TMTC-type O-mannosyltransferases as potential regulators of cadherin-mediated cell adhesion

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Introduction

The function and development of organized tissues in metazoan organisms depends on the complex mechanisms of cell adhesion. Cadherins are a large family of transmembrane glycoproteins which are major contributors to cell adhesion. Their functions are essential for numerous biological and pathological processes, such as neural development, tumor suppression, and epithelial maintenance. Mutations in cadherins lead to a motley of congenital diseases, from neurological abnormalities to cancer. Cadherins are prominently modified with O-mannose within important functional domains by TMTC-type O-mannosyltransferases, however, the in vivo function of these modifications remains poorly understood. Notably, *TMTC* mutations were found to be associated with brain malformations and neurological disorders, including Cobblestone lissencephaly, a severe congenital disorder characterized by defects in neuronal migration, which reveals that TMTC glycosyltransferases play important roles in nervous system development, possibly by affecting the function of neural cadherins. Defects in the O-mannosylation profile of E-cadherin, caused by a loss of TMTC3 activity was found to decrease cell adhesion in vitro, however, the effect of TMTC mutations on cadherin functions has not been characterized in vivo. TMTCs are well conserved in metazoan organisms, which provides the opportunity to study their functions using experimentally amenable model organisms such as *Drosophila*. Our analyses of *Drosophila TMTC1-3* mutants indicated that these genes function in the nervous system development and are required for axon development in a partially redundant manner. Our initial experiments indicated that these mutants show abnormalities in axon wiring and connectivity, disturbed morphology of axonal tracts, and defects in the patterning of the visual system. Unraveling the relationship between TMTCs and cadherin functions in *Drosophila* is expected to elucidate evolutionarily conserved functions of TMTCs in mammalian organisms, including humans. Our results may shed light on the pathomechanisms of human disorders associated with defects in the O-mannosylation of cadherins.

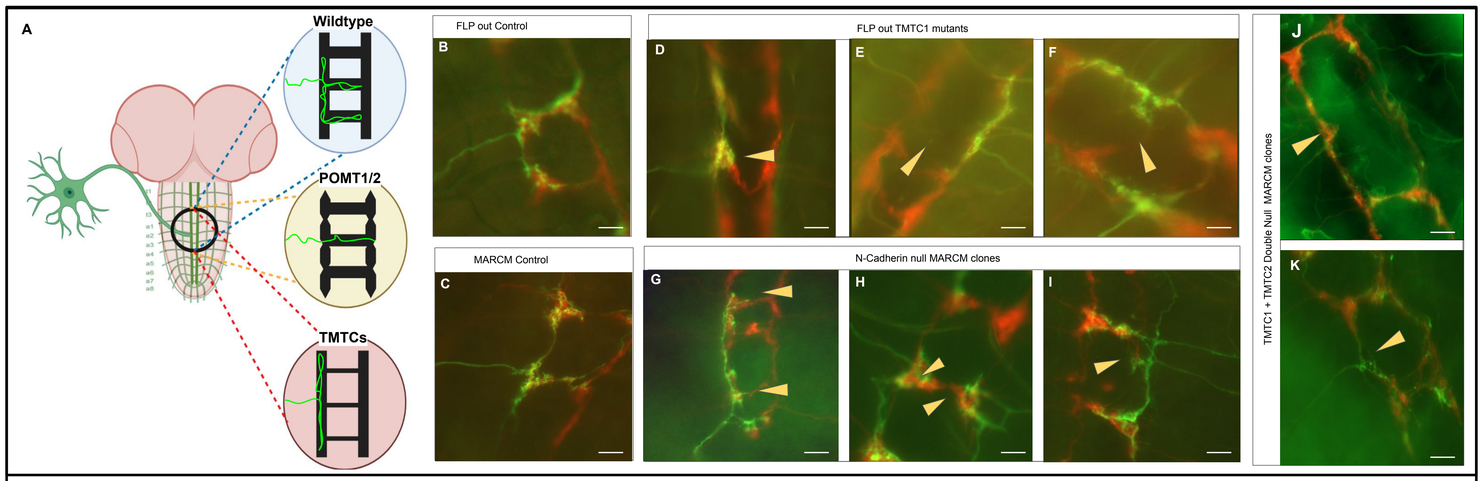

Figure 6: Single cell clones of TMTCs show defects similar to CadN mutant clones. Mosaic Analysis with A Repressible Cell Marker (MARCM) and the FLP-FRT system were utilized to generate or visualize individual mutant axons. MARCM approach produced mutant neurons in heterozygous Drosophila, while FLP-out technique labeled individual neurons in mutants. **A**. A schematic depicting the effects of TMTC mutations on single sensory axons. **B.** Wild Type FLP out control. **D,E,&F.** Three examples of Missing commissural connection is denoted by yellow arrowheads. **C**. MARCM "wild-type"control. **G,H,&I,**. MARCM CadN^{M19} (null mutation) clones displaying abnormal branching and failure to cross the midline. These abnormalities are also induced by TMTC1-2 mutations. The similarity of the phenotypes suggests that N-Cadherin is a functional target of TMTCs. **J&K,** Double mutant (TMTC1 + TMTC2 null) MARCM clones displaying failure to cross the midline and abnormal branching (also found in CadN mutants). Scale bars represent 10µm.

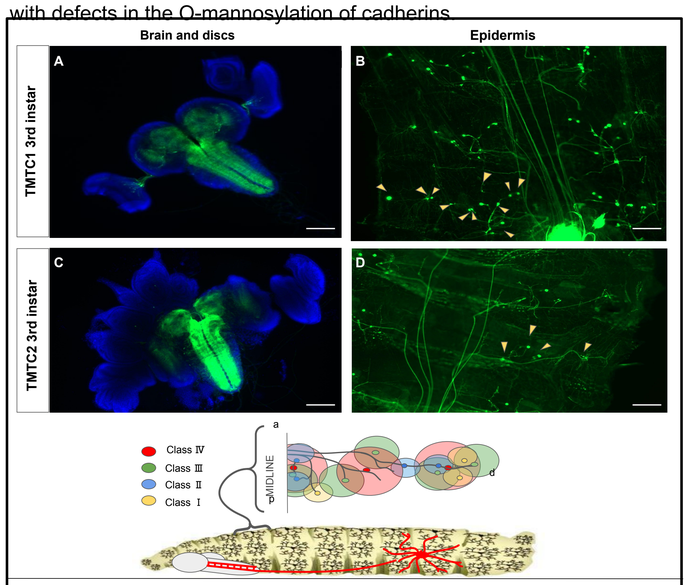

Figure 1: Expression of TMTCs in the developing larval nervous system: Central and peripheral nervous system expression of TMTC1&2 in the 3rd instar larvae show overlapping expression of TMTC1 & TMTC2 restricted to the nervous system. **A.** Drosophila 3rd instar CNS and discs expressing TMTC1 shown in green. **B.** Drosophila 3rd instar peripheral nervous system, arrowheads point to sensory neurons expressing TMTC1, all classes of sensory neurons. **C.** Central nervous system and discs, expressing TMTC2 shown in green. **D.** Peripheral nervous system, arrowheads point to class 2 and 4 sensory neurons, which are selectively expressing TMTC2. **E.** Schematic showing the approximate location and spatial distribution of the CNS (grey) and all 4 classes of sensory neurons in the drosophila 3rd instar larvae, Scale bars (white) are 100µm

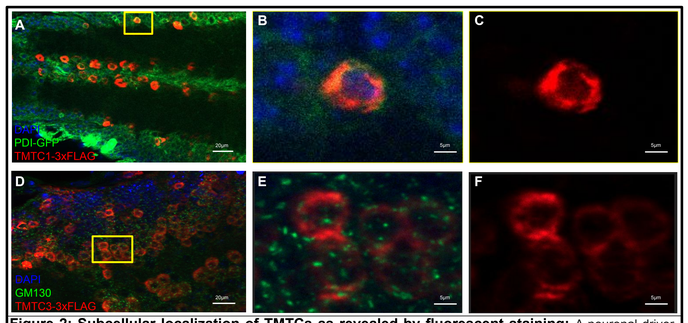

Figure 2: Subcellular localization of TMTCs as revealed by fluorescent staining: A neuronal driver, C155-Gal4, was used to express Drosophila UAS-TMTC1-3xFLAG (**A,B,C**) and Drosophila UAS-TMTC3-3xFLAG (**D,E,F**) respectively. **A.** a 20x image of the posterior tip of the Ventral Ganglion in a 3rd instar larvae, TMTC1 (red) and PDI-GFP (green) a yellow square highlights a region of interest. **B & C.** 40x images of the highlighted ROI seen in **A**. **D.** a 20x image of the middle area of the Ventral Ganglion in a 3rd instar larvae, TMTC3 (red) and GM130 (green), a yellow square highlights a region of interest. **E & F.** 40x images of the highlighted ROI seen in **D**.

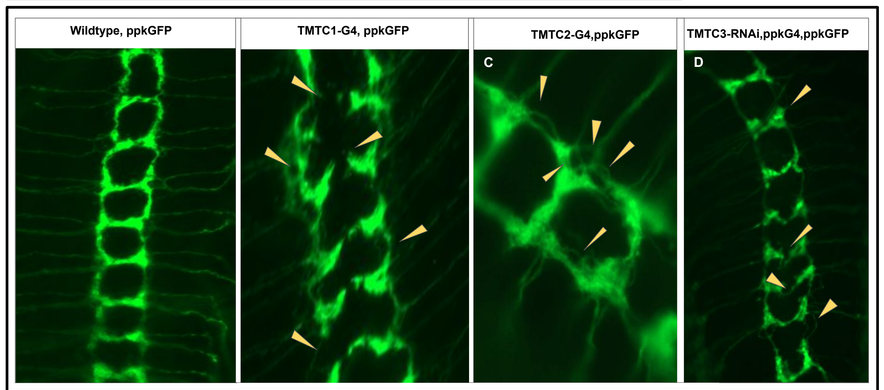

Figure 3: Effects of TMTC 1 and TMTC 2 mutations on sensory axon wiring. Sensory axon termini in the larval ventral ganglion have distinct morphology that is disturbed in TMTC 1 & 2 mutants. Combining TMTC 1 and 2 mutations with a fluorescent marker for sensory neurons, ppk-CD4-GFP, reveals the effect of TMTC1/2 mutations on axon wiring. **A.** WT control **B.** TMTC1-G4,ppk-CD4-GFP, with perturbations in axon wiring and morphology indicated (yellow arrowheads). **C.** TMTC2 mutants show with perturbations in axon wiring (yellow arrowheads). **D.** TMTC3-RNAi knock down in sesnsory neurons show defects in axon wiring.

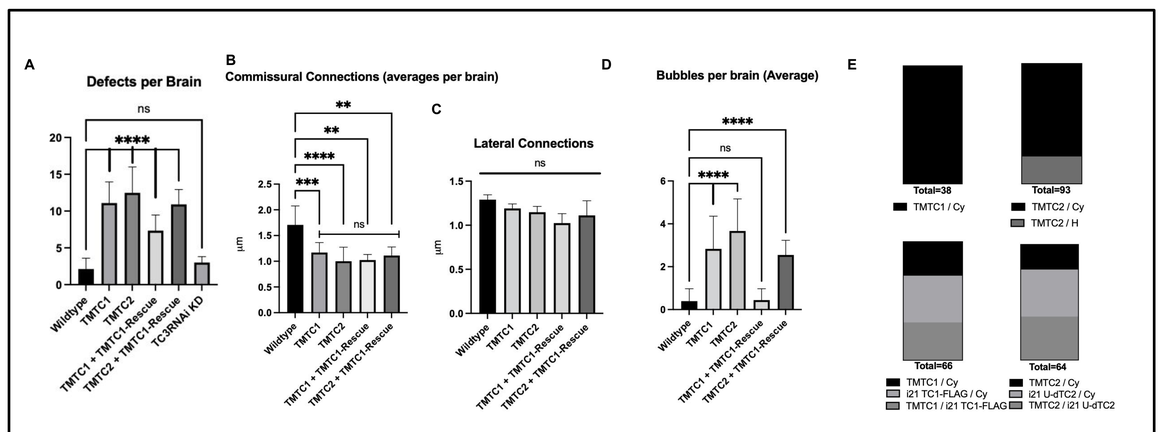

Figure 4: Quantification of Axonal Defects and viability of TMTC1 & 2 mutants. A. TMTC mutants show significantly increased total number of defects per brain in the A3-A7 region of the ventral ganglion. The mutants also show more defects in commissural connections (B) and develop more abnormalities in at the nodes of the sensory "ladder", such as bubble-like morphology (D). No significant defects were found in longitudinal connections (C, width of longitudinal connections) . Rescue with TMTC1-FLAG transgenic construct slightly reduced the number of total defects, but did not rescue the thinning of the observed in commissures (B). **C,** Width of commissural connections. TMTC1 & 2 mutants showed significant decrease in the width of commissural connections, however, transgenic expression of TMTC1 did not rescue the phenotype.. Significance was analyzed by one-way ANOVA and Tukey post-hoc multiple comparisons. Error bars show SD. **E.** Viability of homozygous TMTC mutants compared with that of heterozygous sibling progeny. TMTC homozygous mutants display lethality in late larval stages, and rarely appear as adult flies. Double mutants (not shown here) display larval lethality at earlier stages in development and are not viable as adults. Double mutant axons were analyzed using MARCM in Fig. 6. Transgenic expression of TMTCs in homozygous single mutants partially rescued the lethality phenotype.

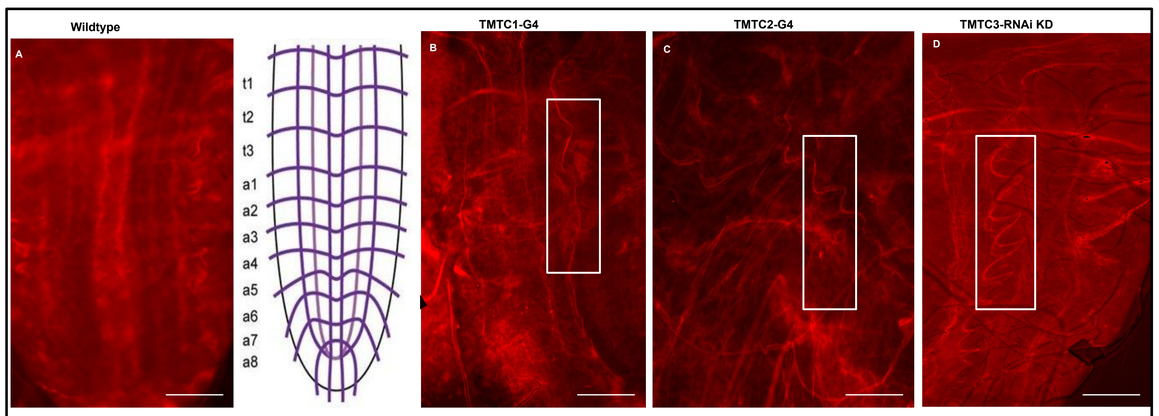

Figure 5: TMTC1 & TMTC2 affect the morphology of main axonal tracts in the larval brain. suggesting that TMTC-mediated O-mannosylation is important for N-Cadherin functions required for proper neuroarchitecture in the developing brain. Anti-Fasciclin 2 (FasII) immunostaining of 3rd instar larval brains (FasII is the Drosophila orthologue of neural cell adhesion molecule) reveals additional abnormalities in TMTC1 & TMTC2 mutants backgrounds. **A.** Wildtype control shows normal distribution of FasII tracts in the ventral ganglion. (right) idealized schematic showing the distribution of FasII tracts in the ventral ganglion. TMTC1 mutant larval brain (B) and TMTC2 mutant larval brain (C) show abnormal distribution and morphology of FasII tracts (white rectangle, region of interest). **D.** TMTC3 RNAi knockdown induced in all neurons using C155-Gal 4 driver. The axonal tracts display more severe phenotype than TMTC1 and 2 mutants. Taken together with our results indicate that TMTC mediated O-mannosylation is crucial for development of proper neuroarchitecture, which is consistent with the scenario that TMTC enzymes modify and modulate different cadherins.

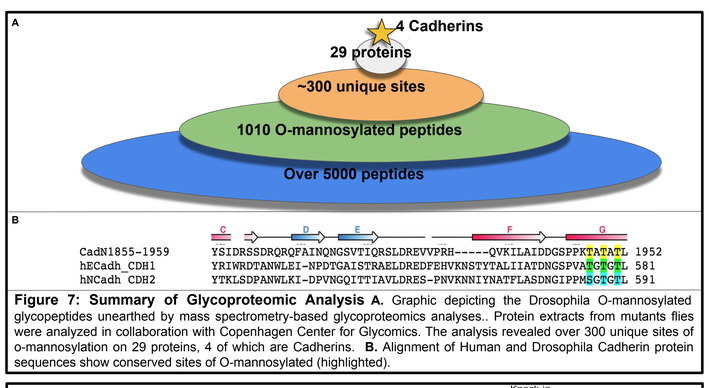

Figure 7: Summary of Glycoproteomic Analysis A. Graphic depicting the Drosophila O-mannosylated glycopeptides unearthed by mass spectrometry-based glycoproteomics analyses.. Protein extracts from mutants flies were analyzed in collaboration with Copenhagen Center for Glycomics. The analysis revealed over 300 unique sites of o-mannosylation on 29 proteins, 4 of which are Cadherins. **B.** Alignment of Human and Drosophila Cadherin protein sequences show conserved sites of O-mannosylated (highlighted).

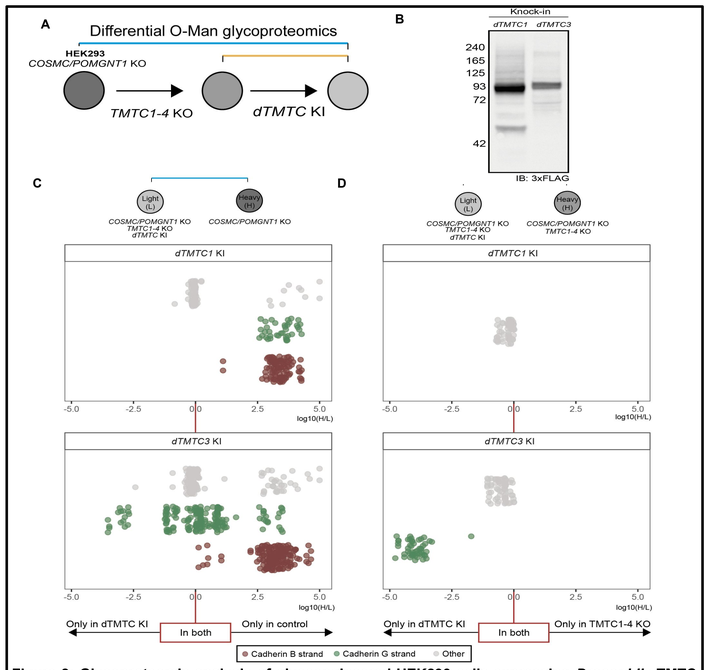

Figure 8: Glycoproteomic analysis of glycoengineered HEK293 cells expressing *Drosophila* TMTC genes. A, HEK293 cells with *COSMC/POMGNT1/TMTC1-4* KO were established before CRISPR/Cas9 knock-in (KI) of *dTMTC1* or *dTMTC3* constructs with FLAG tag. **B,** The expression of dTMTC1 and dTMTC3 was confirmed by Western blot analysis. Differential O-Man glycoproteomic analyses using light (L) and heavy (H) stable diethyl isotopes for quantification of O-Man in glycoengineered HEK293 cells lines. Dotplot depicts O-Man relative abundances in *COSMC/POMGNT1* KO and *COSMC/POMGNT1/TMTC1-4* KO/*dTMTC1* KI cells. **C,** Dotplot depicts O-Man relative abundances in *COSMC/POMGNT1/TMTC1-4* KO and *COSMC/POMGNT1/TMTC1-4* KO/*dTMTC1* KI cells. **D,** Dotplot depicts O-Man relative abundances in *COSMC/POMGNT1* KO and *COSMC/POMGNT1/TMTC1-4* KO/*dTMTC3* KI cells.

Summary

- **TMTC1 & 2 are expressed in an overlapping pattern** in the nervous system throughout development, and are upregulated during larval stages.
- TMTC1 & 2 mutants have **defects in sensory neuron connectivity** affecting the wiring of axon termini and dendrite morphology. These mutants also display defects in the morphology of main axon tracts in the larval brain, suggesting that **TMTC functions are important for proper neuronal connectivity**.
- Glycoproteomics approaches uncovered many potential substrates of TMTCs in Drosophila, including 4 Cadherins, supporting the hypothesis that cadherins represent a major group of functionally important targets of O-mannosylation.
- Analysis of individual mutant TMTC1 and TMTC1+TMTC2 double mutant neurons show a decreased ability to cross the midline, reflective of the 'thinned commissural connection' phenotype.
- N-Cadherin mutant clones also exhibited a decreased ability to cross the midline, supporting our hypothesis that **N-Cadherin may also be a substrate of TMTC1 and TMTC2**.
- TMTC3 function is conserved between Drosophila and humans; Drosophila TMTC3 can O-mannosylate sevearl human cadherins and protocadherins when transgenically expressed in human cells.

Acknowledgements

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