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Evolutionary conservation of Notch signaling inhibition by TMEM131L overexpression

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Human *KIAA0922/TMEM131L* encodes a transmembrane protein, TMEM131L, that regulates the canonical Wnt/β-catenin signaling pathway by eliciting the lysosome-dependent degradation of phosphorylated LRP6 co-receptor. Here, we use a heterospecific Drosophila transgenic model to examine the potential evolutionary conservation of TMEM131L function. Analysis of TMEM131L transgenic flies shows that TMEM131L interference with the Wnt pathway results primarily from a Notch-dependent decrease in Wingless production. Consistently, lentivirus-mediated overexpression of TMEM131L in human $CD34^+$ hematopoietic progenitor cells leads to decreased susceptibility to Notch1 ligation and defective commitment toward the T lineage. These results show that TMEM131L corresponds to an evolutionary conserved regulator of the Notch signaling pathway.

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1. Introduction

The human *KIAA0922/TMEM131L* gene is a complex locus on chromosome 4q31 comprising 35 exons as well as multiple transcription initiation and alternative splicing sites. It encodes a prototypic type I transmembrane protein and a variety of lower molecular weight variants, the majority of which reside in the nuclear compartment. Combining *in vivo* and *in vitro* approaches, we have previously shown that, through its ectodomain (ECD), TMEM131L regulates the canonical Wnt/β-catenin signaling pathway by eliciting the lysosome-dependent degradation of phosphorylated LRP6 co-receptor (pLRP6) [1]. Here, we used a hetero-specific transgenic approach in *Drosophila melanogaster* to test the potential conservation of this function. Unexpectedly, we found that TMEM131L interference with the Wnt pathway proceeds indirectly through Notch signaling inhibition. Consistent with an evolutionary conservation of Notch signaling inhibition, overexpression of human *TMEM131L* conferred intrinsic resistance to Notch1 ligation in human $CD34^+$ hematopoietic progenitor cells.

2. Materials and methods

2.1. Production and analysis of TMEM131L transgenic Drosophila melanogaster

Flies carrying UAS-TMEM131L were generated by P element transformation. Full-length TMEM131L coding sequence was subcloned in frame into a pUAS-expression vector as described [2]. Transgene injection and production of the corresponding transgenic lines were performed by BestGene. The following Drosophila strains were used: w^{1118}, *ptc-GAL4*, and *pnr-GAL4* provided by Bloomington Stock Center, and *hh-GAL4* [3] provided by A. M. Pret (Institut de Biologie Intégrative de la Cellule, Gif-sur-Yvette, France). Flies were raised at 25 °C on standard medium and crosses were performed at 29 °C. Pictures of adult flies were recorded on a Leica MZFLIII stereomicroscope. Microchaetes were enumerated using the image J (NIH) software. Immunostaining on imaginal discs was performed as described [4]. The following antibodies were used: guinea-pig anti-Senseless (1:500) and rat anti-Distal-less (1:200) were kindly provided by H. Bellen (Baylor

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Table 1
Primer sequences.

Gene	forward primer (5′ - 3′)	reverse primer (5′ - 3′)
HPRT	GGCAGTATAATCCAAAGATGGTCAA	TCAAATCCAACAAAGTCTGGCTTATAT
DTX1	AGAATCCCGAGGATGTGGTTCG	TCGTAGCCTGATGCTGTGACCA
HES1	GGAAATGACAGTGAAGCACCTCC	GAAGCGGGTCACCTCGTTCATG
NRARP	CAAGGGCAACACGCAGGAGCT	CCGAACTTGACCAGCAGCTTCA
Notch1	GGTGAACTGCTCTGAGGAGATC	GGATTGCAGTCGTCCACGTTGA
GATA3	ACCACAACCACACTCTGGAGGA	TCGGTTTCTGGTCTGGATGCCT
IKZF1	GCTGCCACAACTACTTGGAAAGC	AGTCTGTCCAGCACGAGAGATC

College of Medicine, Houston, TX, USA) and S. Cohen (University of Copenhagen, Denmark), respectively; mouse anti-Cut (2B10, 1:200) and mouse anti-Wg (4B4, 1:200) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, US). Alexa fluor 647-conjugated goat anti-guinea pig, Alexa fluor 568-conjugated goat anti-rabbit, and Alexa fluor 488-conjugated goat anti-mouse (1:400) were from Molecular Probes. Pictures were recorded on a Leica TCS SPE confocal microscope and processed using Image J (NIH) software.

2.2. In vitro Notch1 stimulation assays of human CD34^{+} hematopoietic progenitor cells

Umbilical cord blood (UCB) collected according to institutional guidelines was processed as described [5,6]. cDNA coding for full-length TMEM131L or a membrane-associated derivative of its cytoplasmic domain (PSTMIC) [1] was cloned into the dual promoter EF1a/PGK pRRL lentiviral vector (gift of Hana Raslova, Institut Gustave Roussy, Paris). Expression of TMEM131L/PSTMIC and EGFP is controlled by the EF1α and PGK promoters, respectively. Production of VSV-pseudotyped lentiviral vectors and lentiviral transduction of CD34^{+} hematopoietic progenitor cells (HPCs) were performed as described [6]. Cultures onto plastic-immobilized Notch ligand Ig-Delta-like4 (IgDll4) were conducted in 24-well plates under serum-free conditions in RPMI medium supplemented with 20% BIT 9500 (Stem Cell Technologies), Stem Cell Factor (SCF; 50 ng/mL), Flt3 ligand (FLT3L: 50 ng/mL), thrombopoieitin (TPO: 10 ng/mL) and Interleukin-7 (IL-7: 10 ng/mL). Plates were pre-coated with IgDll4 or bovine serum albumin (BSA: 10 μM/mL) 24 h before culture initiation.

2.3. Gene expression analyses

Quantification of selected cDNAs was performed as described [6] with relevant primer pairs (Table 1), and the 7500 Fast Real-time PCR system (Applied Biosystems). PCR products were detected by the SybrGreen dye and quantified with the Applied Biosystem Analysis Module.

2.4. In vitro assessment of T and B differentiation potentials

T and B cell differentiation assays have been described elsewhere [5,6]. For assessment of T cell differentiation, CD34^{+} HPCs transduced with the TMEM131L lentiviral vector or an empty vector (pRRL) were seeded onto OP9-Delta-like1 (OP9-DL1) stromal cells in α-MEM (Life Technologies) supplemented with 20% FCS (HyClone), 10 ng/mL SCF, 5 ng/ml FLT3L and 2 ng/mL IL-7, all from Miltenyi. Medium was changed every 6 days, and OP9-DL1 cells were renewed every week. Cells were harvested weekly for FACS analysis. For assessment of B potential, lentivirus-transduced cells were seeded for two weeks onto MS5 stroma cells in RPMI medium supplemented with 10% FCS, 1% penicillin-streptomycin, 1:1000 β-mercapto-ethanol (Life Technologies), SCF (10 ng/mL), TPO (10 ng/mL) and IL-7 (10 ng/mL), before FACS analysis. Monoclonal antibodies against CD45 (HI30), CD1a (HI149), CD8 (RPA-T8), CD14 (M5E2), CD19 (HIB19), CD34 (581), CD127 (A019D5) were from Biolegend; CD4 (RP4-T4), CD5 (UCHT2), CD7 (M-T701) and CD33 (WM53) antibodies were from BD Biosciences. Cells were analyzed and sorted using a FACSCanto II analyzer or a FACSAria III Cell sorter (both from BD Biosciences). Data were processed by the FlowJo software.

3. Results and discussion

To examine whether Wnt signaling inhibition by TMEM131L is evolutionary conserved, we expressed a transgene encoding human full-length TMEM131L in Drosophila, by using the *UAS-GAL4* system [7] (Fig. 1A). A *hedgehog (hh)-GAL4* driver was first used to express TMEM131L in the posterior compartment of the adult wing [8], allowing the anterior compartment to serve as internal control (Fig. 1B). *TMEM131L*-overexpression resulted in the reduction of wing posterior compartment size associated with a prototypic *Notch* mutant phenotype (Fig. 1C). The wing derives from a larval primordium called wing imaginal disc. Its development depends on a complex network of interactions between the Notch and Wingless (Wg) signaling pathways. During the third larval instar, Notch activation in cells abutting the dorso-ventral (D/V) boundary of the imaginal discs regulates wing growth and wing margin formation by upregulating secondary Notch target genes, *wg* and *cut* [9,10]. To assess whether the notching phenotype could be due to interference with Drosophila Notch signaling, we examined whether distribution of Cut and Wg proteins in larval wing imaginal discs was modified by *TMEM131L* expression in the posterior compartment of the disc (Fig. 1D). Immunofluorescence analysis confirmed *TMEM131L* expression in the posterior compartment and disclosed a strong reduction in Cut levels in cells of the posterior part of the D/V boundary (Fig. 1E). Wg levels were also reduced, but to a lesser extent. Consistent with decreased production of Wg ligand, Senseless (Sens), a short range high-threshold Wg target gene product [11], was undetectable in the posterior disc compartment (Fig. 1F, left panel). In contrast, *TMEM131L*-overexpression did not modify levels of Distal-less (Dll), a long-range low-threshold Wg target [12] (Fig. 1F, right panel). These data indicate that TMEM131L affects first the Notch and, thus, the Wg signaling pathway.

To confirm TMEM131L ability to interfere with Drosophila Notch signaling, the *pannier* (*pnr*)-*GAL4* driver was then used to express *TMEM131L* in the dorso-central region of the notum, allowing lateral areas to serve as internal controls [13] (Fig. 2A). The dorsal thorax (notum) is a well-established model system to screen for regulators of the Notch pathway [14]. During larval and pupal stages of Drosophila development, Notch successively acts through both lateral inhibition to restrict the number of external sensory organs within the notum and asymmetric cell division to control downstream fate choices. Consistent with a defect in Notch-

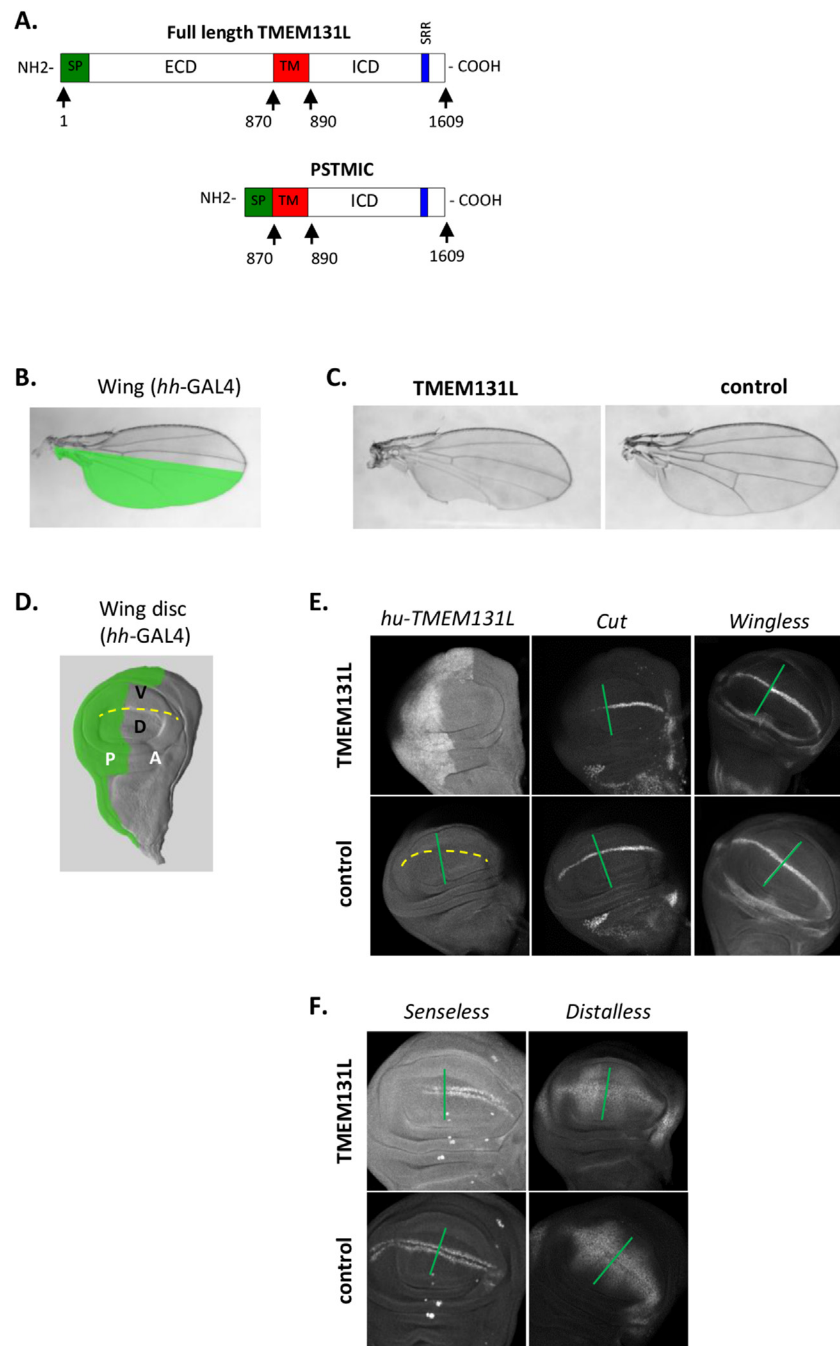
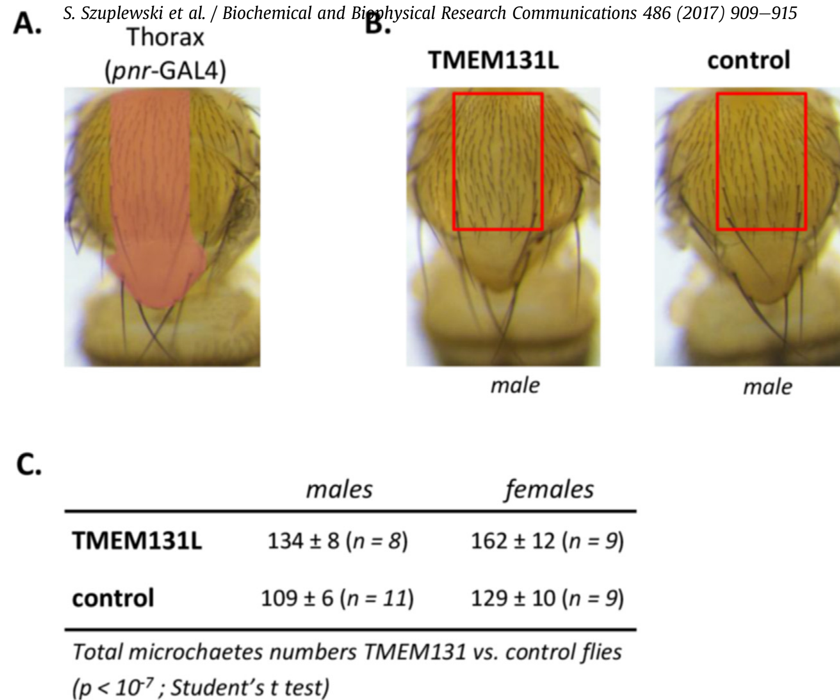


Fig. 1. Effect of human (hu) TMEM131L on Drosophila wing development.

(**A**). Schematic representation of full-length TMEM131L and of the membrane-associated PSTMIC derivative. Signal Peptide (SP; green square), Extra-Cellular Domain (ECD), Transmembrane domain (TM; aa: 870–890; red square), Intra-Cellular Domain (ICD), Serine-Rich Region (SRR; aa: 1302–1331; blue square).
(**B, C**). Effect of hu-TMEM131L on wing development. (**B**) *hh*-GAL4 expression domain in the posterior wing compartment (green area). (**C**) Preparations of adult female wings from *hh-GAL4>UAS-TMEM131L* (TMEM131L) and *hh-GAL4/+* (control) flies.
(**D, E**). Effect of hu-TMEM131L on the expression of canonical components of Notch and Wg pathways. (**D**) *hh*-GAL4 expression domain in wing imaginal discs (green area); the dashed line indicates the dorso-ventral (D/V; yellow dashed line) frontier. (**E**) Immunostaining of hu-TMEM131L and Drosophila Notch targets Cut and Wg gene products in wing imaginal discs from *hh-GAL4>UAS-TMEM131L* (upper panel, TMEM131L) or *hh-GAL4/+* (lower panel) third instar larvae; the thick and dashed lines indicate anterior-posterior (A/P, thick green line) and dorso-ventral (D/V; yellow dashed line) frontiers.
(**F**) Immunostaining of Wg target gene products Senseless and Distalless in wing imaginal discs from *hh-GAL4>UAS-TMEM131L* (upper panel, TMEM131L or *hh-GAL4/+* (lower panel, control) third instar larvae.



	males	females
TMEM131L	134 ± 8 (*n = 8*)	162 ± 12 (*n = 9*)
control	109 ± 6 (*n = 11*)	129 ± 10 (*n = 9*)

Total microchaetes numbers TMEM131 vs. control flies ($p < 10^{-7}$; Student's t test)

Fig. 2. Effect of hu-TMEM131L on Drosophila external sensory organ development.

(**A**) *pnr*-GAL4 expression domain in the dorso-central region of the thorax (red area).
(**B**) Examples of TMEM131L-mediated increase in bristle density in male adult flies. Left panel shows bristle density in a male adult TMEM131L-transgenic fly; right panel shows a *pnr-GAL4/+* male adult control. The *pnr*-GAL4 expression area is shown (red rectangle).
(**C**) Absolute bristle numbers in (n) male and female transgenic *pnr-GAL4>UAS-TMEM131L* or control flies.

dependent lateral inhibition, *TMEM131L*-overexpressing flies from both genders had a significant increase in microchaetes absolute numbers in the dorsocentral notum area compared with the lateral area (Fig. 2B and C). Notably, no change in identity and/or numbers of external cells of each microchaete was observed, which suggests that the human protein does not affect asymmetric cell division. Similar results were obtained in both systems (wing and notum) using several transgenic fly lines.

Since these results indicate that human TMEM131L selectively interferes with the Notch pathway in the Drosophila system, we next examined whether this is also the case in humans. Therefore, UCB CD34$^+$ HPCs were transduced with lentiviral vectors driving expression of either full-length TMEM131L protein or PSTMIC, its membrane-associated intracellular domain (Fig. 1A, lower panel), and cultured for 72 h onto plastic immobilized IgDll4 to activate Notch1 signaling pathway. The cells were then FACS-sorted based on GFP expression and processed for quantitative RT-PCR analysis (Fig. 3). As expected, both constructs inhibited Notch-dependent induction of its most sensitive target *DTX1* (with a stronger effect of the full-length construct) [15], but did not affect *HES1* or *NRARP* transcript levels. Consistent with these data, TMEM131L and PSTMIC also inhibited Notch-dependent upregulation of transcripts coding for GATA3, a transcription factor playing a key role during the early stages of T cell differentiation [16]. In contrast, ectopic TMEM131L or PSTMIC did not affect *IKZF1* transcript levels, attesting the specificity of the effect on Notch target genes. Of note, TMEM131L- and PSTMIC-transduced cells expressed lower levels of Notch1 transcripts, suggesting that down-modulation of surface Notch1 contributes to the reduced susceptibility of CD34$^+$ HPCs to Dll4 stimulation. That PSTMIC inhibited Notch1 signaling suggests in addition that, conversely to interference with the Wnt pathway which takes place at the level of the LRP6 signalosome and depends on TMEM131L extracellular domain [1], Notch inhibition depends in part on the intracellular signaling domain.

To determine whether these data were relevant to the cells' differentiation potential, we finally examined how TMEM131L ectopic expression affected Notch-dependent T cell differentiation *in vitro*. To this end, TMEM131L- or empty vector-transduced CD34$^+$ HPCs were cultured for 2 weeks onto OP9-DL1 cells under the T condition (Fig. 4A and B). Phenotypic characterization of their differentiated progeny showed that enforced TMEM131L expression did not interfere with cell growth (data not shown), but led to significant reduction of T cell development, with 50% decreased CD34$^-$CD7$^+$ T cell precursor percentages and correspondingly increased percentages of CD33^{++}CD34$^-$CD7$^-$ monocytic cells, some of which expressed surface CD11b (Fig. 4C). TMEM131L did not interfere with Notch-independent upregulation of CD4 and the subsequent acquisition of CD8 after passage through β-selection by T cell precursors, which reinforces the view that ectopic TMEM131L selectively interferes with Notch-dependent commitment of CD34$^+$ HPCs toward the T lineage. As expected, TMEM131L did not affect CD19$^+$ B cell output as compared with empty vector-transduced cells, arguing again for the specificity of its interference with T cell development (Fig. 4D). We, therefore, concluded that TMEM131L-dependent inhibition of Notch1 signaling restricts T cell differentiation and promotes myeloid diversion of CD34$^+$ HPCs.

Collectively, these results identify TMEM131L as an evolutionary conserved inhibitor of the Notch signaling pathway. Inasmuch as our unpublished results show that TMEM131L does not interfere with Notch1 cleavage and subsequent nuclear translocation, nor does it affect the transcriptional activity of Notch1 intracellular domain in a luciferase RBPJ reporter assay (data not shown), we speculate that down-modulation of surface Notch1 contributes to TMEM131L interference with Notch signaling pathway.

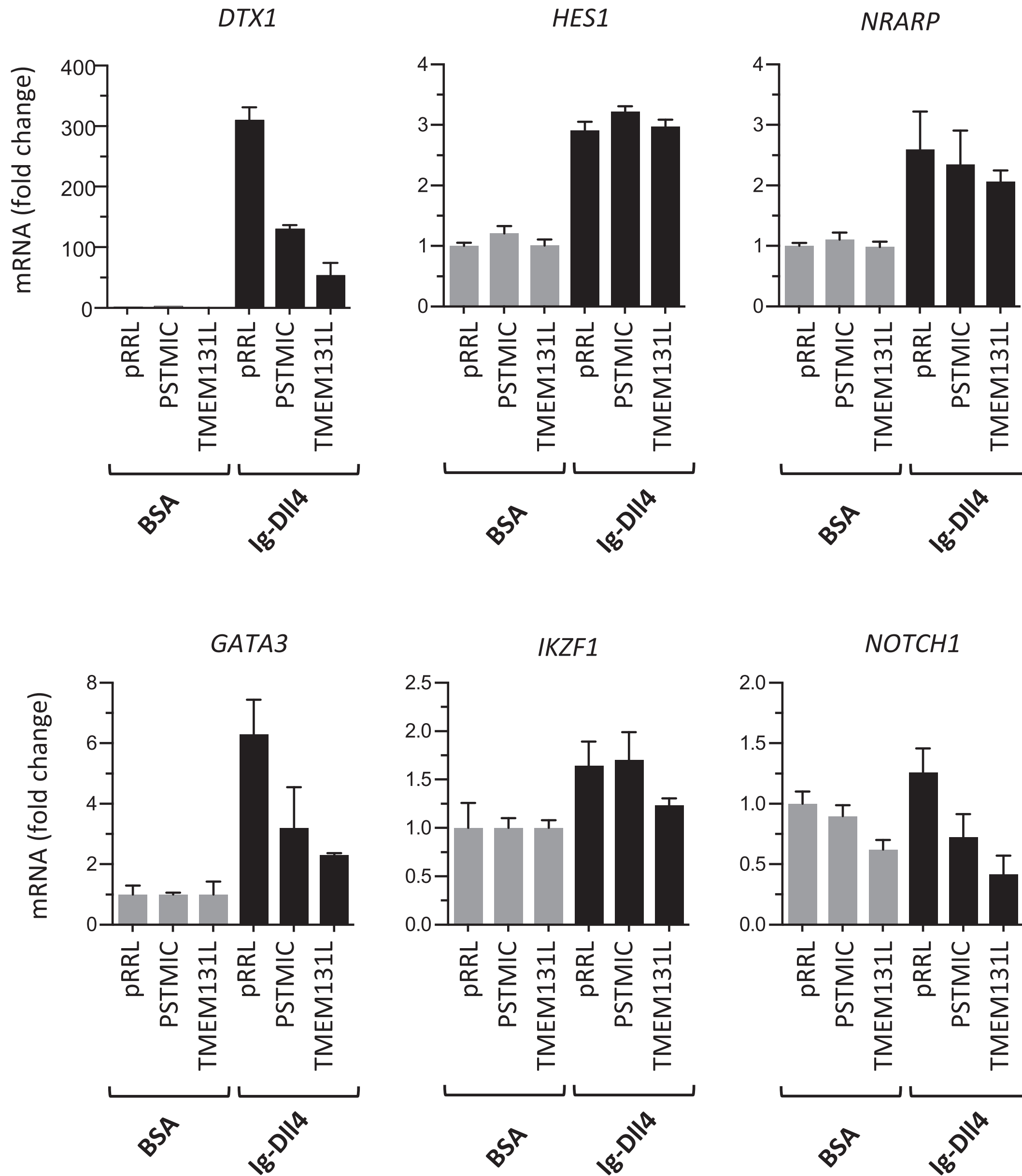


Fig. 3. Effect of TMEM131L on CD34+ HPC transcriptional responses to Notch1 ligation.

CD34+ HPCs transduced with lentiviruses driving ectopic expression of TMEM131L or membrane-associated truncated PSTMIC, or the empty vector (pRRL), were cultured for 72 h onto plastic-immobilized IgDll4 or BSA in the presence of SCF, FLT3L and IL-7. The GFP+ cells were then sorted by FACS and analyzed by RT-qPCR using HPRT as a reference. Transcript levels of the indicated genes are normalized relative to those detected in non-stimulated pRRL-transduced cells and expressed as fold changes. Bar plots show mean ± SD of triplicates. Data are from one experiment out of three.

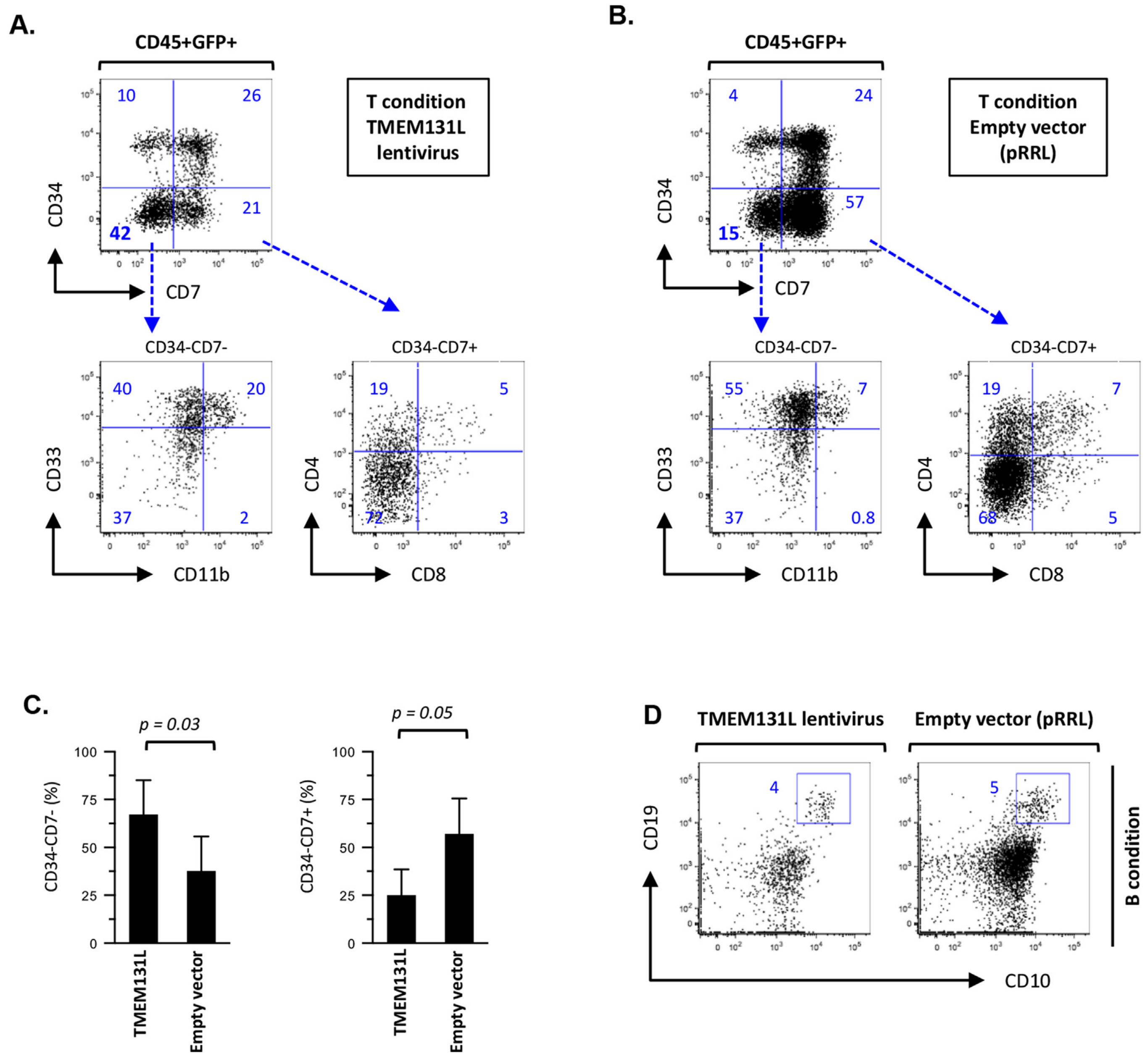


Fig. 4. TMEM131L ectopic expression selectively interferes with T cell development.

(**A**, **B**) Effect of TMEM131L on T cell development. CD34$^+$ HPCs transduced as above with TMEM131L or the empty vector (pRRL) were cultured for two weeks under the T condition before FACS analysis. Gates are set on CD45$^+$GFP$^+$ transduced cells. Dashed arrows show the gating strategy. Bi-dimensional dot-plots show expression of CD34/CD7, CD4/CD8, CD33/CD11b.
(**C**) Bar plots show the percentages of CD34$^-$CD7$^-$ monocytic and CD34$^-$CD7$^+$ T cell precursors. Data are from two independent experiments performed in duplicates; assessment of statistical significance is based on the unpaired Student's *t*-test; the exact p value is indicated.
(**D**) Effect of TMEM131L on B cell development. CD34$^+$ HPCs transduced with TMEM131L or the empty vector (pRRL) were cultured for two weeks under the B condition before being analyzed as above. Gates are set on CD45$^+$GFP$^+$ cells. Bi-dimensional dot-plots show expression of CD19/CD10. Data are from one experiment out of three.

Finally, our results stress the pertinence of using hetero-specific Drosophila transgenic systems to decipher the function of yet poorly characterized human proteins.

Conflict of interest

The authors disclose no potential conflicts of interest.

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