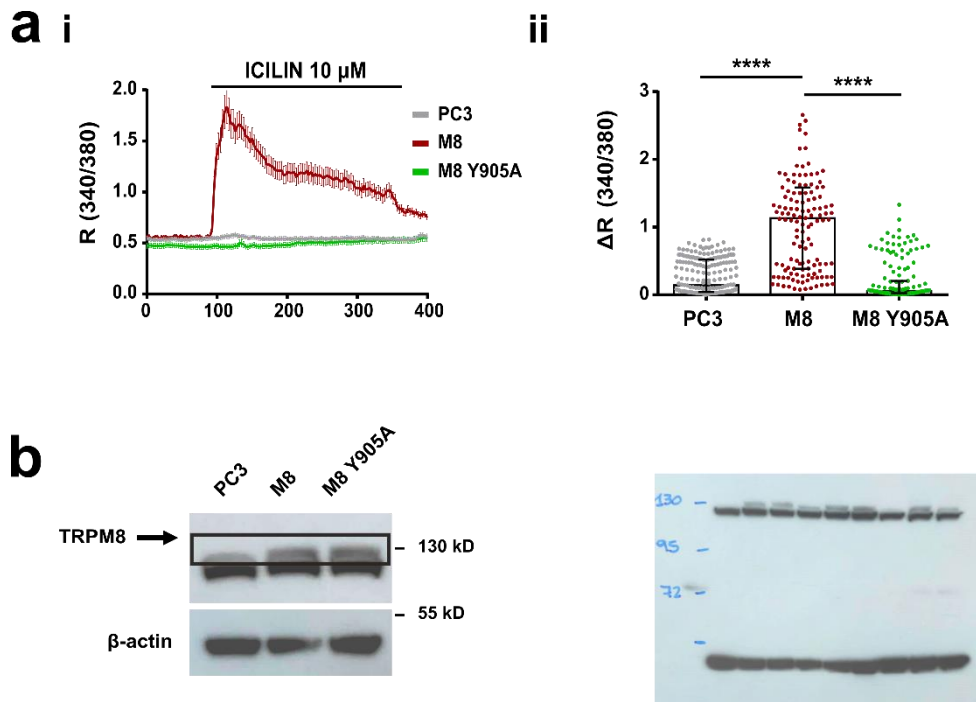


## Supplementary materials

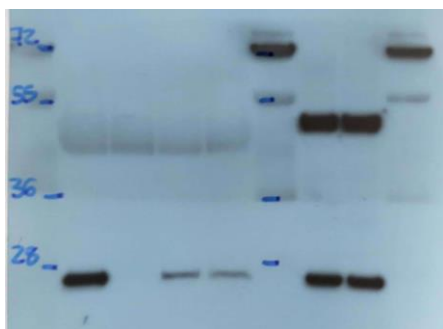


**Figure S1. Validation of TRPM8 pore mutant.**

(a i)  $\text{Ca}^{2+}$ -imaging traces in response to TRPM8 agonist (10  $\mu$ M icilin) in PC3 (grey), PC3 transiently overexpressing TRPM8 (red) and PC3 transiently overexpressing the pore mutant TRPM8 Y905A (green). Traces represent mean  $\pm$  SEM of cells in the recorded field of one representative experiment ( $n=33$  for PC3,  $n=24$  for M8 and  $n=25$  for M8 Y905A). (a ii) Scatter dot plot showing peak amplitude of icilin-mediated  $\text{Ca}^{2+}$  responses (median with interquartile range of different cells in the field from at least 5 independent experiments). All cells were considered for PC3 and PC3 overexpressing TRPM8 Y905A analysis, only icilin-responsive cells were considered for PC3 overexpressing TRPM8 ( $n=225$  for PC3,  $n=135$  for M8 and  $n=159$  for M8 Y905A). Statistical significance versus PC3 or PC3 overexpressing TRPM8, \*\*\*\*:  $P < 0.0001$  (Kruskal-Wallis test with *post-hoc* Dunn's test).

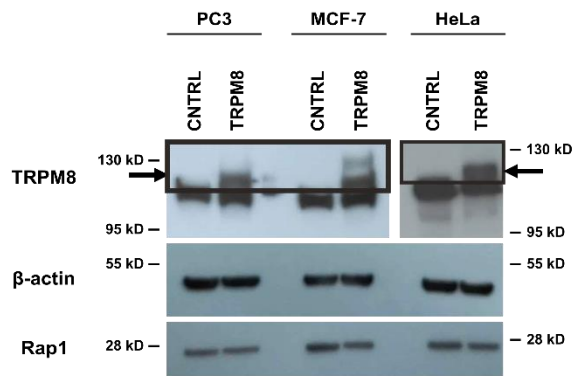
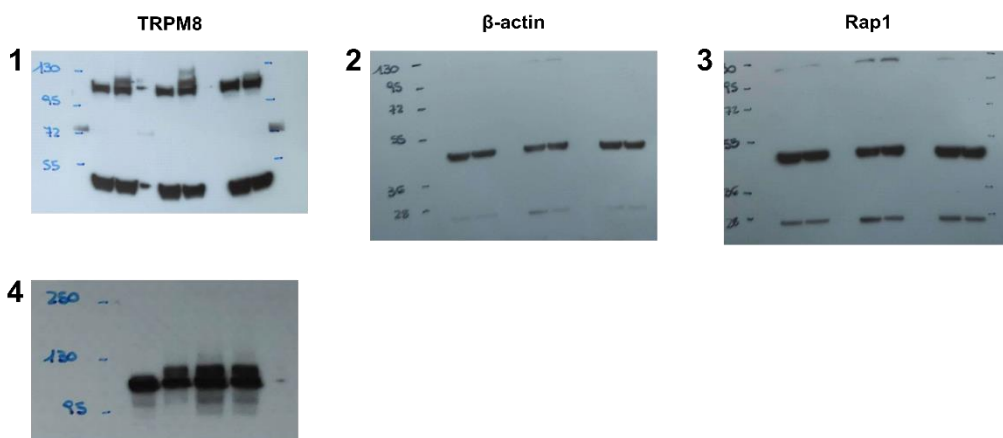
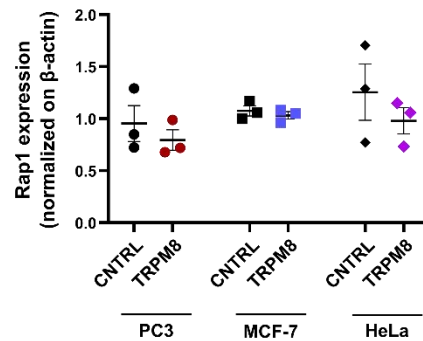
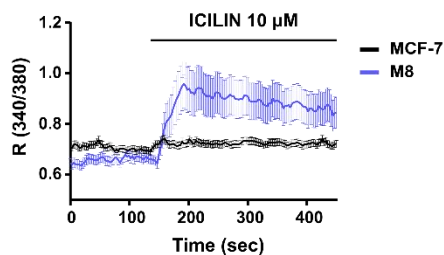
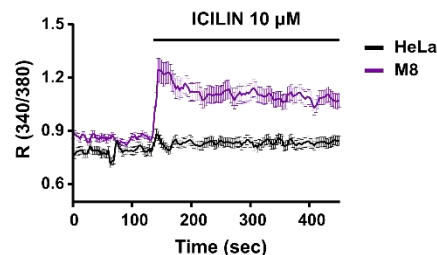
(b) Representative immunoblot showing TRPM8 expression (rabbit anti-TRPM8 Ab109308 1:800) in PC3 cells, PC3 cells overexpressing TRPM8 wt (0.625  $\mu$ g) and PC3 cells overexpressing TRPM8 Y905A (0.625  $\mu$ g).  $\beta$ -actin (mouse anti- $\beta$ -actin Sigma-Aldrich A5316 1:1000) was used as a loading control; the specific bands referring to TRPM8 expression are framed and indicated by the arrows.

The full uncropped blot is reported on the right. Samples order (from left to right): 1) PC3 CNTRL; 2) PC3+TRPM8 wt; 3) PC3+TRPM8 Y905A; 4) MCF-7 CNTRL; 5) MCF-7+TRPM8 wt; 6) MCF-7+TRPM8 E207A Y240A; 7) HeLa CNTRL; 8) HeLa+TRPM8 wt; 9) HeLa+TRPM8 E207A Y240A



**Figure S2. Original uncropped blot referring to Fig. 2ai.**

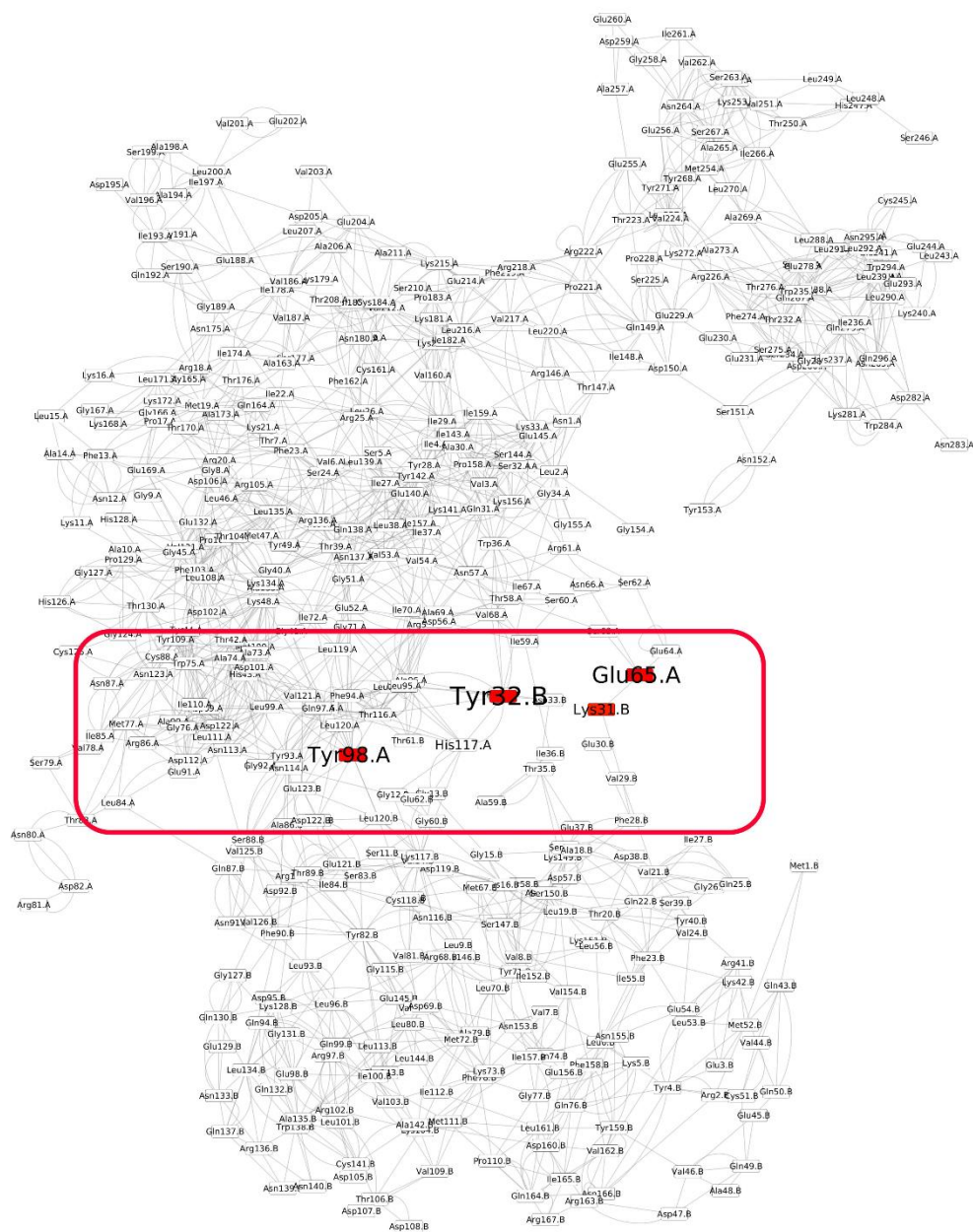
Samples order (from left to right): 1) GTP Ys; 2) GDP; 3) PC3 CNTRL (pulled-down PD); 4) TRPM8 (PD); 5) empty; 6) PC3 CNTRL (total lysates TL); 7) TRPM8 (TL)

**a i****ii****b i****ii**

**Figure S3. TRPM8 and Rap1A basal expression in PC3, MCF-7, and HeLa cell lines.**

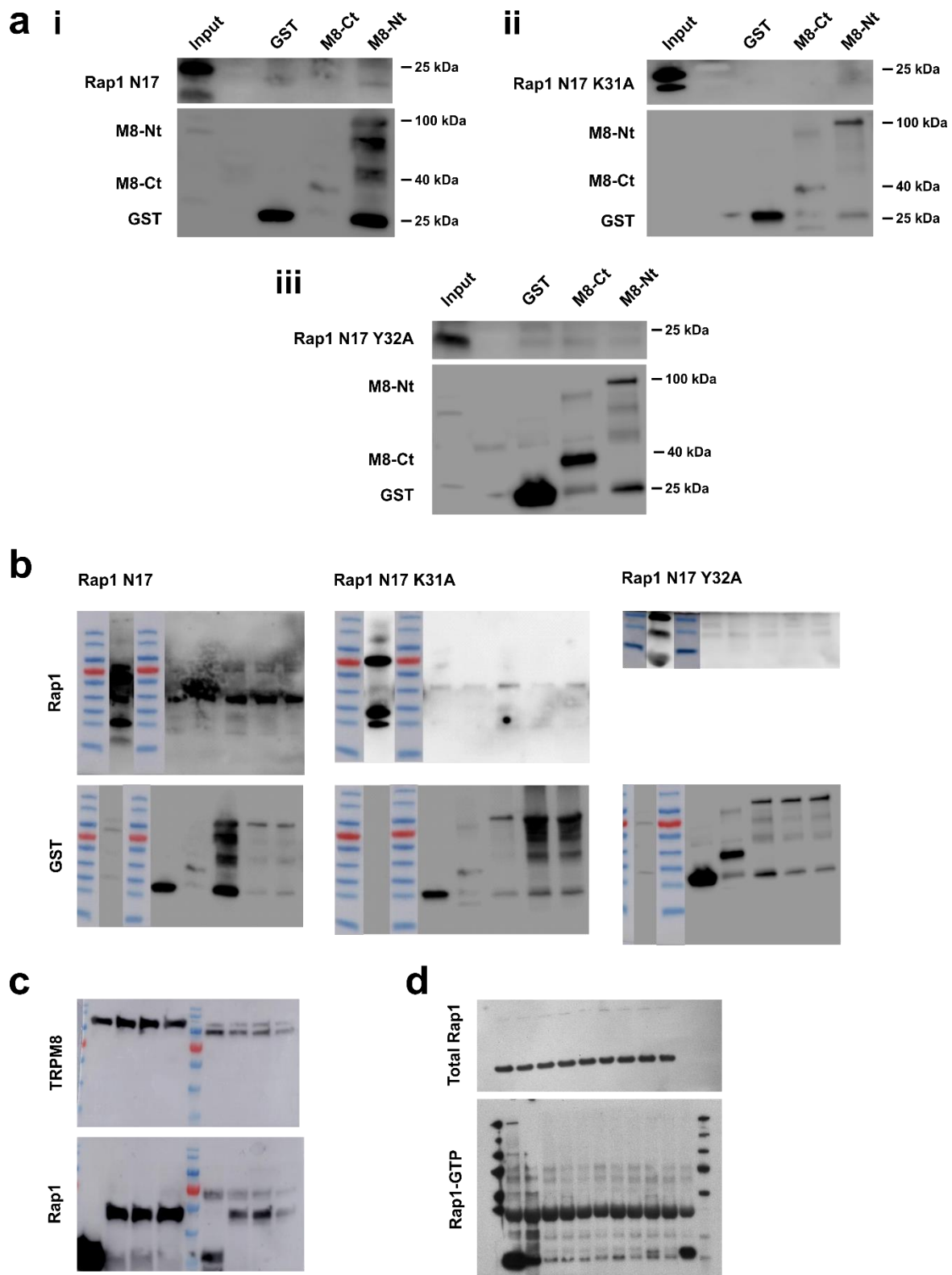
**(a i)** Representative immunoblot showing TRPM8 (rabbit anti-TRPM8 Ab109308 1:800) and Rap1 (rabbit anti-Rap1 Thermo Fisher scientific 1862344) expression in PC3, MCF-7, and HeLa cells wt and overexpressing exogenous TRPM8 wt (0.625  $\mu$ g). The specific bands referring to TRPM8 expression are framed and indicated by the arrows.  $\beta$ -actin (mouse anti- $\beta$ -actin Sigma-Aldrich A5316 1:1000) was used as a loading control and to normalize and quantify **(ii)** the endogenous amount of Rap1 within cells overexpressing or not TRPM8 ( $n=3$ ). The original uncropped blots are reported below; samples order in blots 1-3 (from left to right): 1) PC3 wt; 2) PC3+TRPM8; 3) empty; 4) MCF-7 wt; 5) MCF-7+TRPM8; 6) empty; 7) HeLa wt; 8) HeLa+TRPM8; samples order in blot 4 (from left to right): 1) HeLa wt; 2) HeLa+TRPM8 (10  $\mu$ g); 3) HeLa+TRPM8 (30  $\mu$ g); 4) 3) HeLa+TRPM8 (20  $\mu$ g).

**(b)**  $\text{Ca}^{2+}$ -imaging traces in response to TRPM8 agonist (10  $\mu$ M icilin) in MCF-7 **(i)** and HeLa **(ii)**, transiently overexpressing or not TRPM8. Traces represent mean  $\pm$  SEM of cells in the recorded field of one representative experiment ( $n=38$  for MCF-7 wt,  $n=29$  for MCF-7 M8;  $n=22$  for HeLa CNTRL,  $n=29$  for HeLa M8).



**Figure S4. RCA of the interaction between TRPM8 and Rap1A.**

The numbering here is slightly modified for TRPM8 by 142 thus His117 is actually His259. Colored in red are the residues considered as central ( $Z \text{ score} \geq 3$ ) in at least one of the five selected docking models. Only central residues at the interface are colored. Chain A corresponds to TRPM8 while B is used for Rap1A.



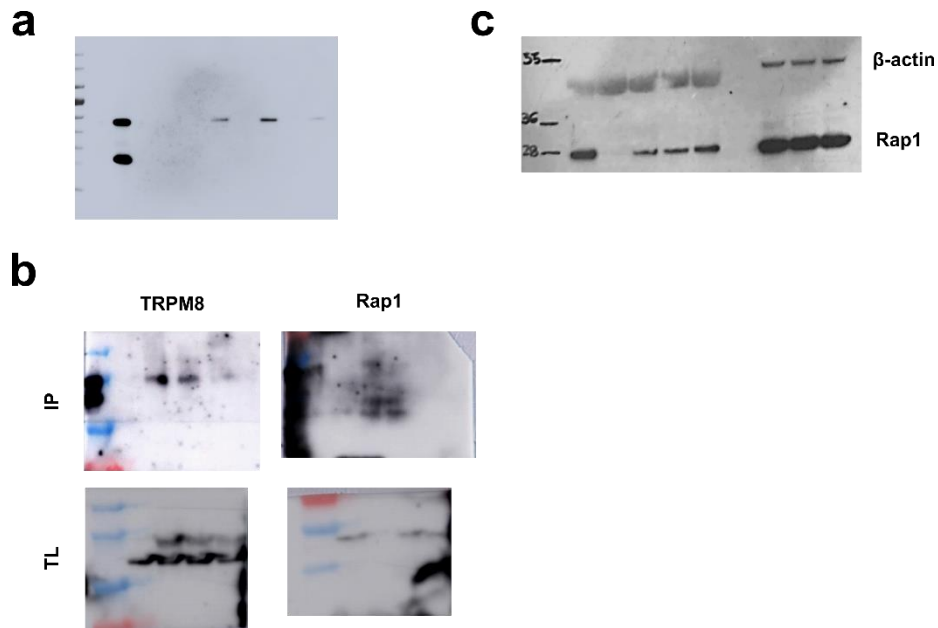
**Figure S5. Role of K31 and Y32 in mediating the direct interaction between the inactive (GDP-bound) form of Rap1 and TRPM8 N terminus.**

a) GST blots relative to GST pull-down assay described in Fig. 5a (GST-TRPM8 N-terminal tail (M8-Nt), GST-C-terminal tail (M8-Ct), or GST incubated with *in vitro* translated Rap1 N17 (i), GFP-Rap1 N17 K31A (ii) and GFP-Rap1 N17 Y32A (iii). The interaction of Rap1 mutants with GST-fused TRPM8 N- and C-terminal tail was detected using anti-Rap1 antibody and anti-GST antibody was used as control for the loading of GST-fused TRPM8 N- and C-terminal tail. One representative experiment of three (the same shown in fig. 5a) is shown.

**b)** Original uncropped blots referring to GST pull-down assays reported in Fig. 5ai, 6ai, aii, and aiii; samples order (from left to right): 1) MW marker; 2) input; 3) MW marker; 4) GST; 5) TRPM8 Ct-GST; 6) TRPM8 Nt-GST; 7) TRPM8 E207A Nt-GST; 8) TRPM8 Y240A Nt-GST.

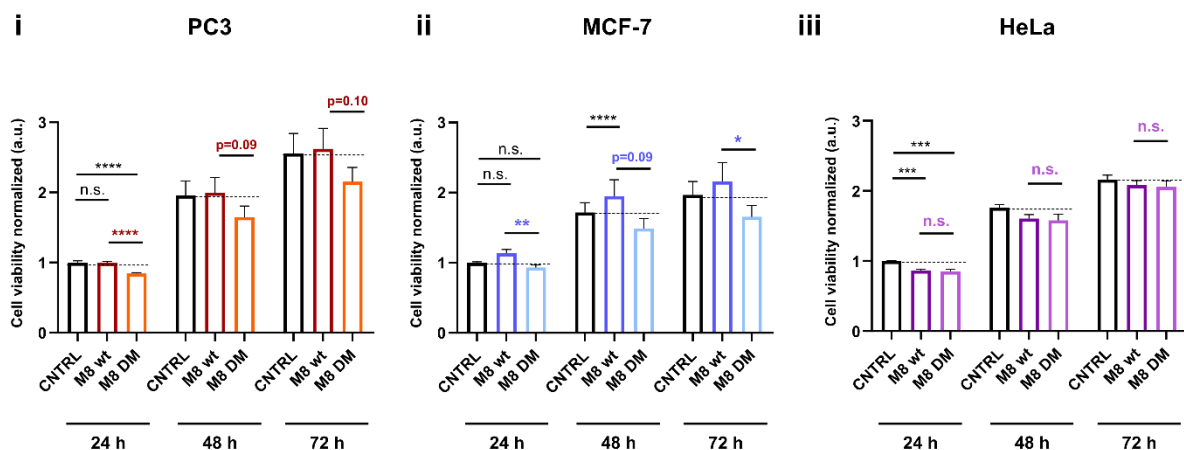
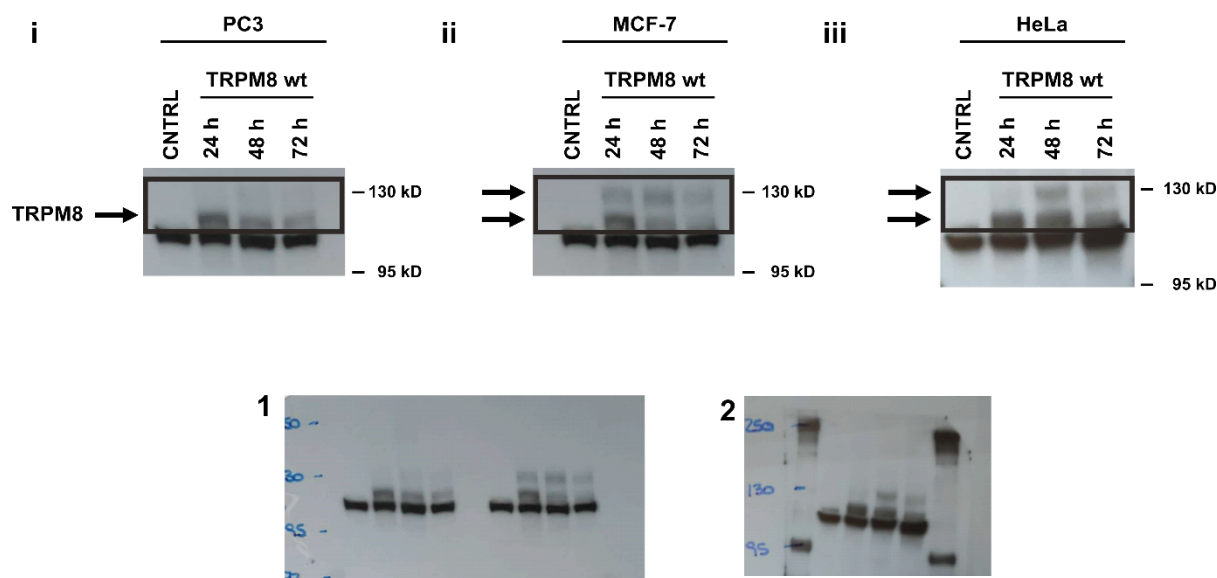
**c)** Original uncropped blots referring to Co-IP experiment reported in Fig. 5bi; samples order (from left to right): 1) MW marker; 2) GFP (total lysates TL); 3) Rap1 N17-GFP (TL); 4) Rap1 N17 K31A-GFP (TL); 5) Rap1 N17 Y32A-GFP (TL); 6) MW marker; 7) GFP (immuno-precipitated IP); 8) Rap1 N17-GFP (IP); 9) Rap1 N17 K31A-GFP (IP); 10) Rap1 N17 Y32A-GFP (IP).

**d)** Original uncropped blots referring to the active Rap1 pull-down assays reported in Fig. 5ci and 5ei; samples order (from left to right): 1) GTP  $\gamma$ S; 2) GDP; 3) TRPM8+Rap1 N17 Y32; 4) TRPM8+Rap1 N17; 5) TRPM8; 6) Rap1 N17 Y32; 7) Rap1 N17; 8) PC3 CNTRL; 9) Rap1 wt; 10) Rap1 K31A; 11) Rap1 Y32A.



**Figure S6. Original uncropped blots referring to Fig. 6 and 7**

- a)** Original uncropped blots referring to GST pull-down assays reported in Fig. 6bi; samples order (from left to right): 1) MW marker; 2) input; 3) GST; 4) TRPM8 Ct-GST; 5) TRPM8 Nt-GST; 6) TRPM8 E207A Y240A Nt-GST.
- b)** Original uncropped blots referring to Co-IP experiment reported in Fig. 6ci; samples order (from left to right): 1) MW marker; 2) Rap1 N17; 3) TRPM8; 4) TRPM8+Rap1 N17; 5) TRPM8 E207A Y240A+Rap1 N17.
- c)** Original uncropped blots referring to the active Rap1 pull-down assays reported in Fig. 7di; samples order (from left to right): 1) GTP Ys; 2) GDP; 3) PC3 CNTRL (pulled-down PD); 4) TRPM8 (PD); 5) TRPM8 E207A Y240A (PD); 6) empty; 7) PC3 CNTRL (total lysates TL); 8) TRPM8 (TL); 9) TRPM8 E207A Y240A (TL)

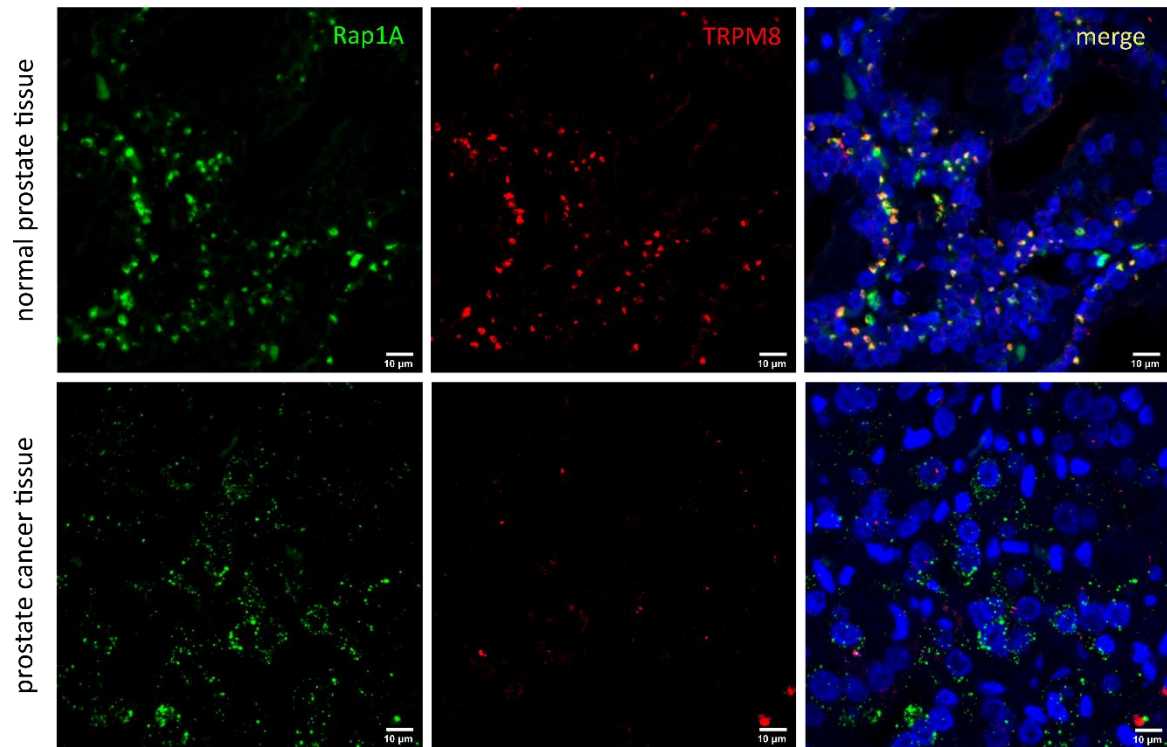
**a****b**

**Figure S7. TRPM8-Rap1A interaction in cancer cells viability.**

(a) Cell viability of PC3 (i), MCF-7 (ii) and HeLa (iii) cells 24, 48, and 72 h after transfection with 0.625  $\mu$ g of TRPM8 wt or TRPM8 E207A Y240A (M8 DM). Data are normalized on the CNTRL (cells transfected with empty vector) at 24 h and are represented as mean  $\pm$  SEM. Data refer to a pool of 3 independent experiments (eight replicates for each experiment). Statistical significance: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  \*\*\*\*  $P < 0.0001$  (Kruskal-Wallis test with post-hoc Dunn's test).

(b) Immunoblot showing TRPM8 overexpression (rabbit anti-TRPM8 Ab109308 1:800) in PC3 (i), MCF-7 (ii) and HeLa (iii) cells 24, 48, and 72 h after transfection with 0.625  $\mu$ g of TRPM8 wt; The specific bands referring to TRPM8 expression are framed and indicated by the arrows; the original uncropped blots are reported below; samples order in blot 1 (from left to right): 1) PC3 wt; 2) PC3+TRPM8 24 h after transfection; 3) PC3+TRPM8 48 h after transfection; 4) PC3+TRPM8 72 h after transfection; 5) empty; 6) MCF-7 wt; 7) MCF-7+TRPM8 24 h after transfection; 8) MCF-7+TRPM8 48 h after transfection; 9) MCF-7+TRPM8 72 h after transfection; ; samples order in blot 2 (from left to right): 1) HeLa wt; 2) HeLa+TRPM8 24 h after transfection; 3) HeLa+TRPM8 48 h after transfection; 4) HeLa+TRPM8 72 h after transfection.





**Figure S8. TRPM8-Rap1A interaction in healthy and cancerous prostate tissues.**

Representative confocal micrographs of healthy prostate tissue and prostate adenocarcinoma (Gleason 5) microarrays (Biomax Inc.) stained for Rap1A (green - anti-Rap1A ABIN2854404 ) and TRPM8 (red - anti-TRPM8 ABIN572229). Nuclei are counterstained with DAPI (blue). Scale bar: 10 µm