Supplementary information

The file contains Supplementary methods and supplementary figures S1-S5 with legends. Supplementary figures show additional data in support of the manuscript's conclusions

SUPPLEMENTARY METHODS

Cell cultures Human primary and metastatic melanoma cell lines derived respectively from primary and metastatic tumor lesions of patients surgically resected at the Istituto Nazionale dei Tumori, Milan, Italy. All cells employed in the current study were designated by either PM or MM, followed by a progressive number. Human peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque (Pharmacia) density gradient of buffy coats from healthy donors. Monocytes were separated from PBMC using CD14 labelled Miltenyi microbeads according to manufacturer's indications and were left to differentiate for 2 weeks at 37°C in RPMI 1640 plus 15% FCS. Remaining peripheral blood lymphocytes (PBL), were obtained after CD14 beads mediated monocyte ablation. CD14+ and CD14 negative PBMC were cultured for two weeks in RPMI 1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 10% FCS in a 5% CO2 environment at 37°C. (All reagents were purchased from Cambrex). Normal human epidermal melanocyte neonatal cells (NHEM-neo) (Lonza) cells were cultured exactly as prescribed by the supplier. Media was Clonetics Melanocyte Basal Medium (MBM-4) BulletKit^{R} with constituents at a final concentration of 0.5% fetal bovine serum, 7.5 μg bovine pituitary extract per ml, 2 ng human recombinant fibroblast growth factor per ml, 16 nM phorbol myristate acetate (PMA), 10 μg insulin per ml, 10 μg hydrocortisone per ml, 100 μg gentamicin per ml, and 100 ng amphotericin-B per ml.

Western Blotting and immunoprecipitation. Whole melanoma cell lysates and CCD-1064SK healthy skin fibroblasts (SantaCruz) were resuspended in SDS sample buffer, denaturated by boiling, separated by SDS-PAGE, and subjected to Western blot. 6xHis tagged protein, GFP, TM9SF4, and GAPDH, were respectively detected with anti 6His mAb

(Sigma), anti-GFP (clone 1E4 MBL), anti-TM9SF4 mouse serum and anti GAPDH (SantaCruz) . TM9SF4 proteins were immunoprecipitated overnight at 4 °C in the presence of protein A+G-Sepharose beads (Pierce) from precleared cell lysates, employing rabbit anti TM9SF4 polyclonal antibody. As a negative control rabbit preimmune serum was used. Actin was detected with anti actin monoclonal antibody (Sigma).

Qproteome Cell compartment fractionation. Cells were harvested and processed according to Qproteome plasma membrane kit protocol (Quiagen) in order to obtain non denatured fractions of cellular compartments corresponding to: purified plasma membranes and cytosol. The latter fractions were then precipitated with acetone and resuspended in RIPA buffer in order to be subjected to immunoprecipitation with rabbit anti-TM9SF4. Residual pellet from cellular compartment fractionation, containing intact cells and organelles, was deprived of the former through centrifugation and subjected to Triton X-100 extraction in order to obtain soluble and insoluble fractions which were immunoprecipitated with rabbit anti-TM9SF4. Following electrophoresis of samples, the nitrocellulose was blotted with TM9SF4 mouse antiserum.

Immunocytochemistry and immunohistochemistry. For immunocytochemistry melanoma cells and macrophages, cultured on glass chamber slides (Falcon), and PBL, cytospun on glass slides, were fixed with 80% methanol 10 min 4°C and stained with TM9SF4 mouse serum or preimmune control serum. Malignant melanoma and corresponding normal skin tissue from Biomax array slides (Biomax) were immunostained with pre-immune serum, for anti-GP100 (Immunotech), for anti-TM9SF4 mouse antiserum. Proteins were visualized using

the peroxidase anti-peroxidase method in single staining (Dako) and counterstained with Mayer's hematoxylin.

Laser Scanning Confocal Microscopy (LSCM). MM2 cells were seeded on microscope slide cover slips placed in 60-mm Petri dishes. After 24 hours cells were fixed with 2% paraformaldehyde and permeabilized (Triton X-100 0.1%). For TM9SF4 and RAB5 double staining, cells were labelled with mouse anti-TM9SF4 serum and rabbit anti-RAB5 (SantaCruz) and respectively revealed with Alexa Fluor 594-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes) For TM9SF4 and LAMP1 detection, cells were labelled with rabbit anti-TM9SF4 antibody and mouse anti-LAMP1monoclonal antibody (BD Pharmingen), respectively stained with Alexa Fluor 594-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG. TM9SF4 and EEA-1 were detected with anti-TM9SF4 rabbit serum and anti EEA-1 goat serum (SantaCruz) and respectively labelled with Alexa Fluor 594-conjugated anti-rabbit IgG and with Alexa Fluor 488-conjugated anti-goat IgG. TM9SF4 and mitochondria were detected staining TM9SF4 with anti-TM9SF4 rabbit serum and labelled with Alexa Fluor 594-conjugated anti-rabbit IgG, while mitochondria were labelled with MitoTracker Green (Invitrogen). After washings, all samples were mounted with glycerol:PBS (2:1) and observations were performed using a Leica TCS SP2 spectral confocal microscopy equipped with argon-helium neon (Ar-HeNe) lasers. The excitation wavelength used was 488nm for indirect staining of EEA1, RAB5, LAMP1, and MitoTracker Green 186 (Invitrogen); the excitation wavelength used was 543nm to evaluate TM9SF4. Emission lines were collected after passage through a double dichroic filter (DD488/543) in a spectral window ranging from 580-783nm. For EEA1 (493-552nm, spectral window range); for RAB5 (496-551nm, spectral window range); for

LAMP1(496-552nm, spectral window range); for MitoTracker (490-560nm, spectral window range) were used. Signals from different fluorescent probes were taken between frames in sequential scan mode, which allows the elimination of channel cross talk. The colocalization was detected in an overlay model. Acquisition parameters were as follow: 63.0/1.4 NA. HCXPLAPOCS objective; image size: 1024x1024; pinhole size: 1 Airy; scan mode xyz, step size: 0.5µm. Images were processed by using LCS (Leica Microsystems. Heidelberg GmbH, Germany) software program.

Lysotracker staining. Human melanoma cells were stained with 50 nM LysoTracker probe (Molecular Probes) for 30 minutes at 37°C and immediately analyzed by FACS. Comparison among different melanoma cell lines was conducted by CellQuest software using the mean values of fluorescence intensity histograms.

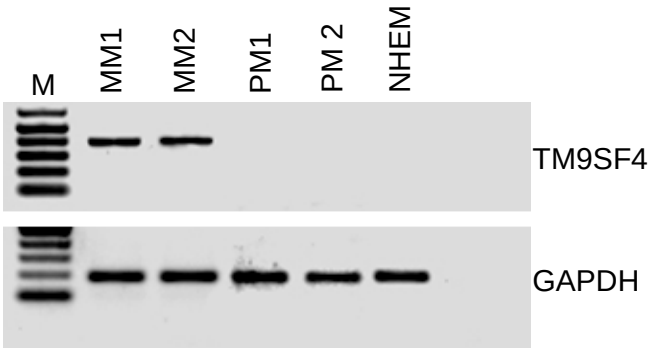

Fig S1 RT-PCR analysis of *TM9SF4* and *GAPDH* on two metastatic melanoma cell lines (MM1-2), two primary melanoma cell lines (PM1-2) and normal human epidermal melanocytes neonatal (NHEM). M: molecular size marker.

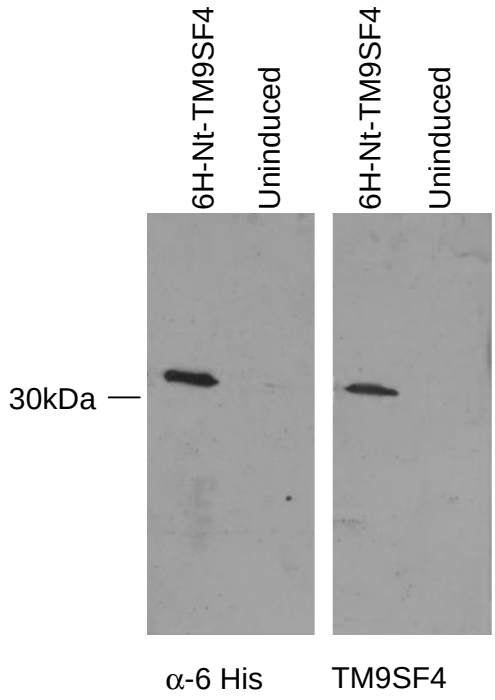

Fig S2 Western blot analysis of 6-histidine tagged TM9SF4-1 peptide (6H-Nt-TM9SF4) and in uninduced bacterial lysated immunoblotted with α-6 His antibody or TM9SF4 mice antisera. Each lane was loaded with 20 ug of proteins.

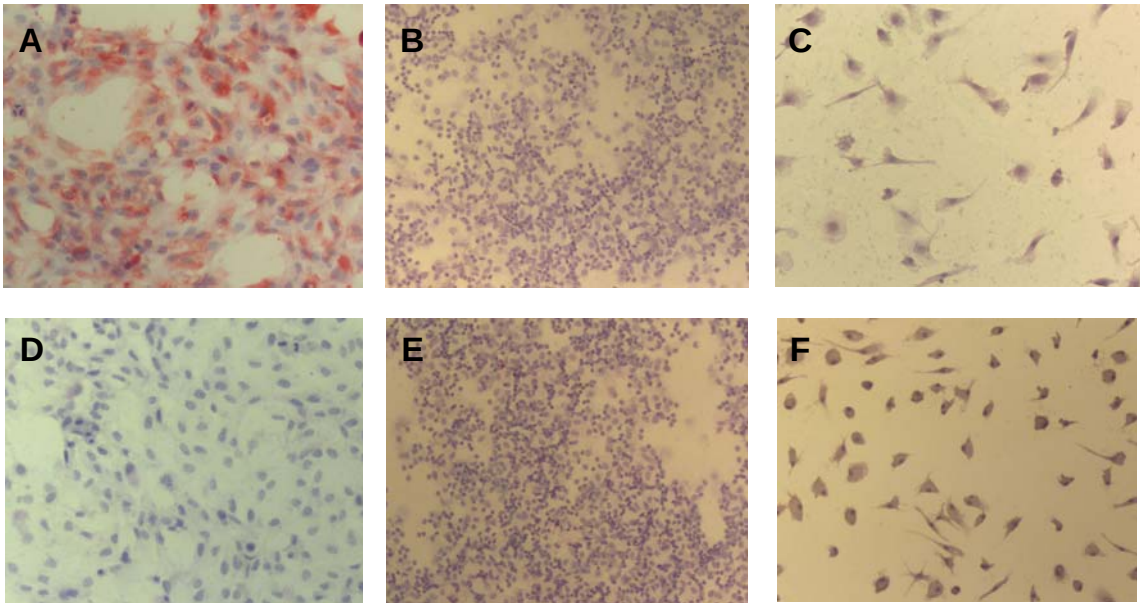

Fig S3 Immunocytochemical analysis of TM9SF4 on: (**A**) MM2 cells; (**B**) peripheral blood lymphocytes (PBL) and (**C**) macrophages. Cells were stained with anti TM9SF4 mouse serum. Immunocytochemical analysis of mouse preimmune serum staining of: (**D**) MM2 cells; (**E**) PBL and (**F**) macrophages. Nuclei were stained with Meyer's ematoxilin. Magnification 10X.

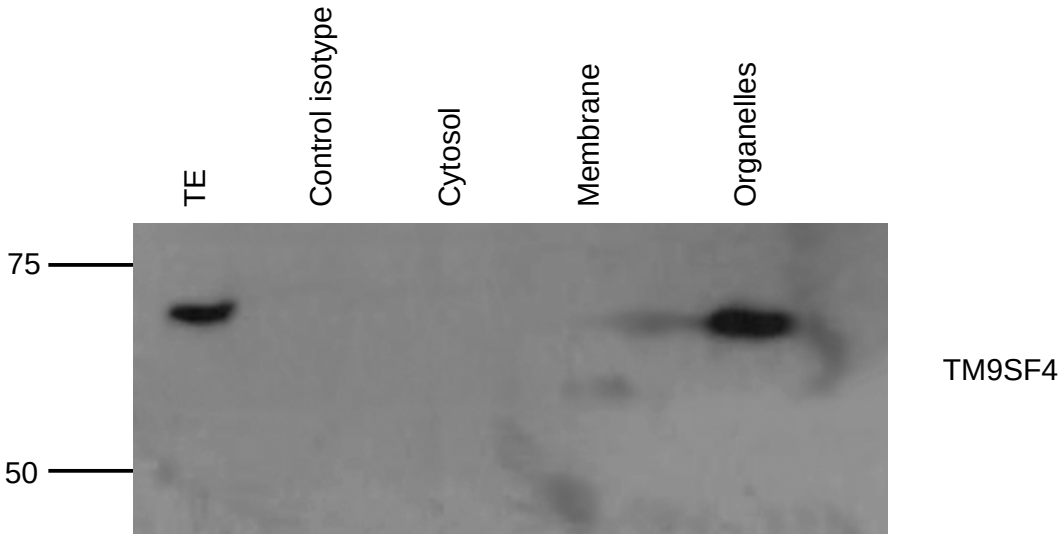

Fig S4. Western blot analysis of subcellular fractions of TM9SF4 immunoprecipitates from MM2 lysates. TE total extracts. MM2 whole cell lysates were immunoprecipitated with anti-TM9SF4 antibodies and various subcellular fractions were separated utilizing the Qproteome plasma membrane kit as described in material and methods. The obtained fractions were analyzed by Western blot.

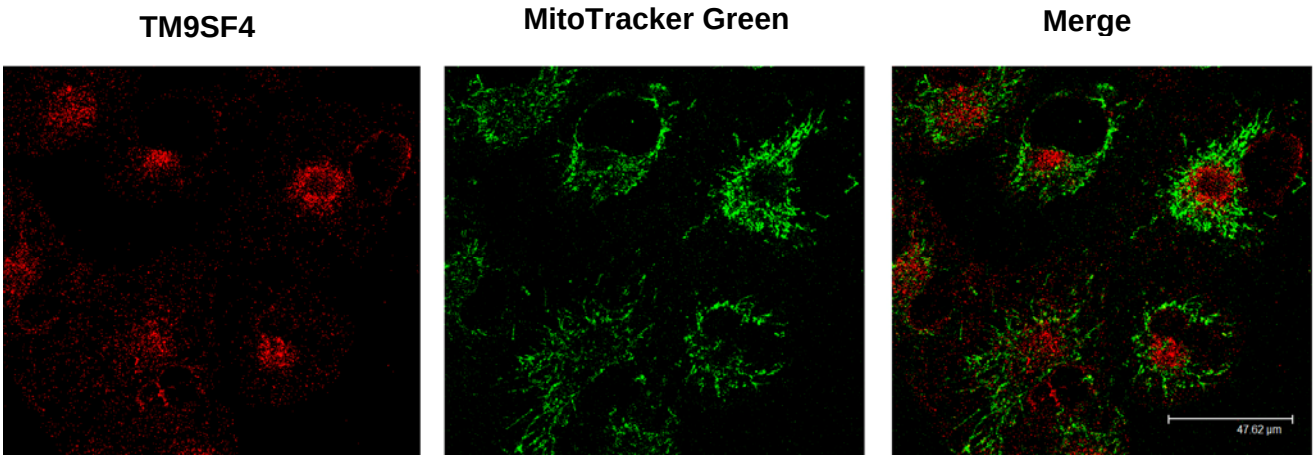

Fig S5. Laser Scanning Confocal Microscopy (LSCM) analysis of TM9SF4 (red) and MitoTracker Green. Lack of yellow/orange areas in merge picture indicate absence of co-localization.