Supporting Information

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SI Materials and Methods

Cell Culture. HEK 293T cells were cultured in DMEM containing 10% FBS, 1% glutamine, and 1% Pen/Strep. All melanoma cells were cultured in RPMI 1640 (with L-glutamine) (Life Technologies) containing 10% Tetracycline-free FBS (Omega Scientific), 1% Pen/Strep. For C32 melanoma cells, 0.5 μg/mL Blasticidin, and 100 μg/mL Zeocin were added to maintain selection of the Tet-inducible MITF. All cells were cultured at 37 °C in 5% CO_2.

***Xenopus laevis* Embryo Assays.** For *Xenopus* mRNA microinjections, mRNAs were synthesized with mMessage mMachine SP6 (Ambion). For Wnt signaling assays, 20 pg of the *TCF SuperTopFlash luciferase* (1) reporter were coinjected with *Wnt8* (2 pg), *MITF* (100 pg), HRS morpholino (4 nL of 0.3 mM HRS MO), or *Vps4EQ* mRNA (500 pg) were coinjected. Equal amounts of total mRNA were injected in all samples by adding *GFP* mRNA. For embryo Wnt signaling assays through chordin in situ hybridizations, suboptimal amounts of *Wnt8* mRNA (0.1 pg) were injected with or without the addition of *MITF-M* mRNA (320 pg). For analyzing the effect of the novel putative GSK3 sites in MITF on protein stability, *Xenopus* embryos were microinjected four times at the four-cell stage with 80 pg of *MITF-WT* or *MITF-GM* mRNA, and lysed at stage 13 with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and Protease inhibitor #10863600 from Roche). *Xenopus* whole-mount in situ hybridizations were carried out according to the protocol described (2).

Knockdown Experiments, DNA Transfections, Reverse Transfection, and Lentiviral Transductions. For HEK 293T cells, DNA constructs were transfected with BioT (Bioland Scientific) 24 h after plating cells. In C32 melanoma cells, RFP-GSK3 DNA was transiently incorporated by reverse transfection with Lipofectamine 2000 following the reverse transfection protocol from Invitrogen. For knockdown of HRS in C32 cells, siRNAs targeting human HRS were ON-TARGETplus SMARTpool siRNAs (L-016835) and the control siRNA pool (D-001810) were from Thermo Scientific. Cells were reverse-transfected with siRNA 24 h before Wnt3a treatment. siRNAs were transfected using Lipofectamine 2000 and following the reverse-transfection protocol from Invitrogen. For Wnt signaling assays, a BAR-firefly luciferase reporter lentivirus containing Puromycin selection marker (3) was transduced into C32 MITF-inducible melanoma cells. After selection with Puromycin, the C32-BAR cells were then transduced with an Ef1α-*Renilla* lentivirus (3) for normalization puposes. For transductions, 1 mL of lentivirus (0.7 μg/mL) was incubated in a six-well plate at 70% confluency for 16 h. After incubation, medium was replaced and 24 h; later cells were trypsinized. One week after transduction, cells were selected with Puromycin.

Flow Cytometric Measurement of Immunostainings. To quantitate immunostaining intensities by flow cytometry, two 10-cm plates of cells per condition were grown to 70% confluency, trypsinized, and collected in a 15-mL conical. Cells were gently spun, supernatant removed, and fixed in suspension in 4% PFA in Eppendorf tubes on an end-over-end rotator at room temperature for 20 min. Cells were then spun at 1,400 rpm in a Eppendorf 5415 D table-top centrifuge (with an F-45-24-11 rotor), supernatant removed, resuspended in freshly prepared DPBS, and rotated in an end-over-end rotator at room temperature for 20 min. This DPBS washing step was repeated three times. Cells were then permeabilized by resuspending them in 0.2% Triton X-100 in DPBS (Gibco), and left rotating for 10 min. Cells were then washed two times in DPBS following the method described above. For antigen retrieval, cells were incubated in 0.5% SDS for 5 min, and washed three times in DPBS. For blocking, cells were resuspended in 5% goat serum with 0.5% BSA in DPBS (blocking solution) for at least 2 h. Cells were then spun down and resuspended with primary antibodies, diluted in 1:4 blocking solution, and left rotating overnight at 4 °C. The following day, cells were washed three times in DPBS as before. Cells were resuspended in secondary antibodies, diluted in 1:4 blocking solution, and rotated for 2 h at room temperature in the dark. The three subsequent washes in DPBS were also performed in the dark. Fluorescence was measured by flow cytometry for 10,000 cells counted. Antibodies used were: anti-CD63 1:400 (BD Pharmingen #556019), anti-LAMP1 1:400 (DSHB #H4A3). Secondary antibodies were Alexa Fluor 488-conjugated AffiniPure Donkey anti-Rabbit IgG and Cy3 conjugated AffiniPure Donkey anti-Mouse IgG (Jackson ImmunoResearch Laboratories).

Flow Cytometry. Quantification of fluorescence by flow cytometry was performed in an LSRII flow cytometer (Becton Dickinson). Ten thousand events per sample were collected by FACSDiva v6.0 and analyzed by CellQuest software.

Western Blot Analyses. For Western blot analyses, cells were cultured in six-well (melanoma cells) or 12-well (HEK 293T cells) plates. Cells were lysed in radioimmunoprecipitation assay buffer (RIPA lysis buffer, 0.1% Nonidet P-40, 20 mM Tris·HCl pH 8, 10% glycerol) supplemented with protease inhibitors (Roche #04693132001) and phosphatase inhibitors (Calbiochem 524629). Western blots were performed using standard procedures. OdysseyTM Blocking Buffer (LI-COR) diluted in PBS (1:1 ratio) was first used to block nitrocellulose membranes for 1 h at room temperature. All antibodies were diluted in PBS:Odyssey Blocking Buffer supplemented with 0.1% Tween 20. Primary antibodies, anti-MITF (DAKO #M3621; diluted 1:1,000), anti-$pMITF^{GSK3}$ serum (diluted 1:5,000), and anti-GAPDH (Cell Signaling Technologies #2118S; 14C10; diluted 1:7,000), were incubated overnight at 4 °C. Membranes were then extensively washed with Tris-buffered saline Tween 20 (TBST buffer) and incubated with fluorescently labeled secondary antibodies for 2 h at room temperature. Fluorescently labeled secondary antibodies used were IRDye 800CW Donkey anti-Rabbit IgG (IgG) (LI-COR Biosciences 926–32213; 1:5,000) and IRDye 680RD Donkey anti-Mouse IgG (LI-COR Biosciences 926–68072; 1:5,000). An Odyssey 9120 infrared imaging system (LI-COR) was used to acquire images.

Cell Viability Assays. C32 cells were plated in opaque 96-well plates at 3,000 cells per well density. Cells were treated in several replicates (eight wells per condition) with four different conditions: ethanol, Wnt3a at 1:500 for 3 d, tetracycline at 1:5,000 for 5 d, and a combination of tetracycline at 1:5,000 for 5 d with Wnt3a at 1:500 for 3 d. After incubation for 120 h total, cell viability was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega), an ATP-based bioluminescent assay, as per manufacturer's instructions. Each experiment was repeated independently at least three times.

Luciferase Assays. For Luciferase measurements of cell lysates, cells from 12-well plates were lysed with 180 μL of Passive Lysis Buffer (Promega). For luciferase measurements of *Xenopus*

ectodermal explant lysates, embryos were coinjected four times with the corresponding mRNAs together with 20 pg of the *TCF SuperTopFlash* reporter (1) at the four-cell stage. Ectodermal explants were excised at midblastula (stage 8.5). Thirty ectodermal explants were harvested per condition and lysed in 100 μL of Passive Lysis Buffer (Promega). Lysates were then spun at maximum speed in a tabletop centrifuge at 4 °C, and luciferase assays were performed on the supernatant with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, using a Glomax Luminometer (Promega).

RT-qPCR. For quantitatively analyzing the expression of endogenous mRNA transcripts, total RNA was extracted from cultured cells (one six-well plate per condition) or *Xenopus* embryos (seven stage 13 embryos per condition), using Absolutely RNA Microprep Kit (Agilent Technologies). cDNA synthesis was carried out using AffinityScript Multi-Temp Reverse Transcriptase (Agilent Technologies) and mRNA levels analyzed using the SYBR green reagent with ROX as reference dye. Relative gene-expression levels were determined using the Comparative C_t method using the housekeeping gene *GAPDH* in the case of cultured cells, or *Ornithine Decarboxylase* (*xODC*) in the case of *Xenopus* samples, for normalizaton. Primers for lysosomal genes and negative controls were those described by Sardiello et al. (4). RT-qPCR conditions and primers for *xODC* can be found in ref. 2. RT-qPCR primers for *x-tyrosinase* were: Fwd: GAAACGTTGACTTTGCCCAT and Rev: CTGCAGACAATCTCCCATGA, and for MART1: Fwd: AGATGCCAAGAGAAGATCTC, and Rev: GCTCTTAAGGTGAATAAGGTGG.

1. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT (2003) Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr Biol* 13(8):680–685.
2. Ploper D, Lee HX, De Robertis EM (2011) Dorsal–Ventral patterning: Crescent is a dorsally secreted Frizzled-related protein that competitively inhibits Tolloid proteases. *Dev Biol* 352(2):317–328.
3. Biechele TL, Moon RT (2008) Assaying β-catenin/TCF transcription with β-catenin/TCF transcription-based reporter constructs. *Wnt Signaling*, ed Vincan E (Humana, Totowa, NJ), pp 99–110.
4. Sardiello M, et al. (2009) A gene network regulating lysosomal biogenesis and function. *Science* 325(5939):473–477.

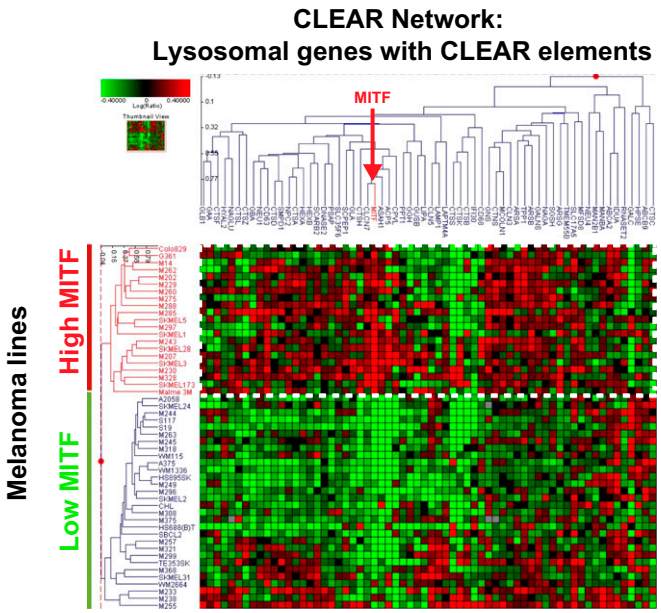

Fig. S1. *MITF* expression correlates with the expression of many lysosomal genes containing a CLEAR promoter element in melanoma cells. Microarray analysis shows that 51 melanoma cell lines clustered into two distinct groups according to their expression signature of 63 CLEAR element lysosomal genes (4). One group had high *MITF* expression (in red) and the other had low *MITF* expression (in blue). The melanoma group with high *MITF* expression up-regulated many, but not all, lysosomal genes containing a CLEAR element in their promoter. Horizontal lines in this heat map correspond to individual cell lines, with lysosomal gene expression shown vertically. *MITF* expression was added as a reference, for it is not a lysosomal gene. The cell lines were allowed to sort according to their lysosomal gene expression signature. Surprisingly, all cell lines with high *MITF* mRNA expression (and *MITF* amplification) clustered as a high lysosomal gene-expressing group.

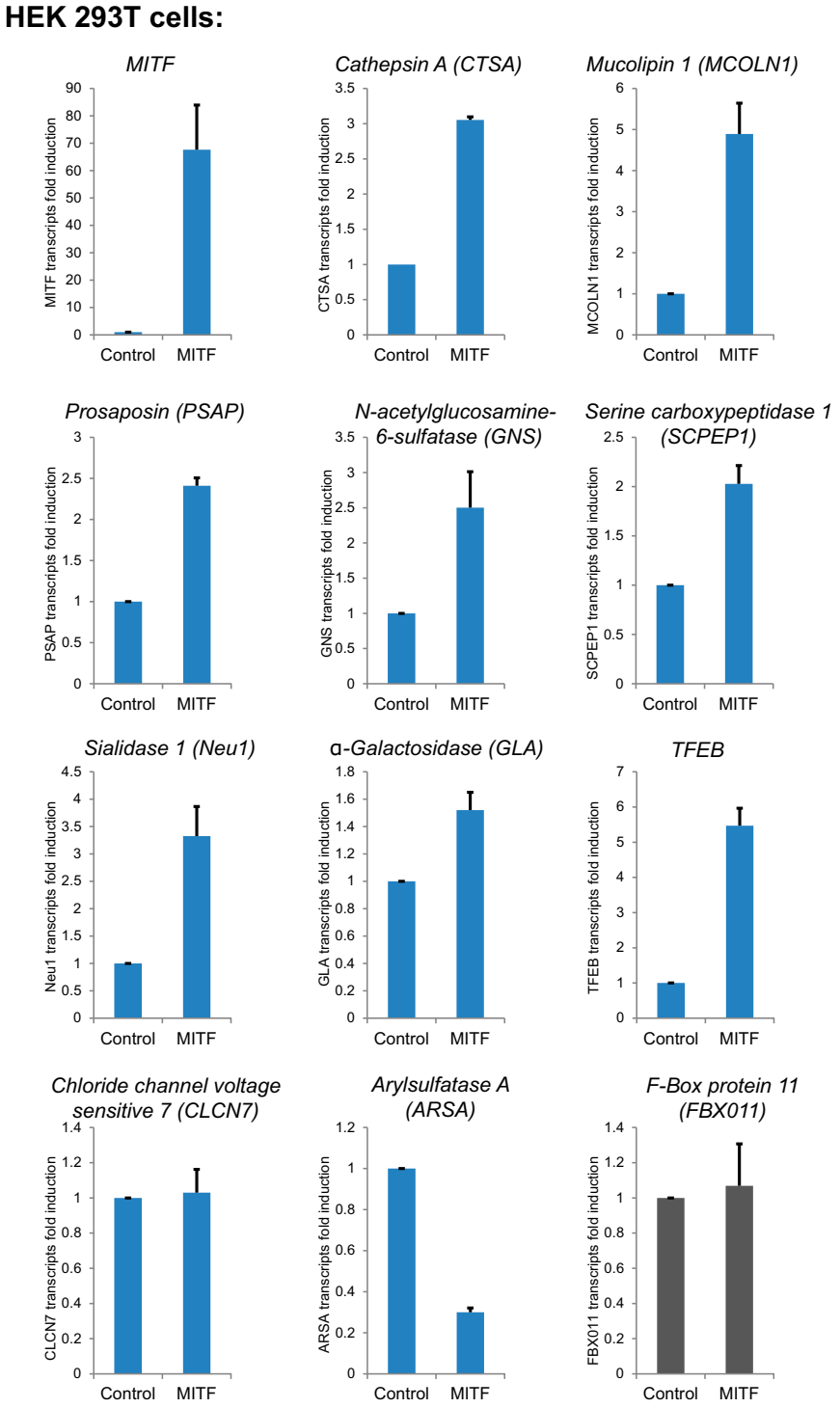

Fig. S2. Transient transfection of MITF into HEK 293T cells increased transcription of many CLEAR element lysosomal genes, as measured by RT-qPCR; *MITF* transcription increased in MITF-transfected 293T cells, as expected. MITF transient transfection up-regulated transcription of the CLEAR element lysosomal genes *cathepsin A* (*CTSA*), *mucolipin 1* (*MCOLN1*), *prosaposin* (*PSAP*), *N-acetylglucosamine-6-sulfatase* (*GNS*), *serine carboxypeptidase 1* (*SCPEP1*), *Sialidase 1* (*NEU1*), *a-galactosidase* (*GLA*), and TFEB. Transcription of *chloride channel voltage sensitive 7* (*CLCN7*) remained unchanged, but it was decreased for *arylsulfatase A* (*ARSA*), by MITF transfection in HEK 293T cells. As a negative control, MITF transfection did not up-regulate transcription of the *F-box protein 11* (*FBXO11*). Error bars indicate the SEM from three independent experiments.

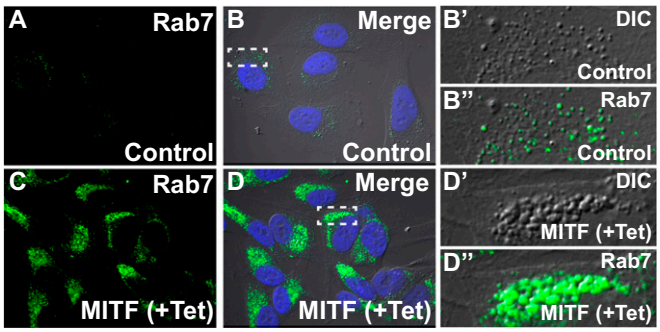

Fig. S3. MITF-induction increases immunostaining of the late endosomal marker Rab7, in MITF-induced vesicles in C32 melanoma cells. (*A* and *B*) Immunostainings for Rab7 in uninduced C32 melanoma cells. (*C and D*) MITF-induced C32 melanoma cells show a striking increase in Rab7 levels. *(B′–B′′* and *D′–D′′)* Note that Rab7 localizes specifically to vesicles that are visible by light microscopy. Upon MITF induction, cells undergo a great expansion of these late endolysosomes marked with Rab7.

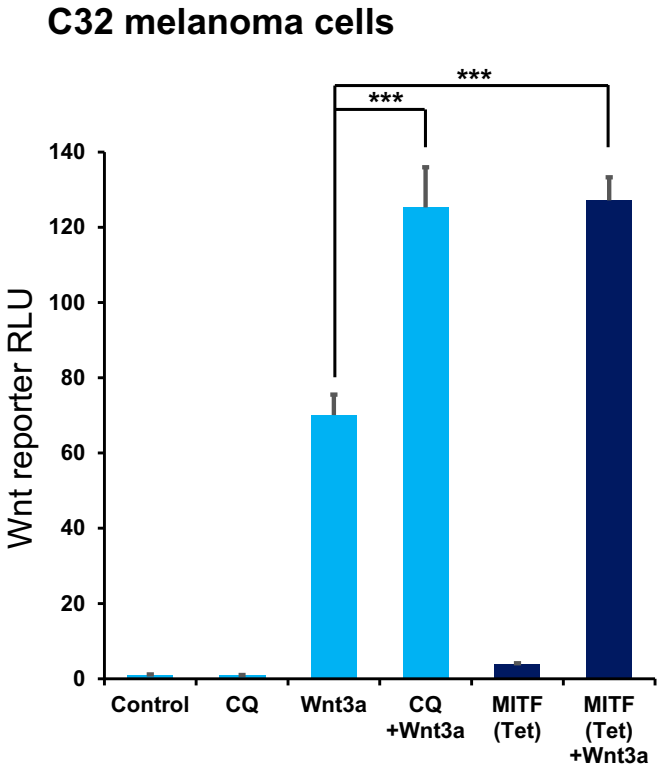

Fig. S4. MITF and CQ enhance Wnt signaling in melanoma cells. In uninduced C32 cells, CQ (100 μM) enhances Wnt3a signaling nearly twofold. Similarly, MITF induction by Tet addition enhanced signaling by Wnt3a (***$P < 0.001$). Signaling was assayed using the Wnt BAR-reporter (*Materials and Methods*) and normalized to *Renilla* under the EF1α promoter control.

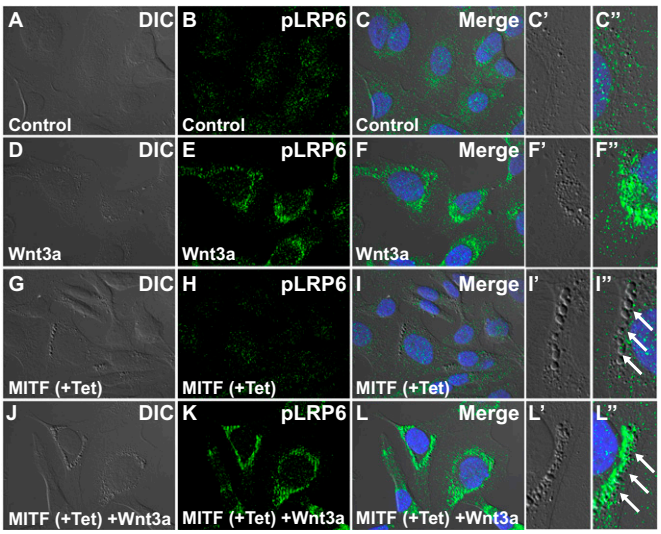

Fig. S5. Activated Wnt receptor pLRP6 localizes to MITF-induced late endolysosomal vesicles when cells are treated with Wnt3a protein. (*A–F′′*) Wnt3a induces phosphorylation of LRP6. Endogenous pLRP6 appeared as punctae with vesicular localization, marking active Wnt signalosomes/MVBs by immunostaining (1, 2). (*G–I′′*) Induction of MITF with Tet increased the number of vesicles, but without Wnt stimulation, these vesicles were devoid of pLRP6 (arrows). (*J–L′′*) Upon Wnt3a treatment, MITF-induced vesicles strongly accumulate pLRP6 (arrows), indicating that the MITF-induced MVB/late endolysosomes participate in Wnt signaling. Note that MITF-induced vesicles contain pLRP6 only when C32 cells were treated with Wnt3a (arrows).

1. Taelman VF, et al. (2010) Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. *Cell* 143(7):1136–1148.
2. Bilić J, et al. (2007) Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science* 316(5831):1619–1622.

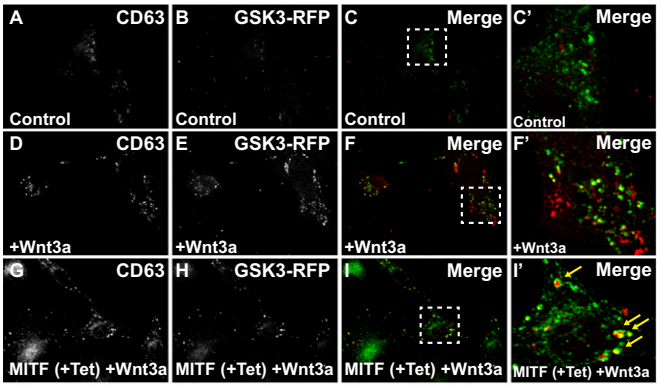

Fig. S6. MITF induction increases the colocalization of Wnt-induced GSK3 puncta and CD63$^+$ MVBs in C32 cells transfected with RFP-GSK3. (*A–F′*) Wnt3a treatment increases the number of GSK3-RFP puncta, which sometimes colocalize with endogenous CD63, a marker enriched in the intraluminal vesicles of MVBs. (*G–I′*) Wnt increases the colocalization of GSK3-RFP puncta together with endogenous CD63$^+$ vesicles in cells induced to express MITF. In these cells, RFP-GSK3 puncta are frequently seen enveloped in CD63$^+$ structures (arrows).

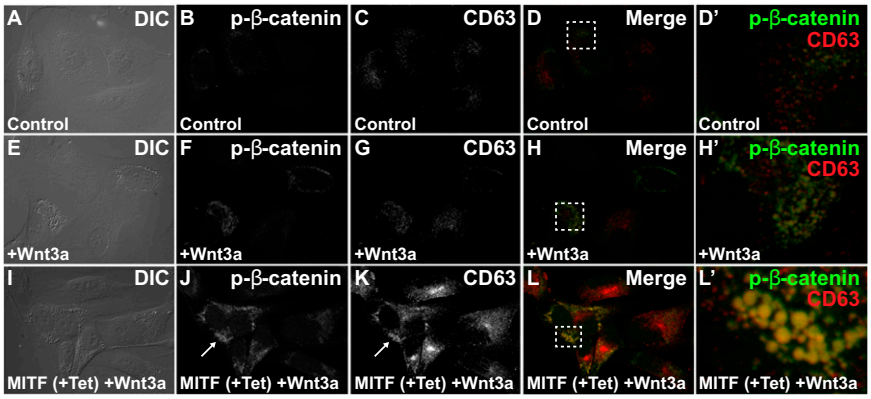

Fig. S7. MITF-induced CD63^{+} MVBs colocalized with the Wnt signalosome component p-β-catenin after Wnt treatment in C32 melanoma cells. (*A*–*H′*) Wnt3a signaling increases p-β-catenin, which localizes to MVBs marked by CD63. (*I*–*L′*) MITF induction causes a further increase in p-β-catenin, which is localized in CD63^{+} MVBs. Note that MITF induction caused a strong expansion of the CD63^{+} MVBs compartment (compare *G* and *K*), and that the increased colocalization of p-β-catenin and CD63 upon Wnt signaling in MITF induced cells resides in vesicles visible through DIC microscopy (*I*, *L*, and *L′*).

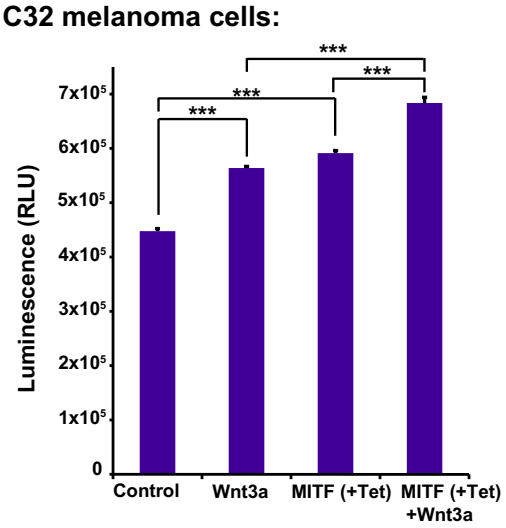

Fig. S8. Wnt3a and MITF increase C32 melanoma proliferation in ATP-based cell viability assays. Wnt3a treatment for 3 d, as well as MITF induction for 5 d, significantly enhanced C32 cell proliferation. Combination of MITF induction and Wnt3a further increased C32 cell growth (***$P < 0.001$).

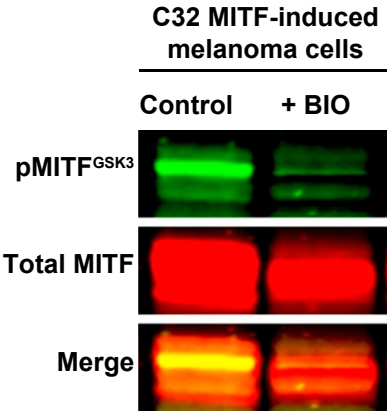

Fig. S9. The novel C-terminal GSK3-regulated phosphorylations take place in MITF in C32 melanoma cells, as monitored by a phospho-specific antibody. In Tet-induced C32 cells, a band corresponding to pMITF^{GSK3} was detected, which disappeared upon treatment with the GSK3 inhibitor BIO.