminus (Pofut2-myc-His), or the empty plasmid pcDNA4 as control by Lipofectamine 2000 (Invitrogen). Culture medium (5 ml) of the transiently transfected cells was dialyzed against 50 mM Tris–HCl pH 7.4 containing 25% glycerol overnight. The dialyzed sample was mixed with 200 µl of the 50% slurry pre-equilibrated Ni–NTA agarose (QIAGEN), rotated at 4 °C for 1 h, and poured into the Poly-Prep Chromatography Columns (BIO-RAD). The resin was washed with 50 mM Tris–HCl pH 7.4 containing 25% glycerol and 0.5 M NaCl, and then with 50 mM Tris–HCl pH 7.4 containing 25% glycerol and 10 mM imidazole. The tagged proteins were eluted with 50 mM Tris–HCl pH 7.4 containing 25% glycerol and 250 mM imidazole. The imidazole in the sample was removed by gel filtration on Sephadex G50 (SIGMA). Protein concentration of the tagged protein was determined by Coomassie staining of samples fractionated on SDS-PAGE using BSA as a standard. After collection of the culture media, the transfected cells were harvested and homogenized in 50 mM Tris–HCl pH 7.4 buffer containing protease inhibitors, 10 mM MgCl₂, and 250 mM NaCl, by Polytron homogenizer for 15 s, and then incubated at 4 °C for 1 h. After the homogenate was centrifuged at 16,000 × g for 20 min, the supernatant was collected as cell extracts and kept at −80 °C until use. Protein concentration was determined by a BCA assay using BSA as a standard.

**Preparation of TSR and EGF repeats**

The third TSR from human thrombospondin 1 (TSP1-TSR3) was expressed in bacteria and purified as described previously (Luo et al., 2006b). The first EGF repeat of human factor VII was a kind gift of Dr. Yang Wang (Wang et al., 1996). Protein concentration was determined by a BCA assay using BSA as a standard.

**Preparation of tissue extracts from adult mice**

Tissues of 5-week-old wildtype and heterozygous male mice were dissected and homogenized in 0.5 ml (approximately 5 volume of tissue sample) of 50 mM Tris–HCl pH 7.4 buffer containing protease inhibitors, 10 mM MgCl₂, and 250 mM NaCl, by Polytron homogenizer for 15 s, and then incubated at 4 °C for 1 h. After the homogenate was centrifuged at 16,000 × g for 20 min and kept at −80 °C until use. Protein concentration was determined by a BCA assay using BSA as a standard.

**Protein O-fucosyltransferase assays**

POFT2 assays were performed with slight modification as previously described (Luo et al., 2006b). Briefly, a 10 µl reaction mixture contained 50 mM HEPES pH 7.0, 10 mM MnCl₂, 10 µM TSR, 2.9 µM (0.05 mcI/ml) GDP-[³H]fucose, 50 µM GDP-fucose, 0.5% NP-40, and 7 µl of tissue extracts. The POFUT2 assays were essentially the same with the POFT2 assays except that the factor VII EGF instead of TSR was used at a concentration of 10 µM as acceptor substrates. The reaction was incubated at 37 °C for 20 min and stopped by adding 900 µl of 100 mM EDTA pH 8.0. The sample was loaded onto a C18 cartridge (100 mg, Agilent). After the cartridge was washed with 5 ml of H₂O, the TSR or EGF repeat was eluted with 1 ml of 80% methanol. Incorporation of [³H]fucose into the TSR or EGF repeat was determined by scintillation counting of the eluates. Reactions without substrates were used as background control.

**Mice and genotyping**

The Pofut2<sup>Gt(RST434)Byg</sup> transgenic mice were generated with stem cell clone RST434 (BayGenomics) at the UC-Davis transgenic facility. For simplicity, we will refer to this allele as RST434 throughout the manuscript. For genotyping, three primers were designed: RST434-forward (GAGGCCGGGAGTACTGGGAT) matches sequence of Pofut2 exon 5, RST434-reverse1 (ATTCCTGCACTTCTTCC) matches the sequence of Pofut2 exon 6 that was deleted by the insertion of gene-trap vector, and RST434-reverse2 (GTTGCCAGAACCGAACTGAA) matches the En2 exon sequence in the gene-trap vector pgTOM1ps. RST434-forward and RST434-reverse1 were used to amplify the wildtype allele-specific band of 955 bp, whereas RST434-forward and RST434-reverse2 amplify the genetrap insertion-specific band of 1344 bp.

The Pofut2<sup>Gt(neo)(699Lex)</sup> transgenic mice were purchased from Lexicon Genetics Incorporated. For simplicity this allele will be referred to as 699Lex throughout the manuscript. For genotyping, 1197-upper (GATCTTAAGTTCCACGGAGACA) and LTR-rev2 (ATAAACCCCTTGAAGTGGCATC) were used to amplify the mutant allele band of 600 bp. 1197-upper and 1197-3′ (GCCCTACTGTA-TATTTAACGTC) were used to amplify the wildtype allele band of 314 bp. Mice heterozygous for both 699Lex and Bat-gal (699Lex/+: Bat-gal+/+) were generated by crossing the Pofut2<sup>Gt(neo)(699Lex)</sup> transgenic mice with Bat-gal transgene reporter (Maretto et al., 2003). The Bat-gal reporter gene was confirmed by PCR with lacZ-up (CGGTATGTCCTGCTGTGAGA) and lacZ-down (ACCACCACAGATA-GAGATT) that amplify 385 bp of the β-galactosidase cDNA.

**LacZ staining and histology**

Embryos in decidua were stained with X-gal as described (Hogan et al., 1994). Briefly, decidualia at E 6.5 and E 7.5 were fixed with 0.2% glutaraldehyde for 25 min and 30 min respectively followed by 3 times of rinses with detergent rinse (15 min for each rinse). The decidualia were then stained at 37 °C for 20 h. After staining, decidualia were rinsed in 0.1 M phosphate buffer pH 7.3 for 15 min followed by post fixation with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. Embryos were subsequently dissected out from decidualia, cleared in 80% glycerol, and photographed. For sectioning, either the embryo in decidua or isolated embryos were then embedded in paraffin and sectioned. The slides were mounted with Gel Mount (Sigma) for LacZ staining photography and photographed. The decidua were photographed. For sectioning, either the embryo in decidua or isolated embryos were then embedded in paraffin and sectioned. The slides were mounted with Gel Mount (Sigma) for LacZ staining photography and photographed.

**Whole-mount embryo in situ hybridization**

The in situ hybridization was carried out as previously described in (Shumacher et al., 1996). To reduce trapping in E 7.5 Pofut2 mutant embryos, tissues were perforated with a tungsten needle. For each gene analyzed, in situ hybridization was carried out with both sense and antisense probes. The cDNAs of Foxa2, Sox2, and Gscoid were amplified from E7.5 mouse embryo cDNA and were cloned into pBluescript SK′ (−) between Xho I and Not I sites. Primers used for cDNA amplification are listed in Supplementary Table 3. Other DNA constructs for probe preparation were kindly provided by Drs. Bernhard Herrman (T), Brigid Hogan (Bmp4), Janet Rossant (Kdr/flk1) and cmosedermian), Hans Scholer (Pou5f1), Stephen Frankenberg (Cubilin), Michael M. Shen (Nodal, Lefty1, and Lefty2), Gail Martin (Fgfr), Rosa Beddington (Cer1), Richard Behringer (Wnt3), Virginia Papaioannou (Tbx6), Nancy Speck (Runx1) and Thomas Gridley (Snail).

To genotype the in situ embryos, the embryos were rehydrated and the crosslinks were reversed in 200 mM NaOH at 60 °C overnight before
adding lysis buffer (200 mg/ml proteinase K, 10 mM DTT, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris–HCl pH 8.5, 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20). Embryo lysates were boiled for 5 min and used directly for PCR. Pofut2-probe-p (5’-GGACTCTAATAGCACCACAAAGAGGAGGAAGAACCAGAGGAGT-3’) and Pofut2-gen-down1 (5’-AAAGAACCCAGCTTCTGAGAGGAGA-3’) were used to amplify the wildtype band, and LacZ-up (5’-CCGTTGATCTGTCGCTTGAGGA-3’) and LacZ-down (5’-ACCACGCACAGTACAGATTCC-3’) were used to amplify the mutant band.

RT-PCR analysis

For Pofut2 expression at adult stage, total RNA from tissues of 5-week-old males was isolated with Trizol reagent (Invitrogen) and was reverse transcribed with Oligo(dt)12–18 primers. Primers used for the semi-quantitative PCR include: Pofut2 forward (5’-TGTAATCCGAGGACAGCAGTGAGT-3’) and Pofut2 reverse (5’-AAAGATGTCTCATGACGATGTCAG-TACGCAATCTTCCAGTGTGTG-3’) were used to amplify Pofut2. GAPDH reverse (5’-ACACACCTGTCGCTGGAATG-3’) and GAPDH forward (5’-TCCACACCTGTCGCTGGAATG-3’) were used to amplify the glyceraldehyde 3-phosphate dehydrogenase (Gadph) cDNA as standards for normalizing samples. Briefly, input of reverse transcription product of different tissues was adjusted to give equal amount of GAPDH PCR product within the linear range of PCR amplification. Subsequently, the adjusted amount of reverse transcription reaction was used for PCR using Pofut2 primers. For the expression of putative POFUT2 target proteins and matrix metalloproteinase-2 and -9, total RNA was isolated from E 7.5 wildtype embryos and was reverse transcribed with random primers (Invitrogen). PCR was carried out with gene specific primers (Supplementary Table 1). To avoid amplification of genomic DNA, the primer pairs span at least one intron. Positive control PCR was carried out with complementary DNA randomly transcribed from total RNAs of adult brain and spleen.

Real time PCR

Total RNA was isolated from E 7.5 homozygous mutant embryos, and E 7.5 wildtype plus heterozygous embryos. Total RNAs were then reverse transcribed using random primers (Invitrogen). Real time PCR was carried out using SYBER Green I Master Mix (Roche). GAPDH was used as endogenous control. The primers of genes tested are listed in Supplementary Table 2. No significant signal was detected when total RNA was used as template.

Immunohistochemistry

For Amnionless, embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Embryo sections (~7 μm) were stained with rabbit anti-mouse Amnionless (a gift from Dr. Elizabeth Lacy) followed by biotinylated anti-rabbit and Vectastain elite ABC kit (Vector laboratories). The peroxidase staining was subsequently developed by biotinylated anti-rabbit and Vectastain elite ABC kit (Vector laboratories). The peroxidase staining was subsequently developed with dianisidine staining as described by O’Brien (1961), with the exception of the anti-His antibody (His-probe (H-15), SantaCruz) (middle panel). POFUT2-myc-His expression levels were detected by Western blot of cell extracts and culture media using anti-His antibody (lanes 5 to 8) were assayed for TSR-specific O-fucosyltransferase activity of mouse POFUT2 (POFUT2-myc-His) and assayed its ability to O-fucosylate the third TSR (TSR3) of human thrombospondin 1 (TSP1) (Fig. 1). O-Fucosylation of TSP1–TSR3 increased linearly with an increasing amount of POFUT2-myc-His (Fig. 1A), donor substrate GDP-[3H]fucose (Fig. 1B), and with increasing amount of TSP1 (Fig. 1C). The POFUT2 ERE motif is essential for O-fucosyltransferase activity using TSP1–TSR3 as acceptor substrate (top panel). POFUT2-myc-His expression levels were detected by Western blot of cell extracts and culture media using anti-His antibody (His-probe (H-15), SantaCruz) (middle panel). β-actin expression provided a loading control (lanes 1 to 4) and the culture media (lanes 5 to 8) were assayed for TSR-specific O-fucosyltransferase activity using TSP1–TSR3 as acceptor substrate (top panel). POFUT2-myc-His expression levels were detected by Western blot of cell extracts and culture media using anti-His antibody (His-probe (H-15), SantaCruz) (middle panel). β-actin expression provided a loading control (bottom panel). Mouse POFUT2 has TSR-specific O-fucosyltransferase activity.

Mouse POFUT2 has TSR-specific O-fucosyltransferase activity

Recombinant Drosophila protein O-fucosyltransferase-2 (Ofut2) transfers fucose from GDP-fucose to a bacterially expressed TSR domain (Luo et al., 2006a). In addition, several mammalian cell lines contain endogenous TSR-specific O-fucosyltransferase activity (Luo et al., 2006a; Ricketts et al., 2007; Wang et al., 2007). To determine whether mouse POFUT2 behaves similarly, we expressed and purified POFUT2 (POFUT2-myc-His) and assayed its ability to O-fucosylate the third TSR (TSR3) of human thrombospondin 1 (TSP1) (Fig. 1). O-Fucosylation of TSP1–TSR3 increased linearly with an increasing amount of POFUT2-myc-His (Fig. 1A), donor substrate GDP-[3H]fucose (Fig. 1B), and with increasing amount of TSP1 (Fig. 1C). The POFUT2 ERE motif is essential for O-fucosyltransferase activity using TSP1–TSR3 as acceptor substrate (top panel). POFUT2-myc-His expression levels were detected by Western blot of cell extracts and culture media using anti-His antibody (His-probe (H-15), SantaCruz) (middle panel). β-actin expression provided a loading control (bottom panel). Mouse POFUT2 has TSR-specific O-fucosyltransferase activity.

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POFUT2 activity was detected in either culture medium or cell extracts. Secretion of some POFUT2 into the medium is observed upon disruption of the gene-trap providing a mechanism for disrupting the gene expression of POFUT2/E396A-myc-His compared to POFUT2-His. O-fucosyltransferase assays were carried out in vitro with an equal amount of purified protein. This assay, POFUT2-myc-His activity increased with an increasing concentration of TSP1–TSR3. In contrast, no activity was detected for POFUT2/E396A-myc-His. These results provided convincing evidence that the ERE motif is essential for the protein O-fucosyltransferase activity of mouse POFUT2.

Gene-trap disruption of Pofut2 abrogates TSR O-fucosylation

Our bioinformatic analysis identified 51 mouse TSR-containing proteins that could potentially be O-fucosylated by POFUT2 (Table 1). These TSR superfamily members are widely expressed and play multiple roles in cell culture and in the adult (Luther and Haltiwanger, 2009; Fiore et al., 2005; Hourcade, 2008; Jones and Riley, 2005; Kramerova et al., 2000; Leask and Abraham, 2006; Meiniel et al., 2003; Porter et al., 2005; Schubert et al., 2006; Shiratsuchi et al., 1997; Zhang and Lawler, 2007; Adams and Tucker, 2000). Similarly, we observed wide spread expression of Pofut2 in adult tissues (Supplementary Fig. 1A). To investigate the biological role of POFUT2 in mice, we characterized two Pofut2 gene trap insertions: Pofut2Gt(neo)699Lex (Fig. 2A) and Pofut2Gt(cre)(neo)699Lex (Fig. 2A) and wildtype (Fig. 2A and Supplementary Fig. 2A, respectively). The RST434 genetrap was identified in an insertional mutagenesis screen to identify components of or proteins traversing the secretory pathway (Mitchell et al., 2001). This is consistent with the prediction that POFUT2 resides in the endoplasmic reticulum (Luo et al., 2006b).

The RST434 insertion occurred within exon 6 and resulted in an in-frame fusion between Pofut2 (exons 1 through 5) and β-gal-neomycin (POFUT2–β-gal) (Fig. 2A, Supplementary Fig. 1B, and BayGenomics). Because the ERE motif essential for the POFUT2 activity is encoded by exon 9 (Fig. 2A), we predicted that the POFUT2–β-gal-neomycin fusion protein would lack TSR-specific O-fucosyltransferase activity. For this reason, the gene-trap provides a mechanism for disrupting Pofut2 gene function as well as a mechanism for reporting Pofut2 gene expression patterns. Consistent with this prediction, we detected half the activity of POFUT2 in RST434 heterozygous cells compared with parent cells (Fig. 2B). In contrast, no significant difference in POFUT1 activity was observed. POFUT2 activity was also reduced in extracts of adult tissues obtained from RST434 heterozygotes relative to wildtype animals, while POFUT1 activity was largely unaltered (Fig. 2C). Combined, these data provided evidence that the RST434 allele produced an enzymatically inactive protein.

Because the RST434 gene-trap insertion resulted in a transmembrane fusion protein that contained 235 amino acids of POFUT2 protein fused to β-geo, the formal possibility existed that the fusion protein retained residual function other than enzymatic activity. For this reason, we characterized a second gene-trap allele, 699Lex, produced by insertion of a retroviral vector VICTR48 into the first intron of Pofut2 (Supplementary Fig. 2A). Our RT-PCR analysis identified a fusion transcript including exon 1 of Pofut2 and neomycin phosphotransferase (Supplementary Fig. 2B). Tissues obtained from animals heterozygous for the 699Lex gene-trap insertion showed reduction in POFUT2 activity similar to that observed in RST434 animals (Supplementary Fig. 2C). Combined, these results provided convincing evidence that gene traps in both strains inactivate one copy of the Pofut2 gene. For this reason, we anticipated that RST434 and 699Lex Pofut2 insertions created null alleles and were valuable tools to determine the function of O-fucosylation of TSR-containing proteins.

Pofut2 is essential for organization of epithelia in pre-gastrula embryos

Consistent with the predicted importance of sugar modification for the function of TSR-containing proteins, no homozygous RST434 animals were obtained from heterozygous intercrosses (Table 2). In contrast, heterozygotes (RST434/+) and wildtype (+/+ ) animals were present in 2:1 ratio at weaning. Heterozygotes showed no overt pathologies and had similar lifespan to wildtype littersmates. These results provided evidence that RST434 disruption of Pofut2 results in either peri- or pre-natal lethality. To distinguish between these possibilities, we collected and genotyped embryos from intercrosses at mid-gestation (E 10.5) (Table 2). Heterozygotes and wildtype embryos were present at the expected 2:1 ratio. Although no homozygous embryos were detected, resorption sites were present at a frequency expected for the homozygotes. Since resorption sites are indicative of embryo implantation and subsequent failure, these data suggested that homozygous embryos died after implantation and before E 10.5, a time period during which gastrulation and early organogenesis occurs.

We took advantage of the Pofut2–β-geo fusion to characterize Pofut2 gene expression as well as identify homozygous mutants at the onset of gastrulation (E 6.5) (Fig. 2). RST434 homozygous embryos were distinguished from wildtype or heterozygote littersmates by the intensity of LacZ staining (Figs. 2D–F). We attributed the increased staining to the presence of two copies of the Pofut2–β-geo fusion and confirmed the genotype by PCR (Figs. 2D–I). LacZ activity, reflecting Pofut2 expression, was detected in both embryonic and extra-embryonic tissues (including extra-embryonic ectoderm, ectoplacental cone, and visceral and parietal endoderm) of heterozygotes and mutant embryos (Figs. 2E–F), with increased activity in the embryonic ectoderm. These data suggested that expression of Pofut2 was elevated in this tissue relative to that of the visceral and parietal endoderm or extra-embryonic ectoderm.

At E 6.5, RST434 homozygous embryos were distinguished subtly from wildtype and heterozygote littersmates by their rounder appearance, shortened proximal-distal axis, and constriction in the junction between the extra-embryonic ectoderm and the ectoplacental cone (Compare Figs. 2D and E with F). To evaluate the tissue composition of mutant embryos, we examined sagittal sections of embryos stained for LacZ activity (Figs. 2G and H) followed by staining with hematoxylin and eosin Y (Figs. 2G and H). At E 6.5, heterozygote littersmates had well-differentiated ectoplacental cone, and embryonic and extra-embryonic ectoderm surrounded by visceral and parietal endoderm (Figs. 2G, H). The composition of tissues in homozygous embryos appeared similar (Figs. 2H, I). However, the mutant embryo was rounder, the embryonic and extra-embryonic ectoderm appeared thickened and disorganized, and there was a constriction between the extra-embryonic ectoderm and ectoplacental cone. This constriction appears to result from abnormal arrangement of the parietal and/or visceral endoderm. Combined, these subtle differences suggested that POFUT2 activity was not
essential for specification of early cell lineages, epithelialization, and formation of the proamniotic cavity in the early post-implantation embryo, but was required for maintaining the organization of these tissues in the pre-gastrula embryo.

Pofut2 is required to restrict expression of signaling molecules early in gastrulation

To determine whether loss of Pofut2 was required for establishment of polarity or for the onset of gastrulation, we looked at gene expression in embryos isolated from RST434 heterozygous intercrosses at E 6.5 (Fig. 3). By E 6.5, the wildtype embryo established anterior posterior polarity and is poised to begin gastrulation (Fig. 3, left embryos). Tissue identity as well as embryo polarity is evidenced by localized expression of Cer1 and Lefty1 in the anterior visceral endoderm (AVE) (Figs. 3A and B), Nodal, Wnt3, Fgf8, T and Eomes in the primitive streak (Figs. 3C–D, F–H), and Eomes and Bmp4 in the extra-embryonic ectoderm (Figs. 3H, I). Wnt signaling activity (Fig. 3E) is similar to Wnt3 mRNA expression and was detected using the BAT-gal transgene in embryos from the 699Lex. Similarly, we detected asymmetric and tissue specific expression of Cer1, Lefty1, Nodal, Wnt3, Fgf8, T, Eomes and BMP4 and Wnt activity in all RST434.
Materials and methods). The relationship between these characteristics and genotype was verified by PCR (Materials and methods).

Wildtype homozygous littermates examined (Fig. 3, right embryo). Because correct localization of the AVE is essential for the posterior localization of primitive streak markers, we do not believe that the apparent thickening of the extra-embryonic endoderm layers observed at the distal tip of some Pofut2 mutant embryos (Figs. 3E, F, and I) results from the failure of the AVE cells to migrate, but rather results from physical constraints that prevent tissue expansion or movement. Combined, these results demonstrated that $O$-fucosylation of TSRs was not essential for establishment of polarity or initiation of gastrulation.

In contrast to wildtype littermates, the expression of Nodal and Wnt3 was significantly expanded and/or displaced in the primitive streak and expression of BMP4 was significantly expanded in the extra-embryonic ectoderm of Pofut2 mutant embryos. Since cross-talk between Nodal, BMP, and Wnt signaling pathways is essential for maintaining normal expression of these genes (Arnold and Robertson, 2009), the expanded expression of the major growth factors in Pofut2 mutants suggested that failure to $O$-fucosylate TSR-containing proteins disrupted the balance of signaling in the pre-gastrula/early gastrula. For this reason, we predicted that loss of Pofut2 would result in altered mesoderm patterning and/or gastrulation defects.

Pofut2 is required to maintain epiblast pluripotency and restrict EMT during gastrulation

By E7.5, gastrulation was well underway, and the RST434 wildtype and heterozygous embryos were easily distinguished from homozygous littermates (Fig. 3). In unaffected embryos (Figs. 4A–C, left and D top), the embryonic and extra-embryonic ectoderm were organized into regular epithelia. The primitive streak, localized at the posterior, had reached its full length and a well-organized mesoderm layer was present. Further, extra-embryonic structures containing mesoderm derivatives (amnion, chorion, and allantois) had formed. Pofut2 expression, as detected by LacZ activity, was similar to that observed at E 6.5, with elevated expression in the distal half of the embryo and additional expression in the mesoderm (Figs. 4A–C, left).

In contrast, RST434 homozygous embryos were unusually dense in appearance, shorter, and characterized by a dumb-bell shape (Figs. 4A–C, right). The ectoplacental cone of mutants easily separated from the embryo. The embryonic ectoderm was present but thicker in structure and not organized into regular epithelia. The extra-embryonic ectoderm was similarly disorganized and separated from the ectoplacental cone by a constriction of visceral and parietal endoderm. In addition, Reichert’s membrane comprised of parietal endoderm cells and ECM failed to expand in RST434 homozygotes, and remained tightly associated with the visceral endoderm. Although Pofut2 mutants lacked organized tissues comprised of mesoderm, mesenchymal cells were abundant, albeit considerably disorganized compared to unaffected embryos (Figs. 4A–D). In addition, the region of the epiblast undergoing EMT transition appeared broader compared with wildtype littermates (compare Fig. 4D, top and bottom). We observed similar abnormalities in 699Lex homozygous and 699Lex/RST434 compound heterozygotes (Table 2 and Supplementary Fig. 2D–G), providing further evidence that the gene-trap insertions resulted in null Pofut2 alleles.

Despite the morphological evidence for a primitive streak and the abundance of mesenchymal cells in Pofut2 mutants, we failed to detect significant expression of the Eomes, T, and Tbx6 in Pofut2 mutants above background (Figs. 4E–G, right; confirmed using real time PCR, Supplementary Fig. 3). In wildtype embryos, Eomes, T, and Tbx6, are expressed in partially overlapping domains within the primitive streak and nascent mesoderm at E 7.5 (Figs. 4E–G). In contrast, expression of Snail1, a classic marker of EMT and newly formed mesoderm during gastrulation, was markedly expanded in Pofut2 mutants (Fig. 4, right) compared to wildtype littermates (Fig. 4, left). These results suggest that lack of Eomes, T, and Tbx6 expression resulted from reduced cell contact within the Pofut2 mutant primitive streak, rather than to a lack of mesoderm differentiation. Together, these data suggested that the Pofut2 mutant epiblast was actively undergoing EMT and that the loose cells were differentiated mesoderm (Fig. 4, right). Consistent with this prediction, we observed a marked reduction in the expression of

Table 2
Disruption of Pofut2 gene resulted in embryonic lethality by E 10.5.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Stage examined</th>
<th>Genotype</th>
<th>Res sites</th>
<th>Litter total</th>
<th>% Hm (p)</th>
</tr>
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<tr>
<td>RST434 × RST434</td>
<td>P 15</td>
<td>Wt 35</td>
<td>Ht 60</td>
<td>Hm 0</td>
<td>95</td>
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<tr>
<td>RST434 × RST434</td>
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<td>Wt 18</td>
<td>Ht 43</td>
<td>Hm 0</td>
<td>80</td>
</tr>
<tr>
<td>RST434 × RST434</td>
<td>E 7.5</td>
<td>Wt 18</td>
<td>Ht 27</td>
<td>Hm 15</td>
<td>60</td>
</tr>
<tr>
<td>RST434 × RST434</td>
<td>E 6.5</td>
<td>Wt 11</td>
<td>Ht 27</td>
<td>Hm 13</td>
<td>51</td>
</tr>
<tr>
<td>699Lex × 699Lex</td>
<td>E 7.5</td>
<td>ND 75</td>
<td>ND 11</td>
<td>Hm 90</td>
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<td>699Lex × 699Lex</td>
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<td>52</td>
</tr>
<tr>
<td>699Lex/RST434</td>
<td>E 7.5</td>
<td>ND 75</td>
<td>ND 11</td>
<td>Hm 90</td>
<td>52</td>
</tr>
</tbody>
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Wt, wildtype; Ht, heterozygous; Hm, homozygous; nd, not determined.

* Genotype determined by PCR (Materials and methods).
** Resorption sites (Res sites) were counted and surviving embryos genotyped by PCR.
* Genotypes were inferred from the intensity of X-gal staining and embryo morphology. The relationship between these characteristics and genotype was verified by PCR (Materials and methods).
* Homozygous genotype inferred from mutant morphology.

zygotes (Fig. 4). In unaffected embryos (Figs. 4A–C, left and D top), the embryonic and extra-embryonic ectoderm were organized into regular epithelia. The primitive streak, localized at the posterior, had reached its full length and a well-organized mesoderm layer was present. Further, extra-embryonic structures containing mesoderm derivatives (amnion, chorion, and allantois) had formed. Pofut2 expression, as detected by LacZ activity, was similar to that observed at E 6.5, with elevated expression in the distal half of the embryo and additional expression in the mesoderm (Figs. 4A–C, left).

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![Image](https://example.com/image.jpg)
epiblast pluripotency markers, Oct4 and Sox2, in Pofut2 mutants compared to the wildtype embryos (Figs. 4H–I).

To confirm that Pofut2 mutant epiblast was undergoing active EMT, we characterized laminin and E-cadherin expression. In wildtype embryos, laminin is decreased in the region of the primitive streak undergoing EMT, compared to levels in surrounding epiblast (Figs. 5A–B). In addition, E-cadherin expression is down regulated in the primitive streak cells undergoing EMT and the mesoderm (Figs. 5E–F, and Ei–Fi). In Pofut2 mutant embryos, the region of decreased laminin expression was slightly expanded compared to wildtype embryos (Figs. 5C, D), corresponding to the region of reduced cell contact. Moreover, E-cadherin expression was markedly decreased throughout the Pofut2 mutant epiblast (Figs. 5G–H and Gi–Hi). These results were consistent with the elevation of Snail1, a negative regulator of E-cadherin, in the Pofut2 mutants. Combined, the expansion of Snail1 expression, decrease in E-cadherin expression, and the abundance of mesenchymal cells in Pofut2 mutant embryos provided strong evidence that O-fucosylation of TSR proteins was essential to restrict EMT within the primitive streak and maintain epiblast pluripotency.

Pofut2 is essential for intercalation of definitive endoderm

Despite the formation of a primitive streak and mesoderm differentiation in Pofut2 mutants, we did not observe a morphological node, the anterior organizing center of the primitive streak. For this reason, we examined the expression of major signals important for primitive streak patterning and markers of node derivatives in E 7.5 embryos. Consistent with our observations at E 6.5, we detected a marked expansion of Nodal, Wnt3, BMP4, and Fgf8 expression, expanded Wnt activity detected by activation of the BAT-gal reporter, and a decrease in Lefty2 expression in Pofut2 mutants compared to wildtype littermates (Figs. 6A–F and Supplementary Fig. 3). Expression of markers characteristic of both posterior mesoderm (Fli1/Kdr) as well as anterior mesendoderm (Gsc) were expanded in Pofut2 mutants (Figs. 6G and H), suggesting that loss of Pofut2 did not block the patterning of the most anterior and posterior mesoderm, but rather affected the abundance of these cells. In addition, we detected expression of the definitive endoderm markers Foxa2 and Cer1 (Figs. 6I and J). These results were consistent with specification of anterior mesendoderm derivatives in mutants, despite the absence of a morphological node. However, in contrast to wildtype littermates, Foxa2 and Cer1 expressing cells remained within the interior of Pofut2 mutants, and did not intercalate into the overlying visceral endoderm. The failure of Foxa2 and Cer1 expressing cells to displace the visceral endoderm in Pofut2 mutants suggested that O-fucosylation of TSRs was essential for directing their localization.

Loss of POFUT2 promotes ectopic differentiation of vascular endothelial cells

In addition to defects in the Pofut2 mutant epiblast, primitive streak and node derivatives, we observed abnormalities in the organization of the parietal and visceral endoderm layers surrounding the embryonic and extra-embryonic ectoderm. The proximal visceral
endoderm overlying the extra-embryonic ectoderm is an absorptive epithelial and is characterized by expression of Cubn and Amn; these markers are normally down regulated in the more squamous visceral endoderm overlying the distal embryonic ectoderm (Figs. 4K and L). Curiously, in Pofut2 mutant embryos we observed expression of Cubn and Amn overlying the epiblast (Figs. 4K and L). In a co-culture assay, proximal visceral endoderm can induce differentiation of blood from anterior epiblast, normally fated to become neural tissue (Belaoussoff et al., 1998). For this reason, the distal expansion of the proximal visceral endoderm raises the possibility that defects in definitive endoderm localization could result in part from defective interactions with the visceral endoderm. In addition, we predict that altered characteristics of the visceral endoderm could dramatically impact the characteristics of the differentiating mesoderm.

Consistent with this prediction, we observed gross alteration in the characteristics of the Pofut2 mutant mesoderm by E 8.5. At this time, wildtype embryos had undergone gastrulation, neurulation, and began organogenesis (Figs. 7A–B). Although the Pofut2 mutant embryos had increased in size, they showed no morphological evidence of neurulation or organogenesis. In addition, clearly defined extra-embryonic- and embryonic ectoderm-derived epithelial structures were lost by E 8.5 [Figs. 7C–D]. In contrast, the mesenchymal cells in Pofut2 mutants appeared to have condensed against the visceral endoderm, producing a structure bearing some resemblance to the visceral yolk sac (compare Figs. 7Bi and Di). In wildtype embryos, the visceral yolk sac develops from proximal visceral endoderm and posterior mesoderm. Posterior mesoderm is characterized by the expression of the VEGF-receptor encoded by Flk1 (Fig. 6G and Ema et al., 2006; Tremblay et al., 2001). A subset of these cells will become the extra-embryonic mesoderm precursors of hematopoietic and endothelial cells in the yolk sac blood islands (Ema et al., 2006; Kabrun et al., 1997; Yamaguchi et al., 1993).

In wildtype embryos, Flk1 expressing cells of the gastrula-staged mouse embryo differentiate into vascular endothelial cells (distinguished by PECAM1 staining) and primitive erythroid cells (characterized by morphology or O-dianisidine staining which detects hemoglobin) (Figs. 7B, Bi, E, and G) (Ema et al., 2006; Motoike et al., 2003). Despite the abundance of Flk1 expressing cells in Pofut2 mutant embryos, there was no morphological evidence for primitive erythrocytes in Pofut2 mutants nor did mutant embryos stain with O-dianisidine (Figs. 7C–Di, and H). In contrast, vascular endothelial cells, marked by PECAM1 expression, were abundant throughout all but the most distal region of Pofut2 mutants (Figs. 7F, Fi, and Supplement Fig. 4). The abundance of Flk1 and PECAM1 positive cells and absence of differentiated primitive erythrocytes suggested that O-fucosylation of TSR proteins by POFUT2 was important for the distinction between the hematopoietic and vascular endothelial cell lineages.

The extracellular environment influences the potential of Pofut2 mutant cells to differentiate

We identified 51 mouse TSR-containing proteins containing the consensus sequence for O-fucosylation by POFUT2 (Table 1). Using
semi-quantitative RT-PCR, we detected transcripts encoding all but 10 putative POFUT2 targets at E 7.5 (Supplementary Table 1). Since POFUT2 facilitates the secretion of target proteins in cell culture experiments (e.g. ADAMTS13 and ADAMTSL1, Ricketts et al., 2007; Wang et al., 2007), the limited capacity for Pofut2 mutant cells to differentiate within the embryo could stem from secretion defects of POFUT2 targets. However, the establishment of anterior/posterior polarity, which is dependent upon BMP, Nodal, and Wnt (not direct POFUT2 targets) signaling pathways provided indirect evidence that secretion was generally unaffected by the Pofut2 mutation. Since

Fig. 6. Loss of Pofut2 resulted in expanded expression of major growth factor signaling molecules. (A–D and F–I) Whole-mount in situ hybridization was used to examine gene expression in E7.5 wildtype (wt) and RST434 homozygous littermates (−/−) using the indicated probes. (E) Wnt signaling activity was measured in wildtype and 699Lex homozygous embryos with one copy of the Bat-gal transgene reporter (Maretto et al., 2003). Wnt activity is expanded toward the anterior (left) in Pofut2 mutants. The number of mutant embryos is represented by panels: Nodal (6), Lefty2 (3), BMP4 (4), Wnt3 (6), Bat-gal (9), Fgf8 (5), Flk1 (10), Gsc (7), Foxa2 (8), Cer1 (3). Anterior is to the left; posterior is to the right. Proximal is up; distal is down. Scale bar indicates 100 μm.

Fig. 7. Mesoderm in Pofut2 mutants preferentially differentiates into vascular endothelial cells. (A–Di) Whole-mount and (B and D) transverse hematoxylin and eosin Y stained sections of E 8.5 wildtype (A–Bi) and Pofut2 mutant (C–Di) embryos. (E–Fi) Immunofluorescence staining with PECAM antibody (green indicated by white arrowhead) and DAPI (blue) in E 8.5 control (E–Ei) and RST434 mutant littermates (F–Fi). The approximate plane of sectioning for panels (B, D, F–G) are indicated by black lines in A and C. Bi and Di represent enlargements of boxed regions in panel B and D. (G, H) O-dianisidine staining of E 8.5 wildtype (G) and mutant (H) embryos. The number of mutant embryos is represented by panels: C (9), D (7), PECAM (9), O-dianisidine (6). For panels A–D and G and H anterior is left; posterior is right. Proximal is up; distal is down. Abbreviations: bi, primitive erythrocytes; ve, visceral endoderm; ven, vascular endothelial cells; *, condensed mesoderm. Scale bar sizes indicated in μm.
many POFUT2 target proteins interact with the ECM or are constitutive components of ECM, the limited capacity for differentiation of Pofut2 mutants more likely resulted from a defect in the extracellular environment or the ability of mutant cells to interact with the environment resulting specifically from absence or altered function of a POFUT2 target(s).

To test this hypothesis, we evaluated the ability of E 7.5 mutant embryos to differentiate in teratomas, where the ECM was provided in part by the environment of the testis (Fig. 8). Wildtype teratomas (n = 6) ranged in diameter from 463 μm to 9819 μm, with the exception of one tumor which only reached 2994 μm in diameter (data not shown). In contrast, Pofut2 mutant embryos (n = 6) formed smaller tumors ranging in size from 551 μm to 4279 μm in diameter. The smaller size suggested a potential cell-autonomous affect on cell proliferation or viability. Although smaller in size, the tissue composition of teratomas was comparable to those derived from wildtype littermates, and contained tissues derived from all three germ layers (Figs. 8A–F). The ability of Pofut2 epiblast to differentiate into teratomas containing a variety of tissues not present in mutant embryos suggested that POFUT2 targets in the extracellular environment rather than cell surface localized-targets or intracellular POFUT2 function was essential for maintaining the pluripotency of the epiblast and influence the patterning of mesoderm.

**Discussion**

Loss of Pofut2 disrupted cell differentiation as well as tissue morphology, boundaries, and relationships during gastrulation. Our biochemical studies demonstrated that POFUT2 specifically adds O-fucose to TSRs. The majority of predicted POFUT2 targets are constitutive components of the ECM or secreted matrix associated proteins capable of influencing cell adhesion/migration, ECM synthesis and remodeling, and modulating growth factor signaling (Table 1). This observation was consistent with the ability of Pofut2 mutant cells to differentiate in the teratoma assay. For this reason, we hypothesize that loss of TSR O-fucosylation resulted in ECM changes that affected gastrulation by modulating growth factor signaling activity and/or altering or restricting cell movement (Rozario and Desimone, 2009).

The gross alteration in the tissue composition of the Pofut2 mutant embryos demonstrated that Pofut2 was a key modulator of gastrulation. During gastrulation the timing, activity, and distribution of growth factor signals must be fine-tuned to generate a diverse array of tissues with limited regions of the embryo. Nodal signaling is central to specification of anterior/posterior polarity, primitive streak formation, and mesoderm patterning (Arnold and Robertson, 2009; Brennan et al., 2001; Camus et al., 2006). In addition, there is considerable cross-talk between the Nodal, BMP, and Wnt signaling pathways during specification of polarity and mesodermal cell fate (Arnold and Robertson, 2009; Fuentealba et al., 2007). Several known and predicted POFUT2 target proteins, including CCN2 (CTGF), CCN6 (WISP3), TSP1, ADAMTS1, ADAMTS8, and ADAMTS2 negatively modulate the activity of TGFβ, BMP, WNT, FGF, BMP4 and VEGF signaling pathways (Abreu et al., 2002; Gupta et al., 1999; Luque et al., 2003; Nakamura et al., 2007; Rodriguez-Manzaneque et al., 2001; Suga et al., 2006). The potential contribution of these proteins to the Pofut2 phenotype is discussed in greater detail below.

The potential importance of O-fucosylation of TSRs in regulating TGFβ3 signaling is underscored by the identification of two amino acid substitutions in the TSRs of ADAMTSL2 in geleophysic dysplasia patients, characterized by brachydactyly, cardiac valvular abnormalities, and short stature (Le Goff et al., 2008). These mutations are within the 6th and 7th TSRs containing conserved sequence for POFUT2 modification. Importantly, increased TGFβ3 and nuclear localization of phospho-SMAD2 are detected in cells isolated from geleophysic dysplasia patients, suggesting that ADAMTSL2 functions as a negative modulator of TGFβ3 signaling (Le Goff et al., 2008). In addition, in Xenopus overexpression of another potential POFUT2 target, CCN2 (CTGF), antagonizes BMP signaling, but potentiates low-level TGFβ3 signaling (Abreu et al., 2002).

Pofut2 mutants shared some phenotypic similarities to mutations that disturb the Nodal (a TGFβ3) pathway. Expansion of gene expression characteristic of the proximal/posterior primitive streak is also observed in Smad2, Nodal-NR (precursor processing mutant), and Drap1 mutant embryos. However, in Smad2 and Nodal-NR mutant embryos, the failure to localize the primitive streak to the posterior results from the inability to specify and localize the AVE and establish...
anterior/posterior polarity (Brennan et al., 2001; Ben-Haim et al., 2006). In contrast, Pofut2 mutant embryos correctly localized the AVE and initially restricted expression of primitive streak markers to the posterior, suggesting that the expansion of markers did not result from a reduction of Nodal signaling or processing of the Nodal precursor or a failure to establish anterior/posterior polarity. In contrast, embryos lacking Drap1, encoding a transcriptional co-repressor, are predicted to enhance expression of Nodal (Iratni et al., 2002). Drap1 mutants are characterized by an expansion of posterior localized Nodal transcripts, loss of Brachyury (T) and Lefty2, enlarged primitive streak, and excess EMT (Iratni et al., 2002). We observed similar defects in Pofut2 mutant embryos, suggesting that the expansion of primitive streak and EMT in Pofut2 mutants could be attributed in part to elevation of Nodal signaling. However, despite the similarities between Pofut2 and Drap1 mutants, differences in the organization of epithelia and pattern of mesoderm migration between Pofut2 and Drap1 mutants, suggested that defects in Pofut2 mutant embryos were not limited to aberrant Nodal signaling.

Likely, loss of TSR O-fucosylation also causes changes in ECM composition that alter cell and/or tissue characteristics (Rozario and Desimone, 2009). Reichert’s membrane consisting of parietal endoderm cells and a dense sheet-like ECM comprised predominantly of laminin-1, collagen type IV, and heparan sulfate proteoglycan, was one of the earliest tissues affected in Pofut2 mutants (Jones and Riley, 2005; Porter et al., 2005; Hogan et al., 1980; Leivo et al., 1980; Wortiavaara et al., 1979). Normally, Reichert’s membrane is physically separated from the visceral endoderm, and the parietal endoderm cells are found widely spaced. In contrast, in Pofut2 mutants this membrane was tightly associated with the visceral endoderm and the parietal endoderm cells were densely packed. These observations suggested that TSR O-fucosylation was essential for expansion of this membrane. The tight association between Reichert’s membrane and the visceral endoderm likely placed considerable physical constraints on the developing Pofut2 mutant embryo. In Xenopus development, increasing mechanical tension promotes early fibronectin fibril assembly and alters tissue characteristics (Dzamba et al., 2009). For this reason, we predict that tension induced alterations in the ECM composition/organization could contribute to the irregular epithelia this reason, we predict that tension induced alterations in the ECM (Belaoussoff et al., 1998; Dyer et al., 2001). Precursors rather than differentiation of neural tissue in explants our data clearly demonstrated that Pofut2 was required during mouse gastrulation to coordinate morphogenetic movements and specify a range of tissues in the mouse embryo. These abnormalities underscored the importance of this unusual O-fucose post-translational modification for TSR protein function. Of the 51 proteins predicted to be modified by POFUT2, most were expressed during mouse gastrulation (Supplementary Table 1). To date, published knockouts of known or predicted targets of POFUT2 do not result in phenotypes similar to Pofut2 mutant embryos (Irvine et al., 2008). Although none of the single Ccn disruptions characterized thus far result in phenotypes similar to Pofut2 mutants (Heath et al., 2008; Ivkovic et al., 2003; Kutz et al., 2005; Hurvitz et al., 1999; Mo et al., 2002), given the high degree of amino acid identity between CCN members (30–50%), it is likely that some CCN cellular functions overlap (Holbourn et al., 2008). CCN members (also POFUT2 targets) modulate cell adhesion by binding to integrins and heparin sulfate proteoglycans (Holbourn et al., 2008). CCN2-deficient mouse embryonic fibroblasts (MEFs) do not adhere well to fibronectin and show delays in cell spreading (Chen et al., 2004). Disruption of CCN6 in human mammary epithelial cells increased SNAIL and decreased expression of E-cadherin, a phenotype remarkably similar to Pofut2 mutant embryos (Irvine et al., 2008). Financial disclosure

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Competing interest

The authors have no financial, personal, or professional interests that could be construed to have influenced this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.07.008.

References


