

# Supplementary information

## **Rab7a is an enhancer of TPC2 activity regulating melanoma progression through modulation of the GSK3 $\beta$ / $\beta$ -Catenin/MITF-axis**

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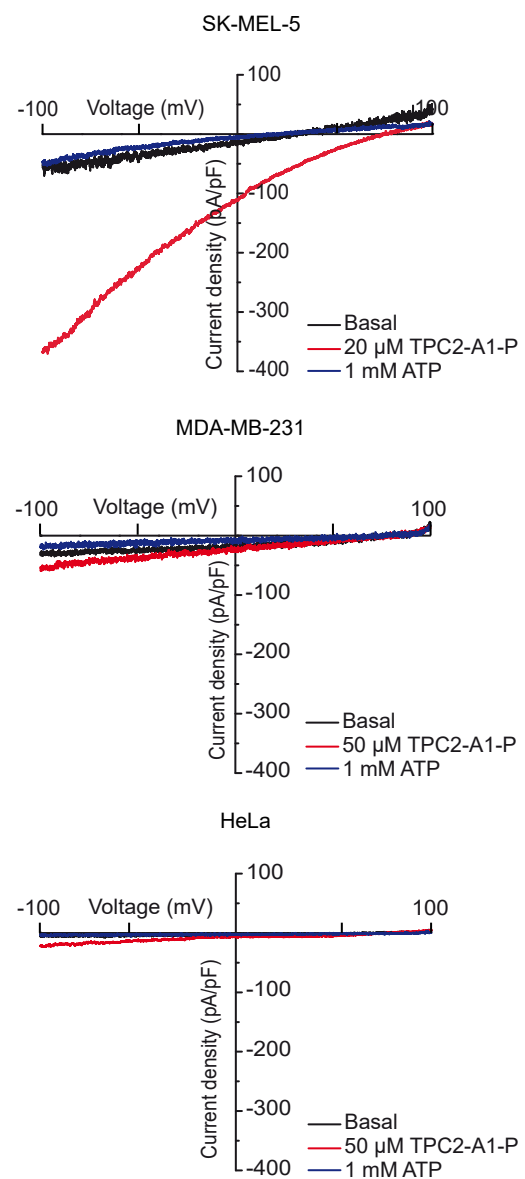
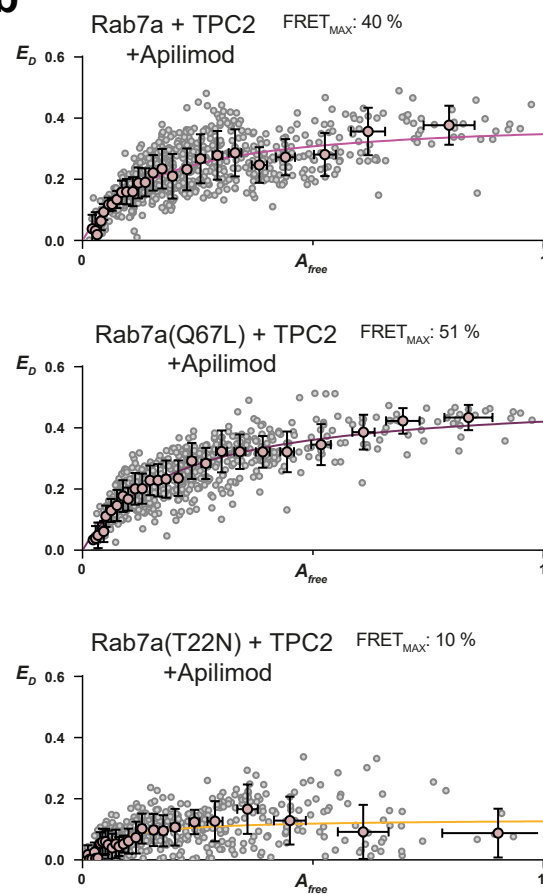
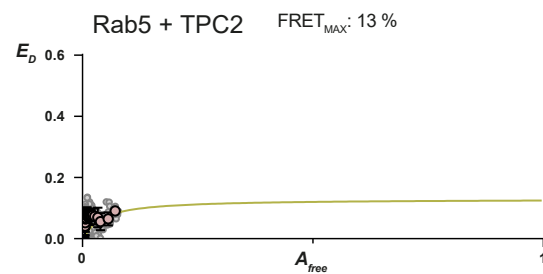
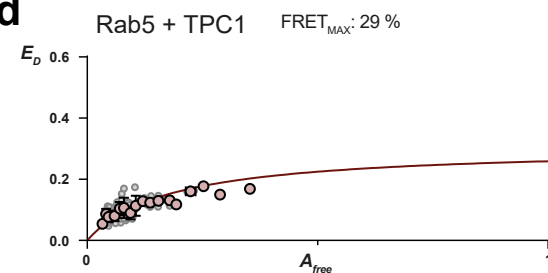
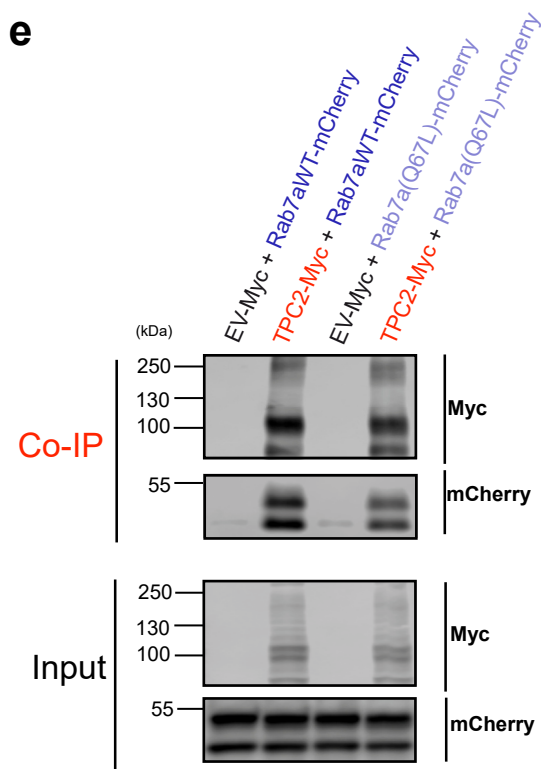
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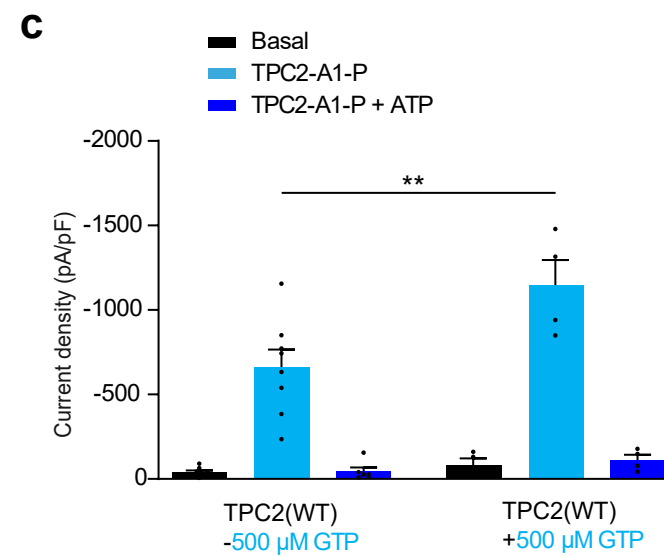
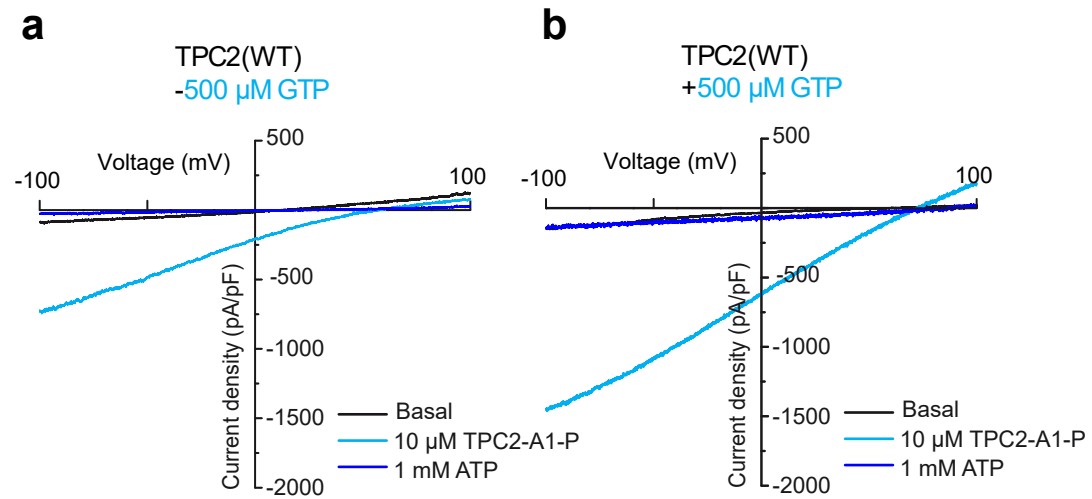
<sup>8</sup>Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei, Taiwan.

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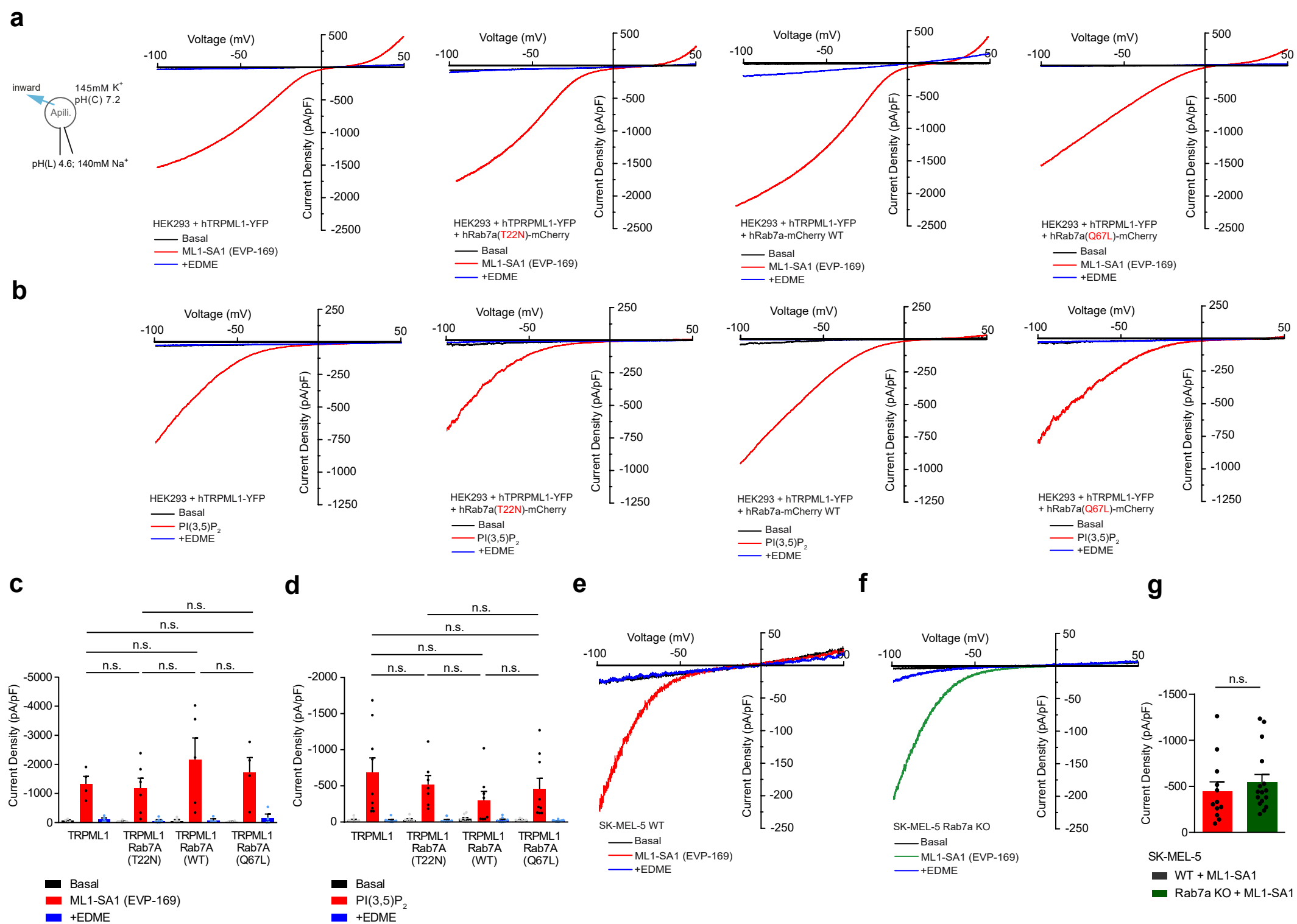
<sup>10</sup>Immunology, Infection and Pandemic Research IIP, Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Munich/Frankfurt, Germany

**a****b****c****d****e**

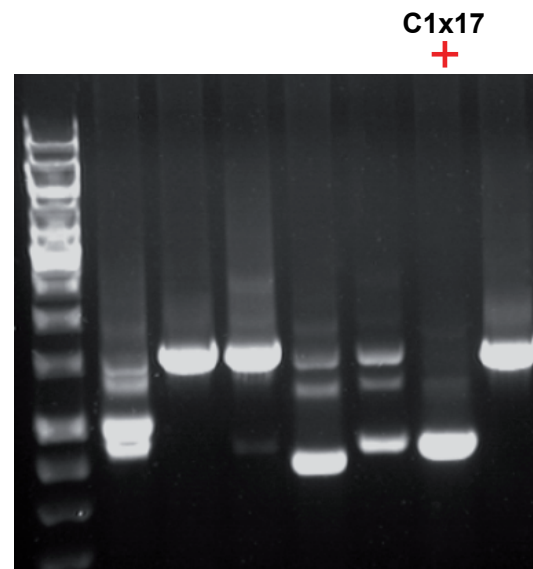
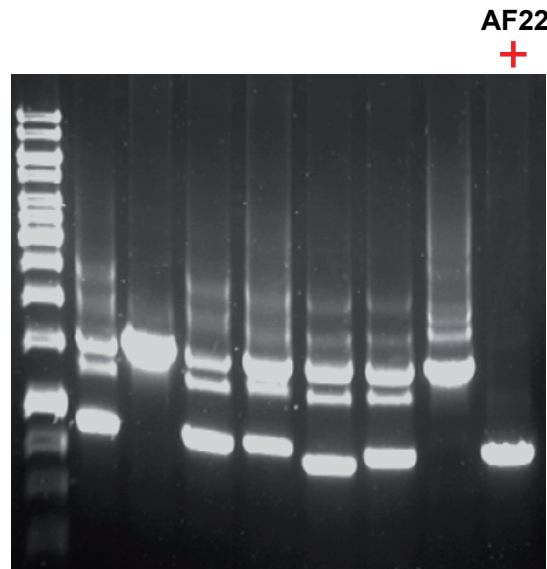
**Figure S1. Endolysosomal patch-clamp, FRET and co-immunoprecipitation experiments.** (a) Endolysosomal patch-clamp experiments showing effect of the TPC2 agonist TPC2-A1-P and the TPC2 blocker ATP on different cancer cell lines. (b-d) FRET experiments showing FRET efficiencies in HEK293 cells expressing hTPC2<sup>WT</sup> + hRab7<sup>WT</sup> (n = 767), hTPC2<sup>WT</sup> + hRab7<sup>Q67L</sup> (n = 676), or hTPC2<sup>WT</sup> + hRab7<sup>T22N</sup> (n = 588) in presence of apilimod (a). Shown in c and d are the controls Rab5<sup>WT</sup> + TPC2<sup>WT</sup> (negative) (n = 536) and Rab5<sup>WT</sup> + TPC1<sup>WT</sup> (positive) (n = 87) (n represents biological replicates, error bars are SEM). (e) Representative co-immunoprecipitation experiment indicating an interaction between both hRab7a<sup>WT</sup>-mCherry and hTPC2<sup>WT</sup>-YFP and hRab7a<sup>Q67L</sup>-mCherry with hTPC2<sup>WT</sup>-YFP<sup>1</sup>.



**Figure S2. Effect of GTP preincubation on TPC2 activity.** (a-b) Effect of GTP and TPC2-A1-P (10  $\mu$ M) on apilimod-treated, enlarged endolysosomal vesicles expressing hTPC2. Shown are representative current density-voltage relationships from -100 to +100 mV with basal currents in black, TPC2-A1-P activated currents in the absence or after preincubation (5 min) with GTP in light blue and ATP (1 mM) blocked currents in dark blue. (c) Statistical summary of data comprising average current densities (mean  $\pm$  SEM) at -100 mV measured in endolysosomal patch-clamp experiments as shown in a-b. Each dot on the bar graph represents a single current density value measured from one endolysosome (n = 4-8 biological replicates). Data were tested for statistical significance with one-way ANOVA test followed by Tukey's post-test, \*\*p < 0.01.



**Figure S3. Effect of Rab7a on TRPML1 activity.** (a) Effect of lipophilic small molecule agonist of TRPML1 ML1-SA1 in endolysosomal vesicles coexpressing human TRPML1 and Rab7a or mutant variants of Rab7a. Shown are representative current density-voltage relationships from -100 to +100 mV with basal currents in black, 10  $\mu$ M ML1-SA1 activated currents in red and EDME (10  $\mu$ M) blocked currents in blue, measured from apilimod-treated, enlarged endolysosomal vesicles expressing either hTRPML1<sup>WT</sup>, hTRPML1<sup>WT</sup> + hRAB7a<sup>WT</sup>, hTRPML1<sup>WT</sup> + hRAB7a<sup>Q67L</sup> (constitutively active Rab7a) or hTRPML1<sup>WT</sup> + hRAB7a<sup>T22N</sup> (dominant negative Rab7a). (b) Analogous experiments for PI(3,5)P<sub>2</sub> (1  $\mu$ M). (c and d) Statistical summary of data comprising average current densities (mean  $\pm$  SEM) at -100 mV measured in endolysosomal patch-clamp experiments as shown in (a) and (b). Each dot on the bar graph represents a single value current density measured from one endolysosome (for (a) n = 4-6 and for (b) n = 7-9 biological replicates). Data were tested for statistical significance with a one-way ANOVA test followed by Tukey's post-test. (e and f) Representative current density-voltage relationships from -100 to +100 mV showing basal, ML1-SA1 activated and EDME (10  $\mu$ M) blocked currents, measured from apilimod-treated, enlarged endolysosomal vesicles, in SK-MEL-5 WT cells (e) and Rab7a KO (f). (g) Statistical summary of data comprising average current densities at -100 mV measured in endolysosomal patch-clamp experiments as shown in (e) and (f) elicited with 10  $\mu$ M ML1-SA1 (WT and Rab7a KO SK-MEL-5 lines, respectively). Each dot on the bar graph represents a single value current density measured from one endolysosome (n = 12-16 biological replicates). Data were tested for statistical significance using a two-tailed unpaired t-test.

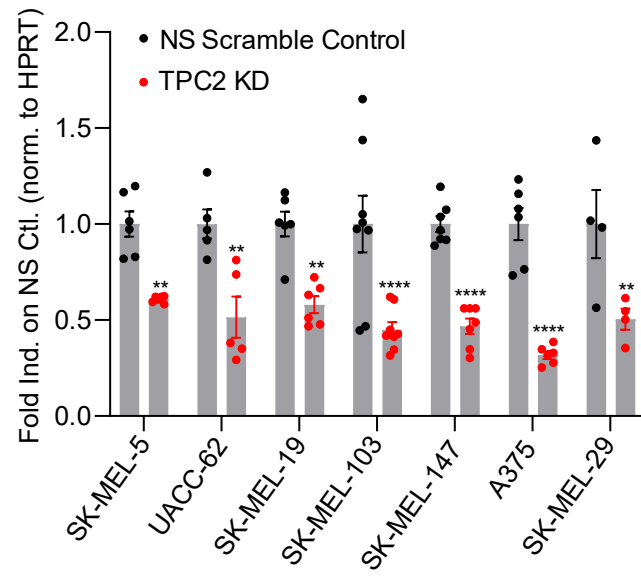
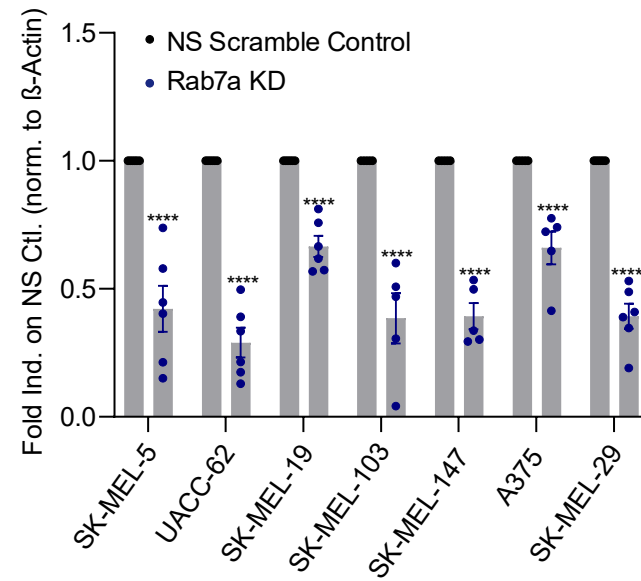
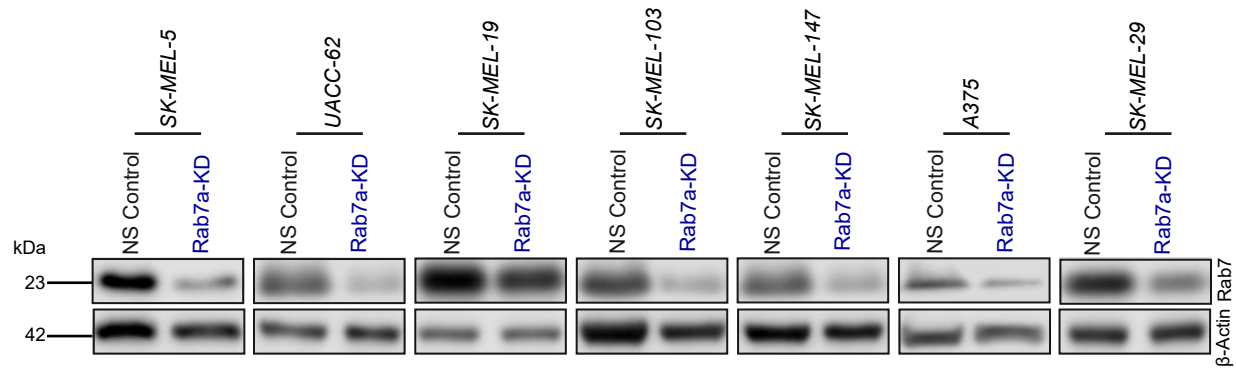


TPC2 KO

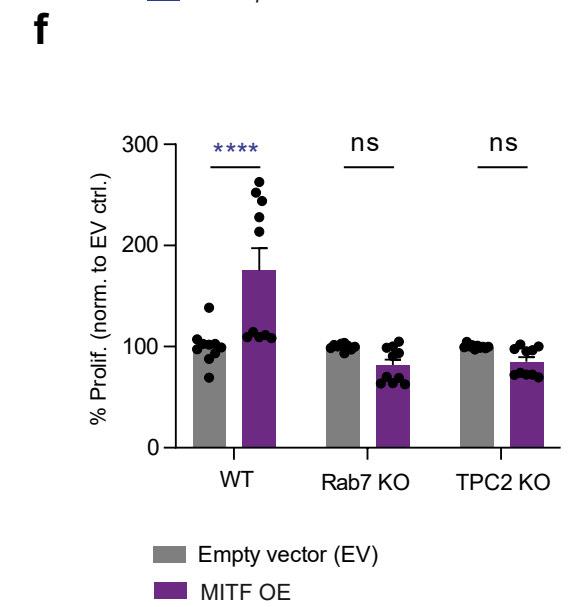
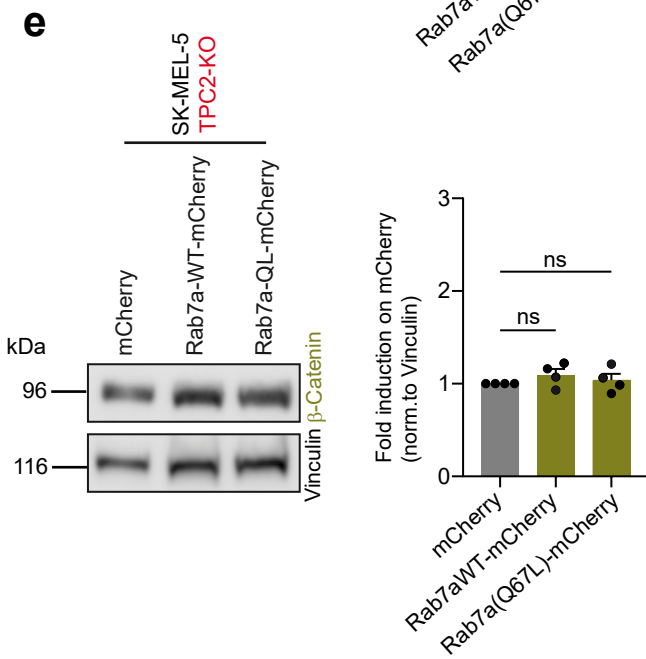
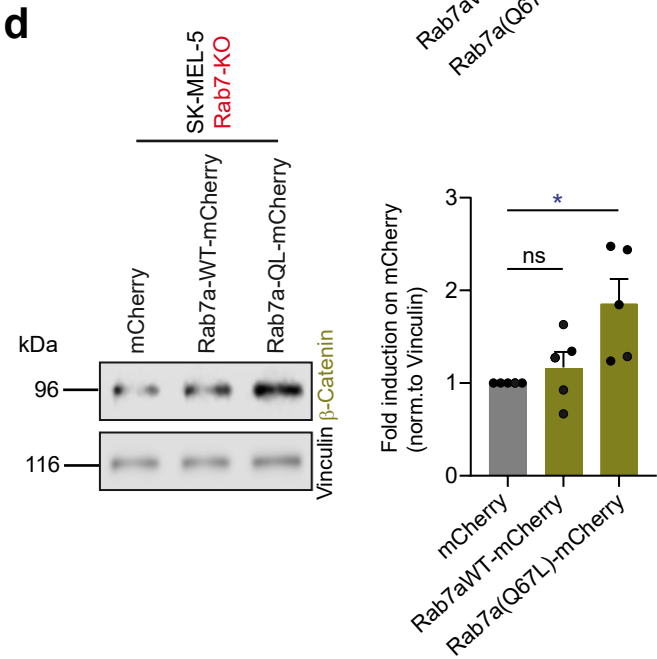
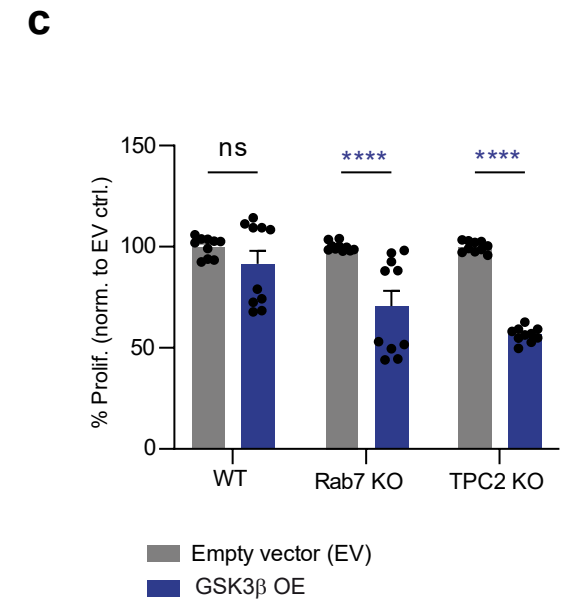
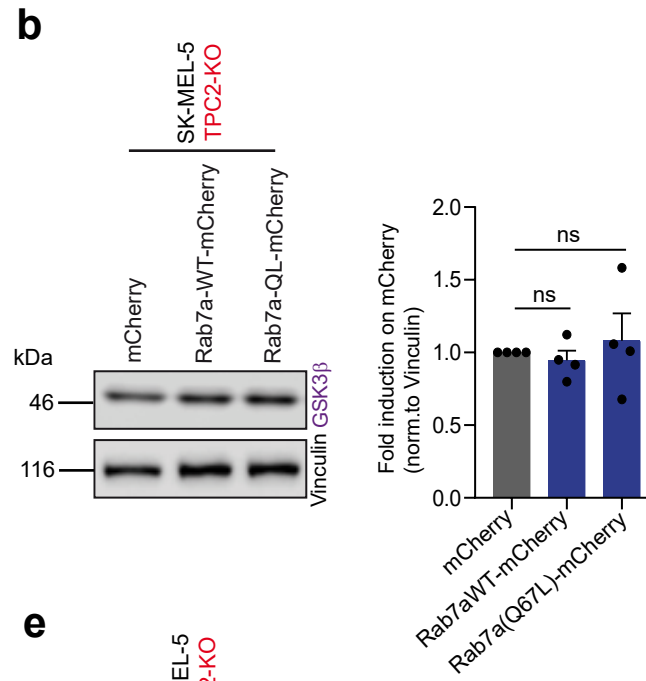
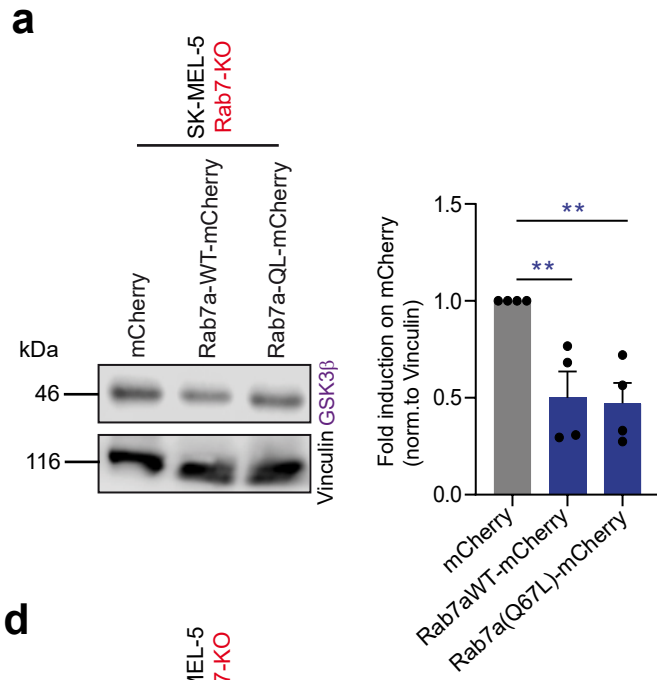
Rab7a KO

Genotyping (SK-MEL-5)

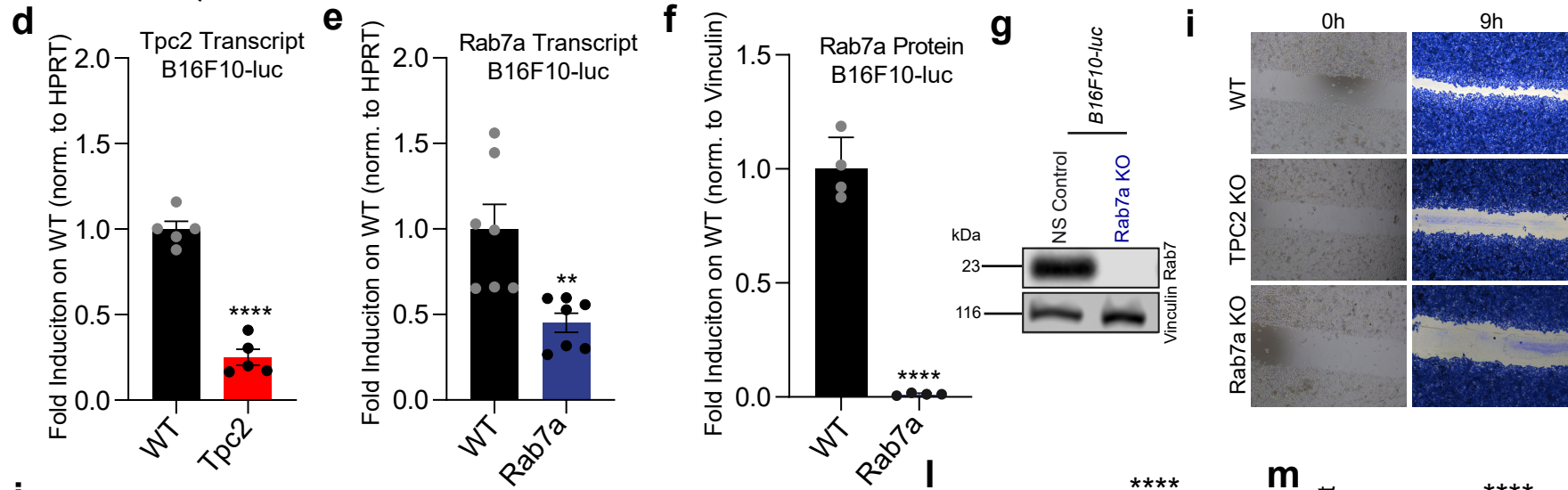
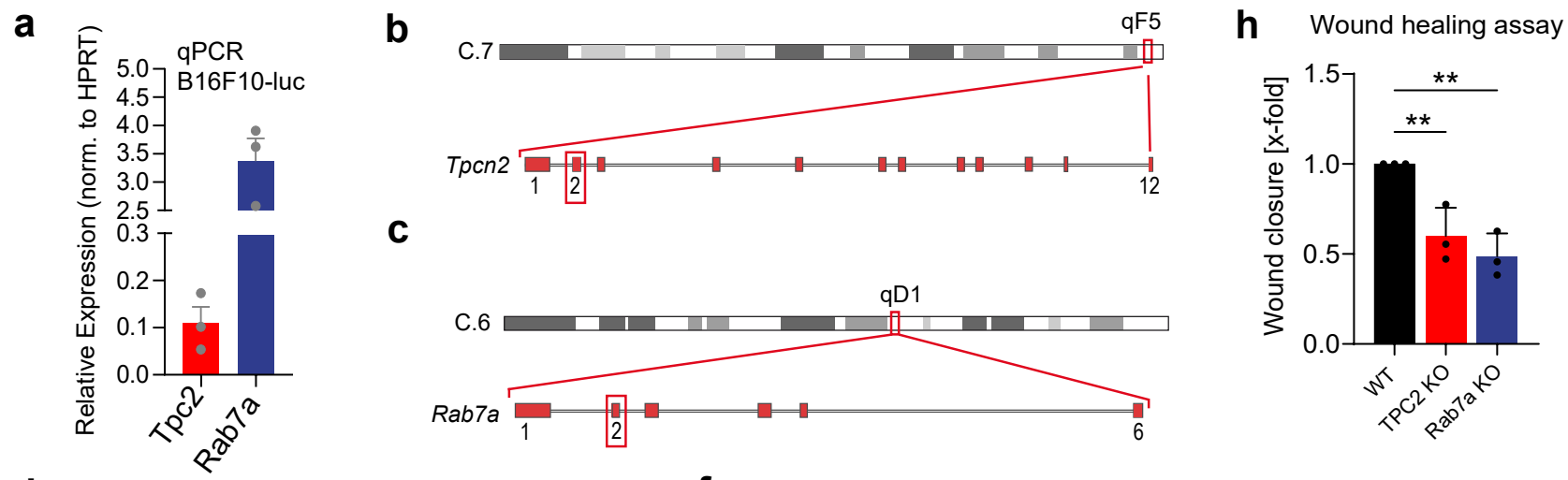
**Figure S4. Genotyping results for Rab7a and TPC2 KO SK-MEL-5 cell lines.** Agarose gels showing KO clone selection. Potential clones were sequenced. For all clones, homogeneity within the deletion amplicons was determined with Sanger sequencing and the CRISPR-ID *in silico* tool as described by Dehairs et al., 2016<sup>2</sup>. Genotyping results for the other two TPC2KO clones have been reported previously in Yuan et al., 2022<sup>3</sup>.

**a****b****c**

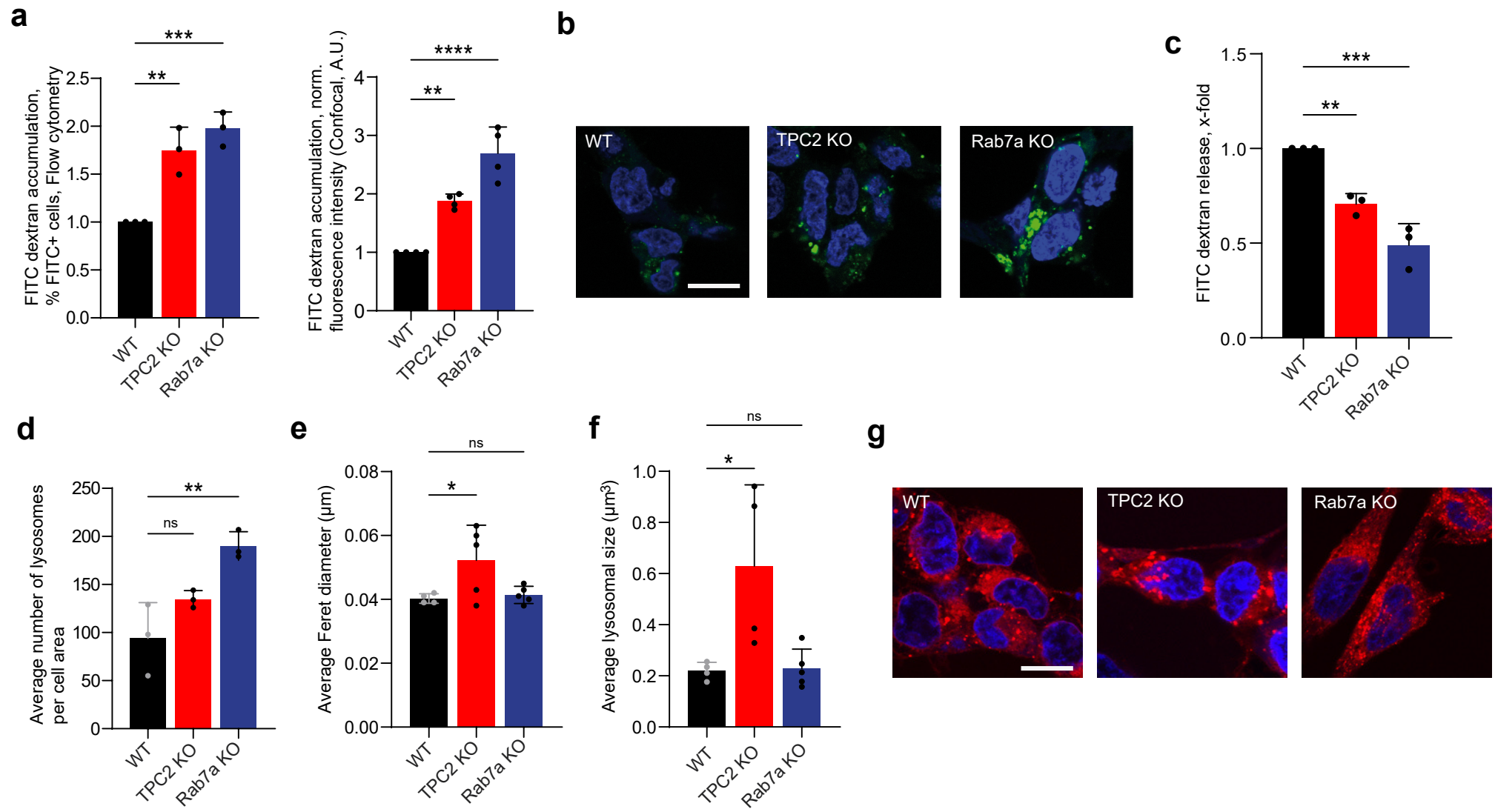
**Figure S5. Knockdown efficiencies for different melanoma lines.** (a) TPC2 KD in different melanoma lines determined by transcript levels using qPCR, fold induction on NS control, normalized to the house-keeping gene HPRT (n = 4-8 biological replicates). (b) Rab7 KD efficiency determined by Western blot experiments showing reduced proteins levels, fold induction on NS control, normalized to  $\beta$ -Actin (n = 5-6 biological replicates). (c) Representative blots for Rab7 KD experiments as shown in b. Statistical significance in a and b was determined using one-way ANOVA followed by Bonferroni multiple comparisons test. Shown are mean values  $\pm$  SEM. \*\*p < 0.01, \*\*\*\*p < 0.0001.



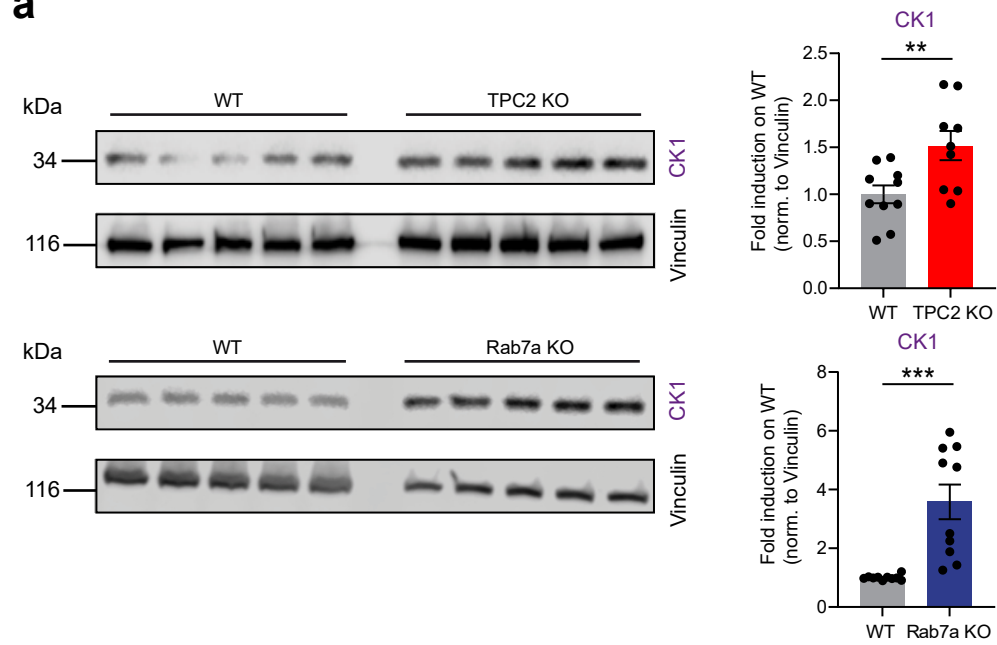
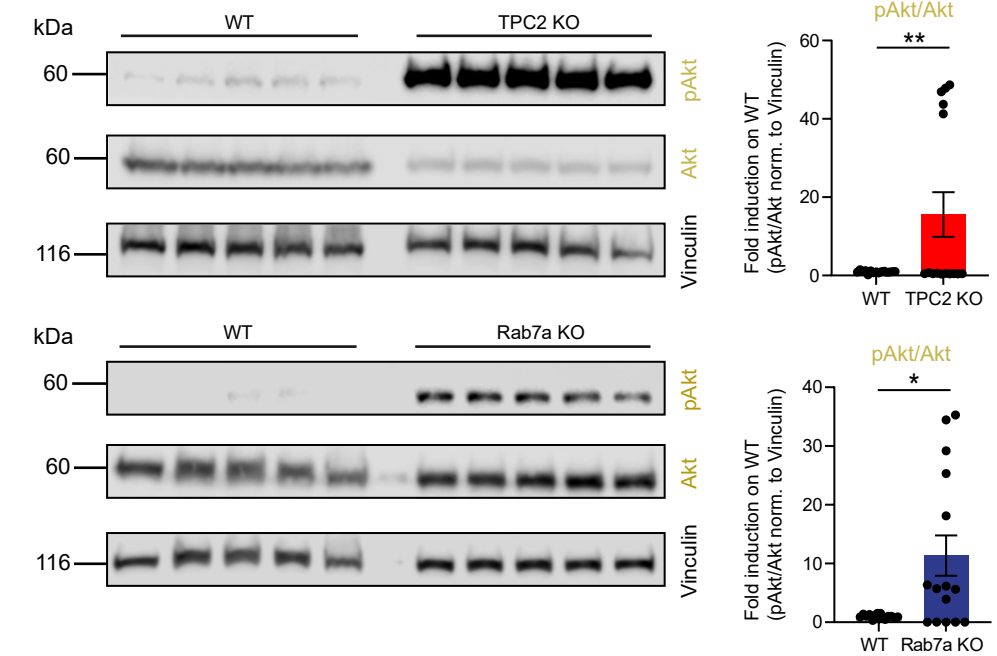
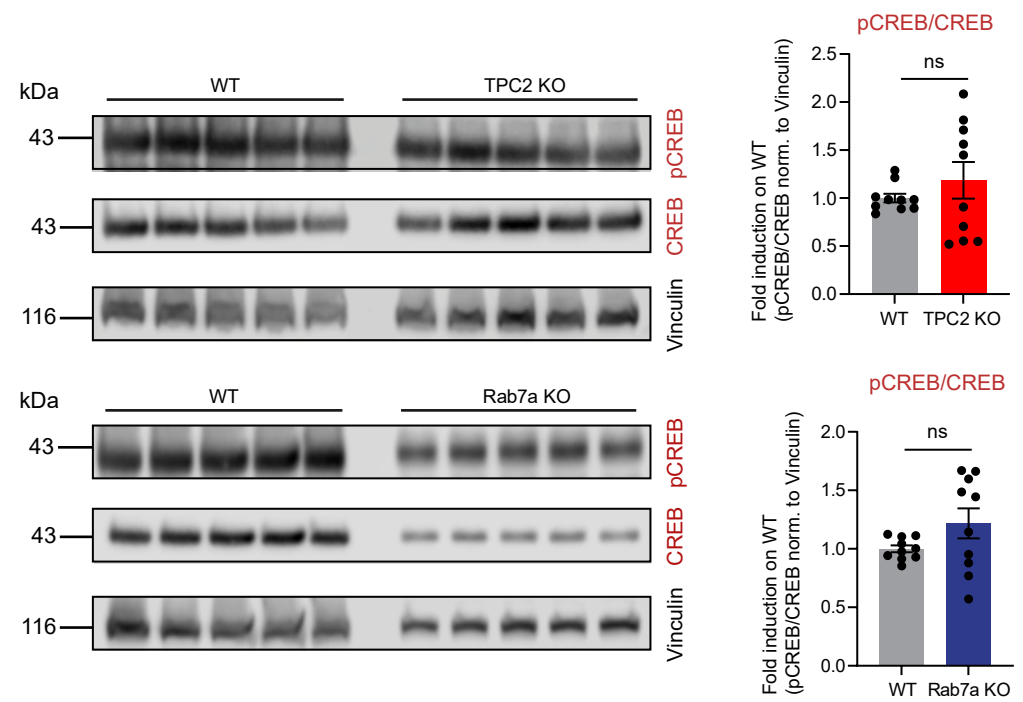
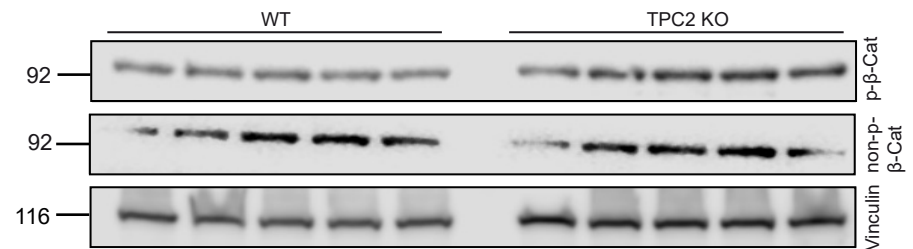
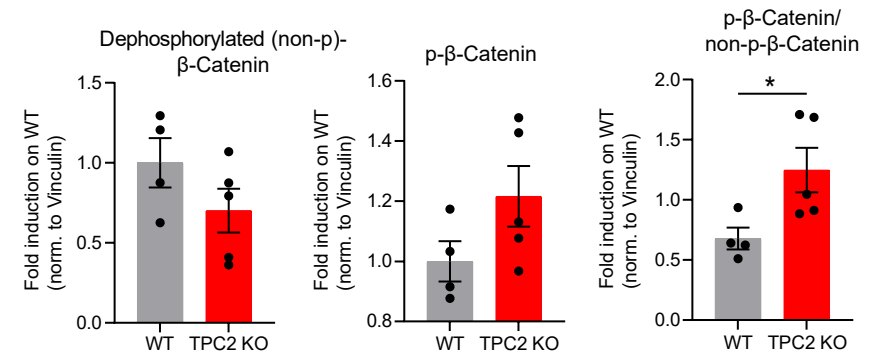
**Figure S6. Effect of Rab7a OE on GSK3 $\beta$  expression in Rab7a and TPC2 KO and effect of GSK3 $\beta$  overexpression on Rab7a and TPC2 KO cell proliferation.** (a) Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in decreased GSK3 $\beta$  protein levels in Rab7a KO SK-MEL-5 cells, respectively (n = 4 biological replicates). (b) Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in unchanged GSK3 $\beta$  protein levels in TPC2 KO SK-MEL-5 cells, respectively (n = 4 biological replicates). (c) Effect of overexpression of GSK3 $\beta$  in either WT, Rab7a or TPC2 KO SK-MEL-5 cells on proliferation (n = 10 biological replicates). (d) Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in increased  $\beta$ -Catenin protein levels in Rab7a KO SK-MEL-5 cells, respectively (n = 5 biological replicates). (e) Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in unchanged  $\beta$ -Catenin protein levels in TPC2 KO SK-MEL-5 cells, respectively (n = 4 biological replicates). (f) Effect of overexpression of MITF in either WT, Rab7a or TPC2 KO SK-MEL-5 cells on proliferation (n = 10 biological replicates). Statistical significance in a-f was determined using one-way ANOVA followed by Bonferroni multiple comparisons test. Shown are mean values  $\pm$  SEM, \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001.



**Figure S7. Characterization of B16F10-luc Tpc2 and Rab7a knockout lines.** (a) Gene expression profile of TPC2 and Rab7a in the B16F10-luc cell line (n = 3 biological replicates). (b and c) CRISPR/Cas9 gene editing strategy used for the knockout of Tpcn2 (b) and Rab7 (c) in the B16F10-luc cells. (d) qPCR experiments showing reduced Tpcn2 transcript levels in the Tpc2 KO B16F10-luc cell line (n = 4-5 biological replicates). (e) qPCR experiments showing reduced Rab7a transcript levels in the Rab7a KO B16F10-luc cell line (n = 7 biological replicates). Statistical significance in d-e was determined using a two-tailed unpaired t-test. Shown are mean values  $\pm$  SEM. \*\*p < 0.01, \*\*\*\*p < 0.0001. (f-g) Western blot results for the Rab7a KO in B16F10-luc cells (n = 4 biological replicates). (h-i) Wound healing assay in B16F10-luc WT, Tpc2 KO and Rab7a KO showing reduced migration in Kos (n = 3 biological replicates). Statistical significance in f was determined using one-way ANOVA. Shown are mean values  $\pm$  SD. \*\*p < 0.01. (j-k) Confocal images of *ex vivo* B16F10-luc tumors. Tumours were fixed in paraffin, cut into 10  $\mu$ m thin slices and stained for MITF or  $\beta$ -Catenin (green) and nuclei (blue), respectively. (l-m) Quantitative analysis of confocal images shown in j-k. Data were obtained from 3 different tumors (duplicates), each. In total 10,676 (WT), 7675 (TPC2 KO) and 4299 (Rab7 KO) cells were analyzed for MITF and 3946 (WT), 4022 (TPC2 KO) and 4793 (Rab7 KO) cells were analyzed for  $\beta$ -Catenin. Statistical significance in l-m was assessed with one-way ANOVA followed by Bonferroni multiple comparisons test. Shown are mean values  $\pm$  SEM, \*\*\*\*p < 0.0001. Scale bar = 100  $\mu$ m.



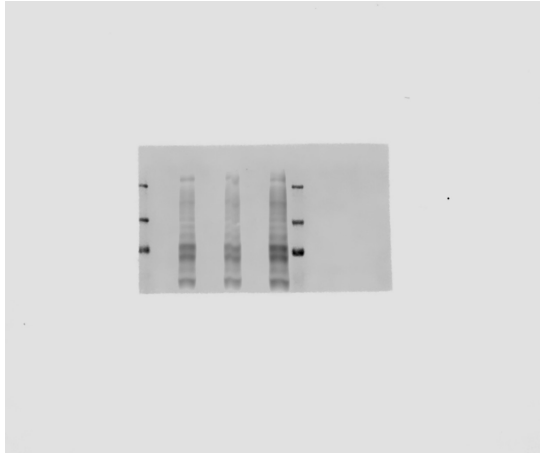
**Figure S8. Lysosomal defects in Rab7a and TPC2 KO SK-MEL-5 cell lines.** (a) Flow cytometry and confocal analysis of endocytosed FITC dextran (200  $\mu$ g/ml) after an incubation time of 2 h (n = 3-4 biological replicates). (b) Confocal images showing internalized FITC dextran (200  $\mu$ g/ml, incubated for 2 h) and nuclei. Fluorescence intensities were measured by ImageJ and normalized to the number of cells per image. Scale bar = 10 $\mu$ m. (c) Lysosomal exocytosis assay of released FITC-dextran (200  $\mu$ g/mL, incubated for 24 h) upon calcium treatment (50 mM). The results were normalized to the WT level (n = 3 biological replicates). (d) Average amount of lysosomes per cell area (n = 3 biological replicates). (e) Average Feret diameters (n = 5 biological replicates). (f) Average size of lysosomes per cell. Data were pooled from four independent experiments (d-f). One dot represents one experiment. A total of 33189 (WT), 40905 (TPC2 KO) and 42414 (Rab7a KO) lysosomes were evaluated. (g) Representative images of WT, TPC2 KO and Rab7a KO SK-MEL-5 cells treated with LysoTracker Red and Hoechst (nuclei). Statistical significance in a, c, d, e, f was assessed by one-way ANOVA followed by Bonferroni multiple comparisons test. Shown are mean values  $\pm$  SEM. p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. \*\*\*\*p < 0.0001. Scale bar = 10  $\mu$ m.

**a****b****c****d****e**

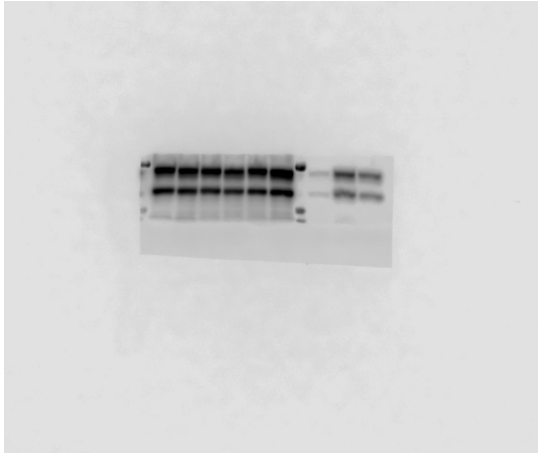
**Figure S9. Effect of Rab7a and TPC2 KO on CK1, pCREB/CREB and pAkt/Akt. (a-c)** Representative Western blots for CK1 (n = 9-10 biological replicates), pCREB/CREB (n = 15 biological replicates) and pAkt/Akt (n = 10 biological replicates) protein expression in SK-MEL-5 WT, Rab7a KO and TPC2 KO lines and respective statistical analysis: significance was determined using a two-tailed unpaired t-test. Shown are mean values  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. **(d-e)** Representative Western blots for dephosphorylated and phospho- $\beta$ -Catenin in TPC2 KO compared to WT SK-MEL5 cells (n = 4-5 biological replicates). Significance was determined using a two-tailed unpaired t-test. Shown are mean values  $\pm$  SEM. \*p < 0.05.

1. Feldmann, C., et al., *Protocol for deriving proximity, affinity, and stoichiometry of protein interactions using image-based quantitative two-hybrid FRET*. STAR Protoc, 2023. **4**(3): p. 102459.
2. Dehairs, J., et al., *CRISP-ID: decoding CRISPR mediated indels by Sanger sequencing*. Sci Rep, 2016. **6**: p. 28973.
3. Yuan, Y., et al., *Segregated cation flux by TPC2 biases Ca(2+) signaling through lysosomes*. Nat Commun, 2022. **13**(1): p. 4481.

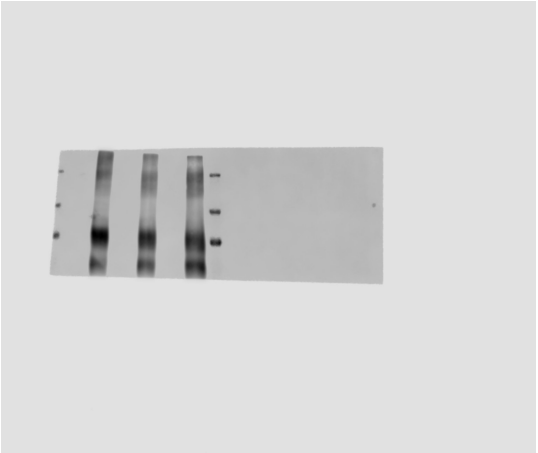
**Figure S1e - myc  
HEK293**



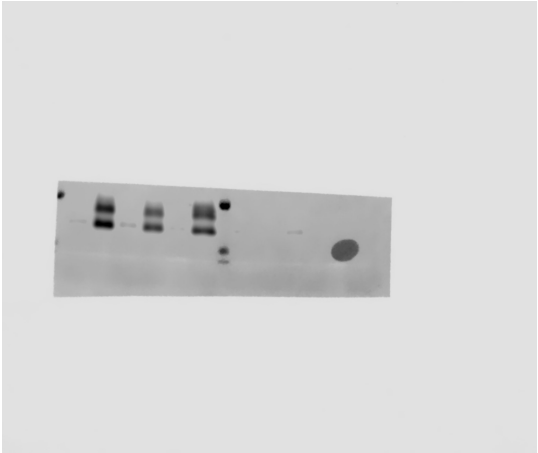
**Figure S1e - mcherry  
HEK293**



**Figure S1e - myc  
HEK293**

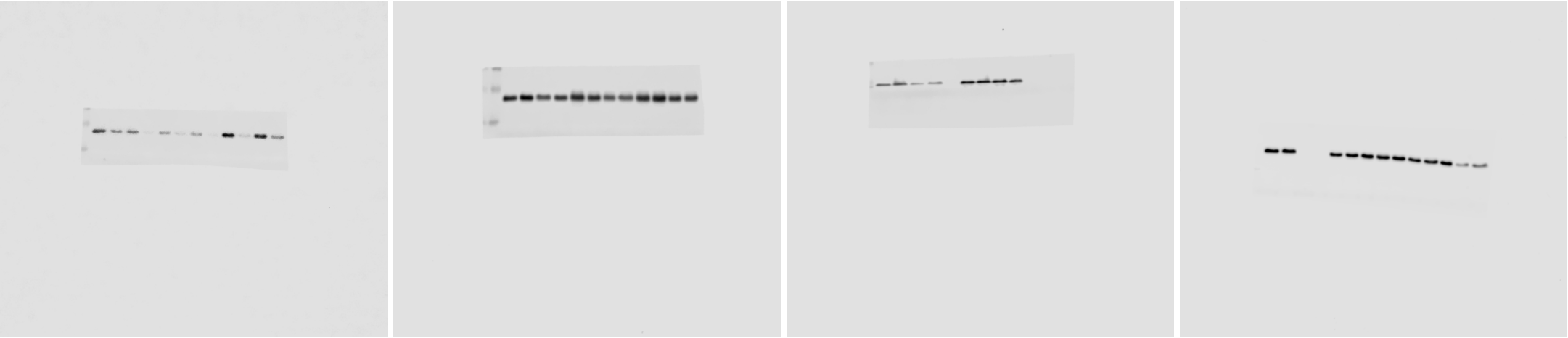


**Figure S1e - myc  
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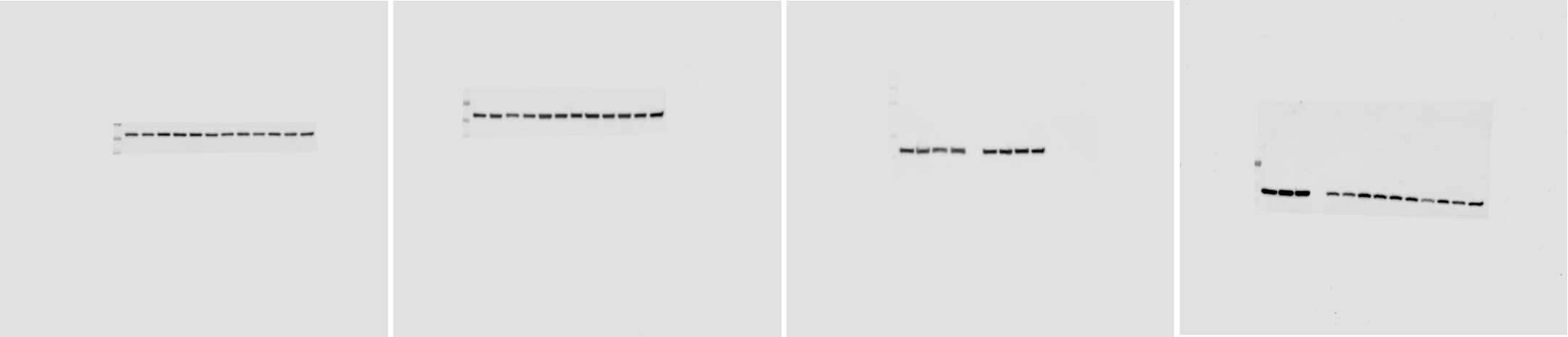


**Figure S10. Unprocessed western blots of figure S1e.** Co-immunoprecipitation experiment indicating an interaction between both hRab7aWT-mCherry and hTPC2WT-YFP and hRab7aQ67L-mCherry with hTPC2WT-YFP1.

**Figure S5c - Rab7**



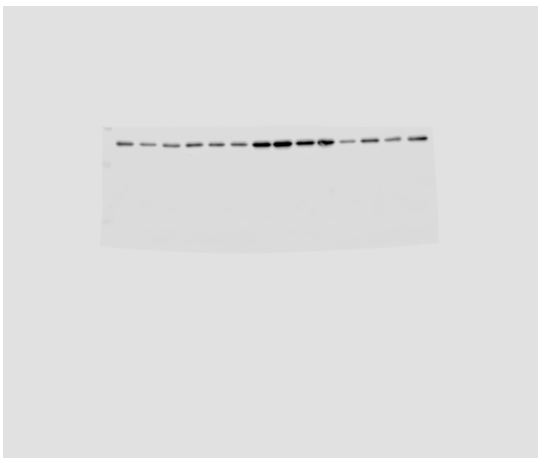
**Figure S5c - B-Actin**



**Figure S6a - Vinculin**



**Figure S6a - GSK3b**



**Figure S6b - Vinculin**



**Figure S6b - GSK3b**



**Figure S11. Unprocessed western blots of figures S5c, S6a and S6b.** Representative blots for Rab7 KD experiments (S5c). Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in decreased GSK3 $\beta$  protein levels in Rab7a KO SK-MEL-5 cells, respectively (S6a). Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in unchanged GSK3 $\beta$  protein levels in TPC2 KO SK-MEL-5 cells, respectively (S6b).

Figure S6d - beta-Catenin

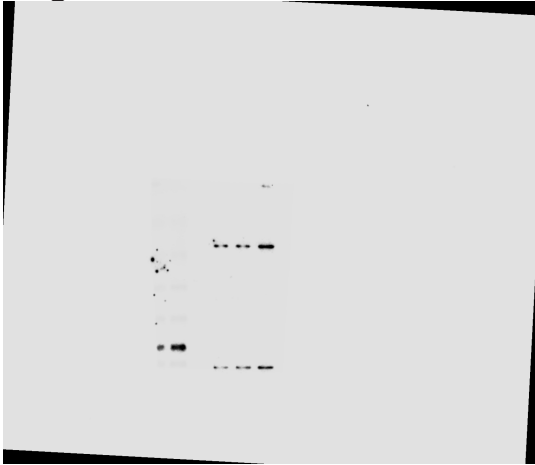


Figure S6d - Vinculin



Figure S6e - beta-catenin



Figure S6e - Vinculin



**Figure S12. Unprocessed western blots of figures S6d and S6e.** Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in increased  $\beta$ -Catenin protein levels in Rab7a KO SK-MEL-5 cells, respectively (S6d). Overexpression of Rab7a-WT-mCherry and Rab7a-QL-mCherry results in unchanged  $\beta$ -Catenin protein levels in TPC2 KO SK-MEL-5 cells, respectively (S6e).

Figure S7g - Rab7



Figure S7g - Vinculin



Figure S9a - CK1, TPC2 KO



Figure S9a - Vinculin, TPC2 KO



Figure S9a - CK1, Rab7 KO

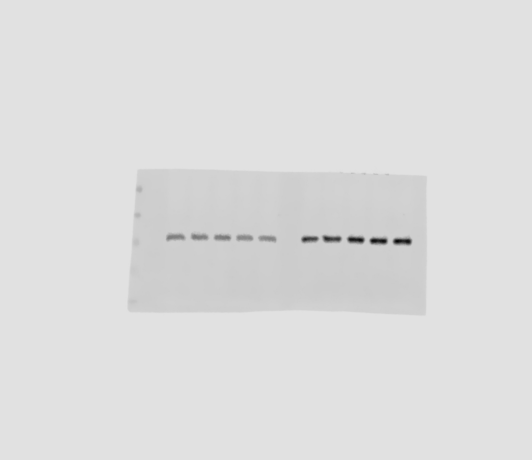


Figure S9a - Vinculin, Rab7 KO

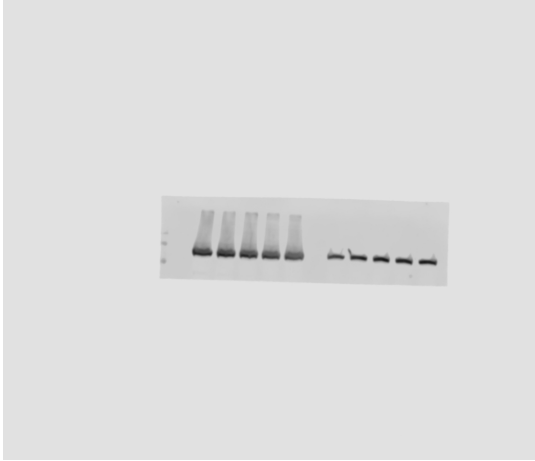


Figure S9b - p-Akt, TPC2 KO



Figure S9b - Akt, TPC2 KO



Figure S9b - Vinculin TPC2 KO

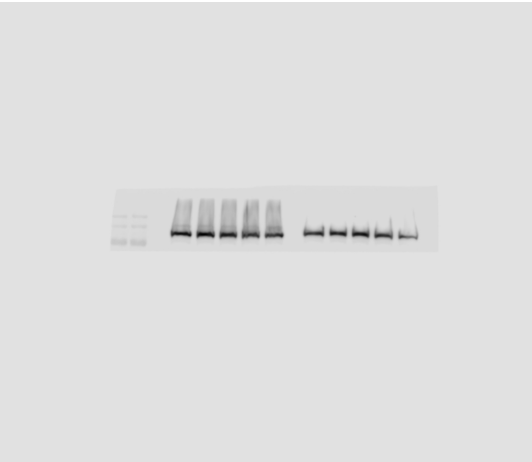


Figure S9b - p-Akt, Rab7 KO



Figure S9b - Akt, Rab7 KO



Figure S9b - Vinculin, Rab7 KO



**Figure S13. Unprocessed western blots of figures S7g, S9a and S9b.** Western blot results for the Rab7a KO in B16F10-luc cells (S7g). Western blots for CK1 (S9a) and pAkt/Akt (S9b) protein expression in SK-MEL-5 WT, Rab7a KO and TPC2 KO lines.

Figure S9c - p-CREB, TPC2 KO

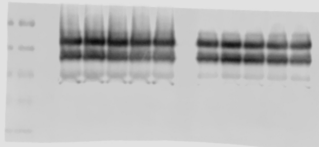


Figure S9c - CREB, TPC2 KO

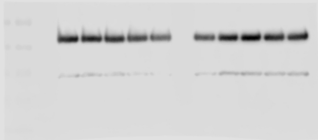


Figure S9c - Vinculin, TPC2 KO

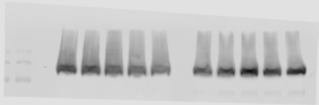


Figure S9c - p-beta-Catenin



Figure S9c - p-CREB, Rab7 KO

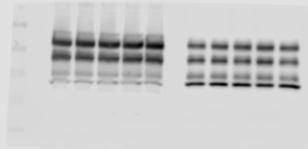


Figure S9c - CREB, Rab7 KO



Figure S9c - Vinculin, Rab7 KO

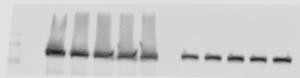


Figure S9d - non-p-beta-Catenin

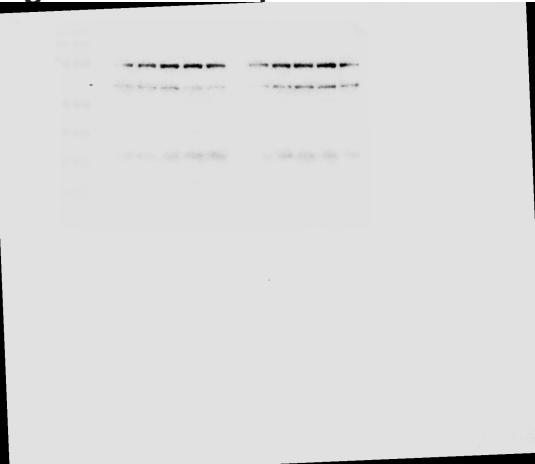


Figure S9d - Vinculin



**Figure S14. Unprocessed western blots of figures S9c and S9d.** Western blots for pCREB/CREB protein expression in SK-MEL-5 WT, Rab7a KO and TPC2 KO lines (S9c). Western blots for dephosphorylated and phospho- $\beta$ -Catenin in TPC2 KO compared to WT SK-MEL5 cells (S9d).