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Supplemental Information

TMEM88 Inhibits Wnt Signaling by Promoting Wnt Signalosome Localization to Multivesicular Bodies

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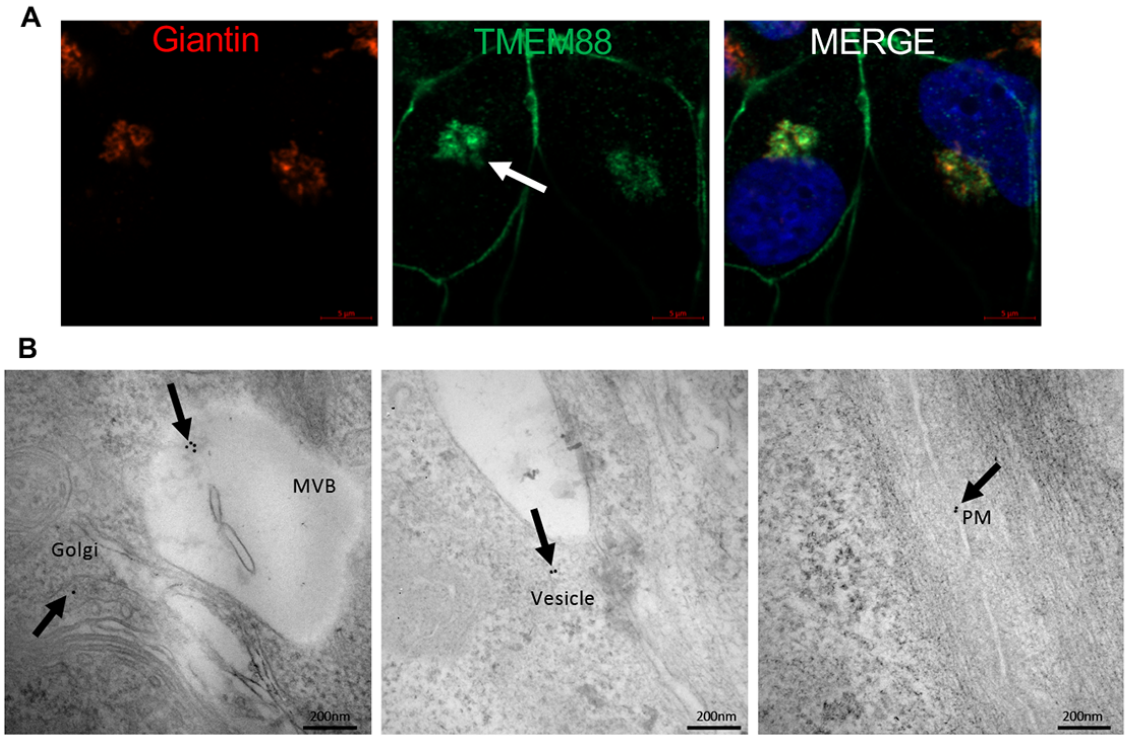
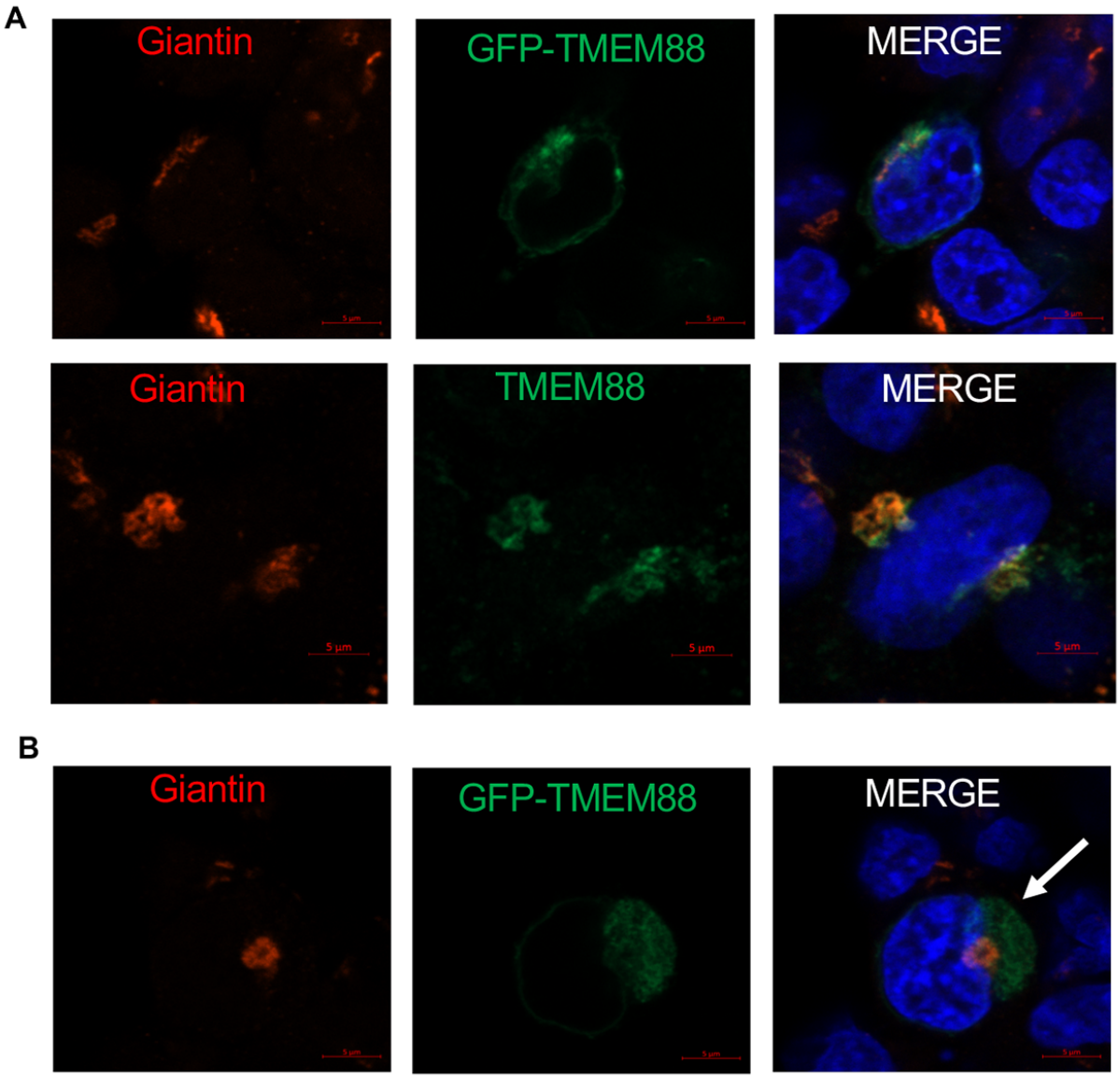
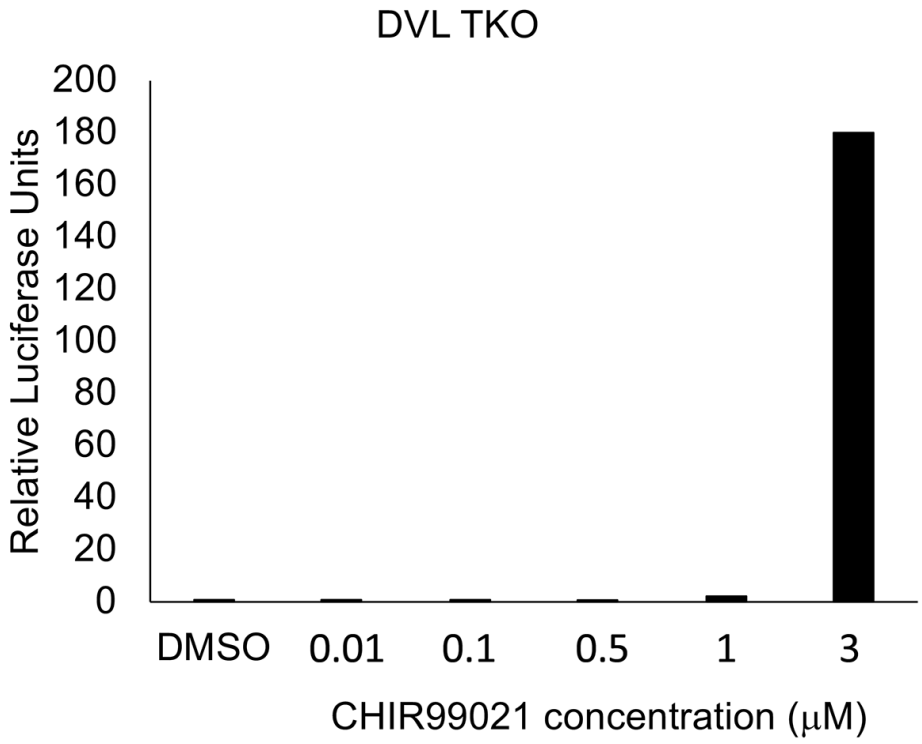


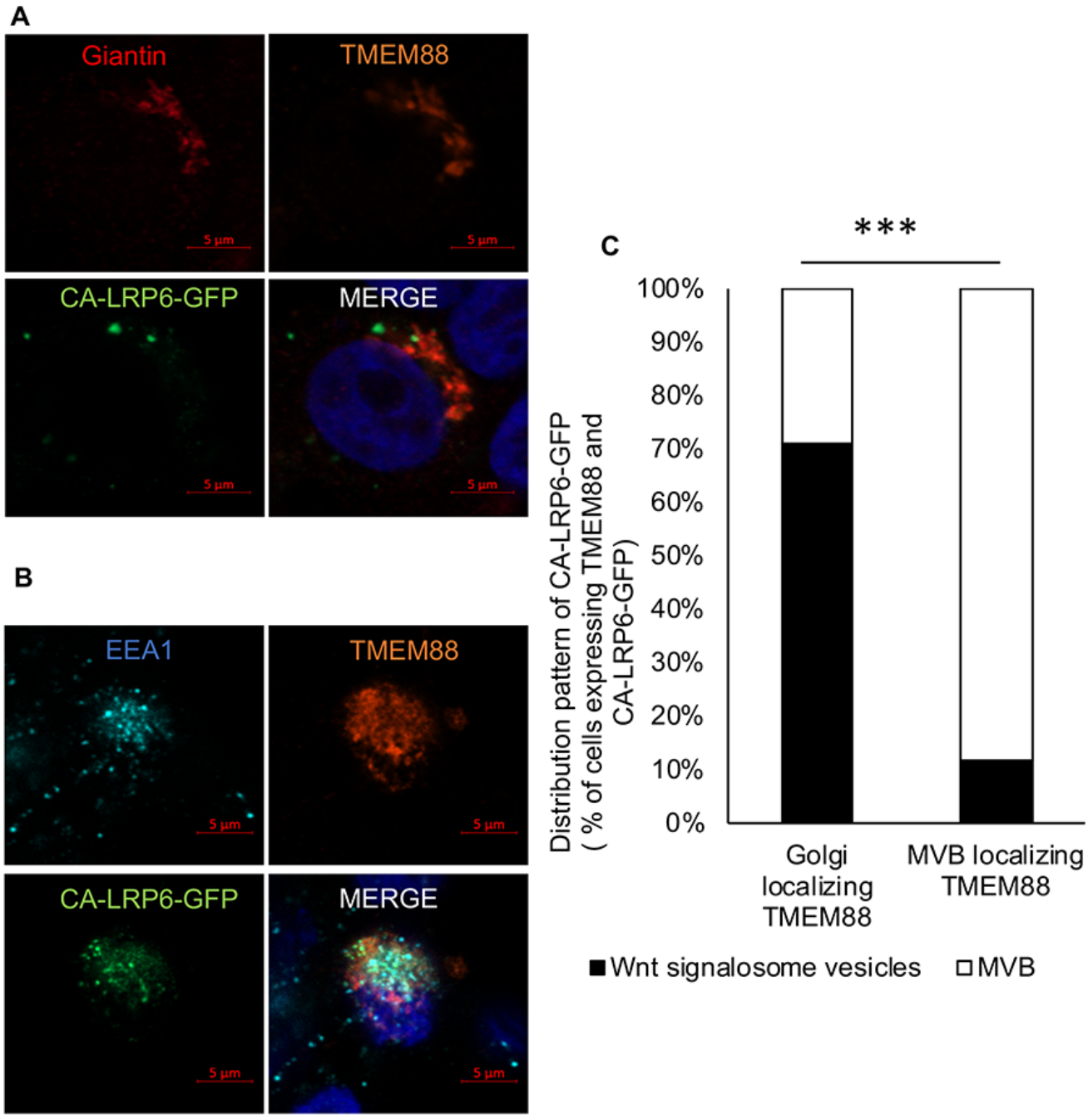
Supplementary Fig. S1. In cardiac progenitor cells TMEM88 is found in Golgi and perinuclear MVB (Refers to Fig. 2). A) Immunofluorescence of the Golgi marker Giantin (left), TMEM88 (center, white arrow indicating Golgi staining) and merged panels also showing the nucleus (right) in hESC-derived day 7 differentiating cardiac progenitor cells. B) Representative immunoelectron microscope images demonstrating endogenous TMEM88 (black arrows) in MVB and Golgi (left), vesicle membrane (center) and plasma membrane (right) in hESC-derived day 7 cardiomyocyte progenitor cells.



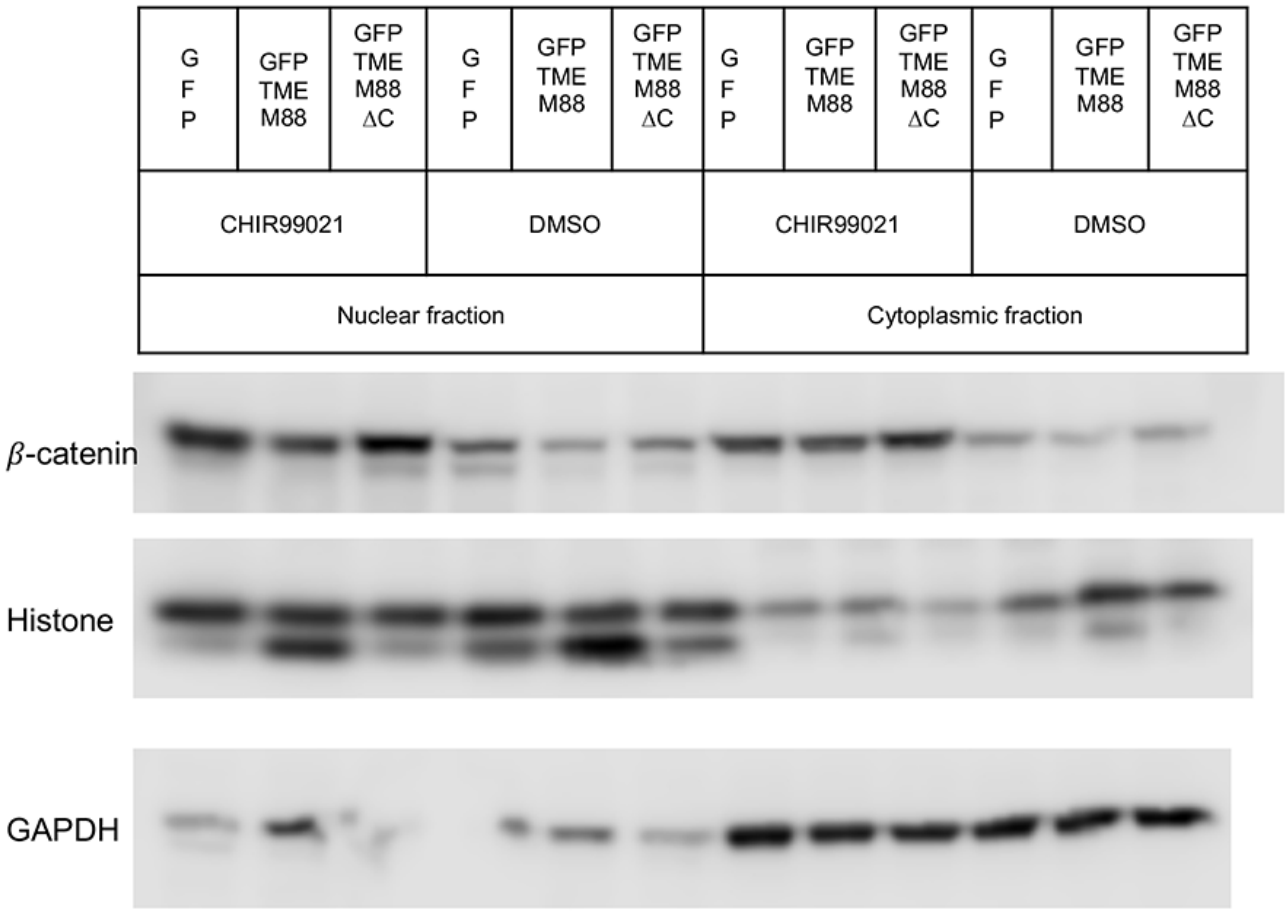
Supplementary Fig. S2. In 293T cells, exogenously expressed TMEM88 is found in Golgi and perinuclear MVB (Refers to Fig. 3). A) 293T cells were transfected with plasmid vectors expressing GFP-TMEM88 or non-tagged TMEM88 and immuno-stained with anti-Giantin antibodies. Panels show Giantin, TMEM88, and the merge, left to right. B) 293T cells were transfected with a vector expressing GFP-TMEM88 and analyzed by immuno-fluorescence with the indicated antibodies. The right panel is a merge, with a white arrow indicating perinuclear TMEM88 that does not co-localize with Giantin.



Supplementary Fig. S3. Titration experiment to define concentration of CHIR99021 required for robust activation of the TOPflash reporter (Refers to Fig. 4). Transfections were carried out in DVL TKO cells with the TOPflash luciferase and Renillla luciferase reporters as described in Methods. CHIR99021 was added at the indicated concentration (or DMSO as negative control) and after 24 hr cells were lysed and luciferase activity measured relative to the Renilla internal control. Shown is a representative result of 3 independent experiments that generated equivalent results.



Supplementary Fig. S4. The LRP6-induced signalosome co-localizes with TMEM88 (Refers to Fig. 6). A) 293T cells were transfected with plasmid vectors expressing non-tagged TMEM88 and CA-LRP6-GFP and immuno-stained with Giantin and TMEM88 antibodies. B) To identify localization to MVB, 293T cells were transfected with plasmid vectors expressing non-tagged TMEM88 and CA-LRP6-GFP and permeabilized with 65 µg/ml digitonin containing RB buffer, fixed, permeabilized with 0.2% triton and stained with anti-EEA1 antibody and anti-TMEM88 antibody. C) Quantification of A and B. Chi squared p value: $2.12E^{-86}$. For cells with TMEM88 localizing to Golgi, N=52. For cells with TMEM88 localizing to MVB, N=26.



Supplementary Figure S5. Expression of TMEM88 shifts localization of b-catenin predominantly to the cytoplasmic MVB (Refers to Fig. 7). 293T cells were transfected with indicated plasmid vectors. After treatment with CHIR99021, cell lysates were fractionated into cytoplasmic and nuclear fractions and probed by western blotting using the indicated antibodies.

Supplemental Movie S1 (Refers to Fig. 3). The movie shows TIRF live imaging of 293T cells stably transfected with an expression vector for GFP-TMEM88. The GFP+ puncta demonstrate membrane localized GFP-TMEM88. The pattern is very dynamic, suggesting that GFP-TMEM88 is moving onto and then off of the plasma membrane (endocytosis).

Transparent Methods

Plasmids, Antibodies, and Other Reagents

Antibodies used included rabbit anti-TMEM88 (abcam151166), mouse anti-beta-actin (Sigma Aldrich A1978), mouse anti-EEA1 (BD Biosciences 610457), mouse anti-NKX2.5 (Santa Cruz sc-376565), mouse anti-Giantin (Abcam ab37266), mouse anti-beta-catenin (Cell signaling technology 2677S), rabbit anti-beta-catenin (Cell signaling technology 9562S), rabbit anti-sodium-potassium-ATPase (Abcam EP1845Y), mouse anti-VPS4 (Santa Cruz sc133122), mouse anti-cardiac-troponin-T (ThermoFisher MA5-12960), rabbit Histone H3 (Cell signaling technology 9717S), GAPDH (Invitrogen), ER-tracker red (Bodipy TR glibenclamide E34250 Thermo Fisher). Secondary antibodies were goat anti-mouse HRP (Biorad), goat anti-rabbit HRP (Biorad), goat anti-mouse Alexa 488 (Invitrogen), goat anti-rabbit Alexa 647 (Invitrogen), anti-rabbit Alexa 568 (Invitrogen). All cytokines were purchased from R&D systems. CHIR99021 was purchased from Stem Cell Technologies. The details on the expression plasmids are: pCS2-CA-LRP6-GFP (Addgene 29682), pCS2-GSK3-RFP (Addgene 29679), pCAG-mGFP (Addgene 14757), pIRESpuro3 vector (Clontech631619), pEGFP-C1. A full-length human *TMEM88* cDNA was purchased from Open Biosystems. Various mutants were generated by PCR and subcloned into the mammalian expression vectors.

Cell culture, transfection, and stable cell lines

H1 human ESCs and Heus8 ESCs were plated on Matrigel-coated plates and maintained in mTeSR1 media (Stem Cell Technologies). Human embryonic kidney 293T cells were cultured in DMEM (VWR) supplemented with 10% FBS, L glutamine (VWR), and penicillin/streptomycin (VWR). HCT116 (ATCC CCL247) was cultured in McCoy's 5A media (ATCC) supplemented with 10% FBS. The Dishevelled triple knock out 293T (DVL TKO) cell line was a kind gift from Dr. Stephane Angers. Plasmid transfections were performed using Lipofectamine LTX (Invitrogen), according to the manufacturer's protocol. To generate the GFP-TMEM88 stable cell line, pIRESpuro3 GFP-TMEM88 expression plasmid was linearized and transfected into HEK 293T cells cultured in the presence of 1.5 μg/ml of puromycin for two weeks.

Immunofluorescence and microscopy

For immunostaining, all cells were fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized for 10 min using 0.05% saponin or 0.2% TritonX100, blocked for 1 hr with 1% goat serum, incubated with primary antibody for 1 hr at RT, washed and subsequently incubated with fluorescent secondary antibodies for 1 hr at RT. Cells were washed again and incubated for 10 min with DAPI for nucleus identification. Cells were mounted in ProLong Gold (Invitrogen) and visualized using a Zeiss fluorescence microscope. For nuclear β-catenin quantification, ZEN image analysis software was used to calculate mean fluorescence intensity of selected areas.

Quantitative RT PCR

For quantitative RT-PCR, total RNA was extracted using RNeasy miniprep Kit (Qiagen) and first strand cDNA was synthesized using SuperScript VILO cDNA synthesis kit (ThermosFisher). Quantitative RT-PCR was performed using light cycler 480 SYBR-green (Roche). The expression level for each gene is normalized to the expression level of HPRT.

Primers used are listed in the table below.

Gene name	Forward Primer	Reverse Primer
HPRT	ACCAGTCAACAGGGGACATAA	CTTCGTGGGGTCCTTTTCACC
Brachyury	ACCCAGTTCATAGCGGTGAC	CCATTGGGAGTACCCAGGTT
EOMES	CTGCCCACTACAATGTGTTCG	GCGCCTTTGTTATTGGTGAGTTT
MIXL1	GGCGTCAGAGTGGGAAATCC	GGCAGGCAGTTCACATCTACC

Immunoelectron Microscopy

Day 7 H1 ESC-derived cardiomyocyte progenitor cells were washed with serum-free media and fixed with 2.5% glutaraldehyde, 4% paraformaldehyde, 0.02% picric acid in 0.1M buffer as above followed by secondary fixation with 1% OsO_4-1.5%K-ferricyanide. *En bloc* staining and dehydration with 1.5% Uranyl Acetate in water 60 min, in the dark, then 50, 70, 85, 95, 100,100,100% EtOH treatments for 5-15 minutes each step, followed by infiltration with resins. Antigenic sites were opened by using saturated Na-

periodate, unreacted aldehydes were quenched with 50 mM glycine in PBS, and blocked for host of secondary antibody. Primary antibody incubation in PBS-c (PBS 0.2% BSA-c (Aurion, EMS) overnight at 4C. A control lacking primary antibody was also included. After washing in PBS-c, secondary antibody incubation was carried out in Aurion gold conjugate 1:100 in blocking buffer and fixed at 2.5% glutaraldehye in 0.1M buffer and contrast with uranyl acetate followed by water wash and air dry. All steps were carried out on clean parafilm in a humid chamber.

Reporter assays

For cell-based luciferase assays, HEK 293T, HCT116, DVL TKO HEK 293T were plated, co-transfected with TOPflash and Renilla expression plasmids (Promega), GFP-TMEM88 expression plasmids or control plasmids, and after 24 hr lysed using the Dual-Glo Assay (Promega). Luciferase assays were conducted according to the manufacturer's protocol (Promega). Titration experiments demonstrated that 3 μM CHIR99021 was necessary and sufficient to generate robust and reproducible luciferase signals (Sup. Fig. S3) and this concentration was used in all experiments. Luciferase signal was normalized to Renilla expression. All data were normalized to the signal obtained from a negative control. Assays were performed in duplicates and repeated at least three times.

Flow cytometry

Cells were harvested using Accutase (Sigma) and resuspended in FACS buffer (10% FBS, DMEM). After fixing in 2% paraformaldehyde at RT for 30 minutes, cells were

incubated with cTnT antibody for 1 hr at RT, followed by incubation with appropriate secondary antibody for 1 hr at RT.

Gene editing with the CRISPR/Cas9 system

H1 derived TMEM88 KO ESC lines were generated using the iCRISPR method previously described (Gonzalez et al.2014). A target sequence (GCTGTCACCATGCTGGGCTT) was inserted into a single stranded T7-gRNA *in vitro* transcription template. The ssDNA was PCR amplified, *in vitro* transcribed using the MEGAshortscript T7 kit (Life Technologies), and the sgRNAs were purified by using the MEGAclear kit (Life Technologies) and stored at -80C until use. iCas9 hESCs were treated with 2 μg/ml doxycycline for one day before and during transfection to induce expression of Cas9 protein. For transfection, cells were replated on Matrigel coated plates and transfected in suspension with sgRNAs using lipofectamine RNAiMAX (Life Technologies). After transfection, cells were single cell plated onto a 96 well plate and clones were screened by genotyping, and then confirmed by genomic DNA sequencing and western blotting. Similar lines were also established using HUES8 parental lines.

Human ESC directed differentiation

The cardiac differentiation protocol was adapted from a previously described protocol (Kattman et al., 2011). In brief, from day 1 to day 5 medium contains RPMI1640 (Thermo scientific), 0.5X B27(ThermoFisher), (1mM) Ascorbic acid (Sigma), (2mM) L-Glutamine (VWR), Transferrin (Roche), and ($4x10^{-4}$ M) MTG (Sigma). From day 5 to 14, the 0.5X B27/RPMI1640 medium contained Ascorbic acid, L-Glutamine, and MTG. 20 ng/ml BMP,

30 ng/ml Activin A, and 5 ng/ml bFGF was added from day 1 to day 2. 5uM XAV and 5 ng/ml VEGF was added from day 3 and 4. In a subset of experiments, XAV939 (Sigma) was removed on day 4. From day 5 to day 9, 5 ng/ml VEGF was added. From day 10 onward, VEGF was removed. For a subset of experiments using HUES8 TMEM88 KO cell line, 9 μM CHIR was added in RPMI1640/B27 (minus insulin) from Day 0 to 1 and 5 μM of IWP2 (Tocris) was added from Day 3 to 5.

Western Blotting

Cells were harvested in in lysis buffer containing 10mM Tris pH7.5, 150mM NaCl, 50mM NaF, 1% NP40 and protease inhibitor. Samples were separated by Bis-Tris gel and transferred to PVDF membrane followed by blocking with 5% milk in Tris-based saline with 0.1% Tween-20. The membrane was incubated with primary antibodies overnight at 4C, followed by incubation with HRP-conjugated secondary antibodies.

Cellular Sub-fractionation

Cells were fractionated as previously described (Holden and Horton, 2009). Briefly, Digitonin soluble fractions were lysed in 25 μg/ml digitonin, 150 mM NaCl, and 50 mM HEPES (pH7.4), and digitonin resistant fractions were lysed in a buffer containing 150 mM NaCl, 50mM HEPES (pH 7.4), and 1% NP40. Nuclear and cytoplasmic fractionation was performed by following the manufacturer's protocol for NE-PER™ Nuclear and Cytoplasmic Extraction reagents (ThermoFisher #78833).

Total Internal Reflection Fluorescence microscope imaging

Membrane localizing GFP-TMEM88 was observed using a total internal reflection fluorescence inverted microscope equipped with an Andor EMCCD camera.

Statistics

Unpaired, two tailed Student's t-test with equal variances were run using Excel. Chi-squared tests were used for a subset of experiments where indicated.