

Expanded View Figures

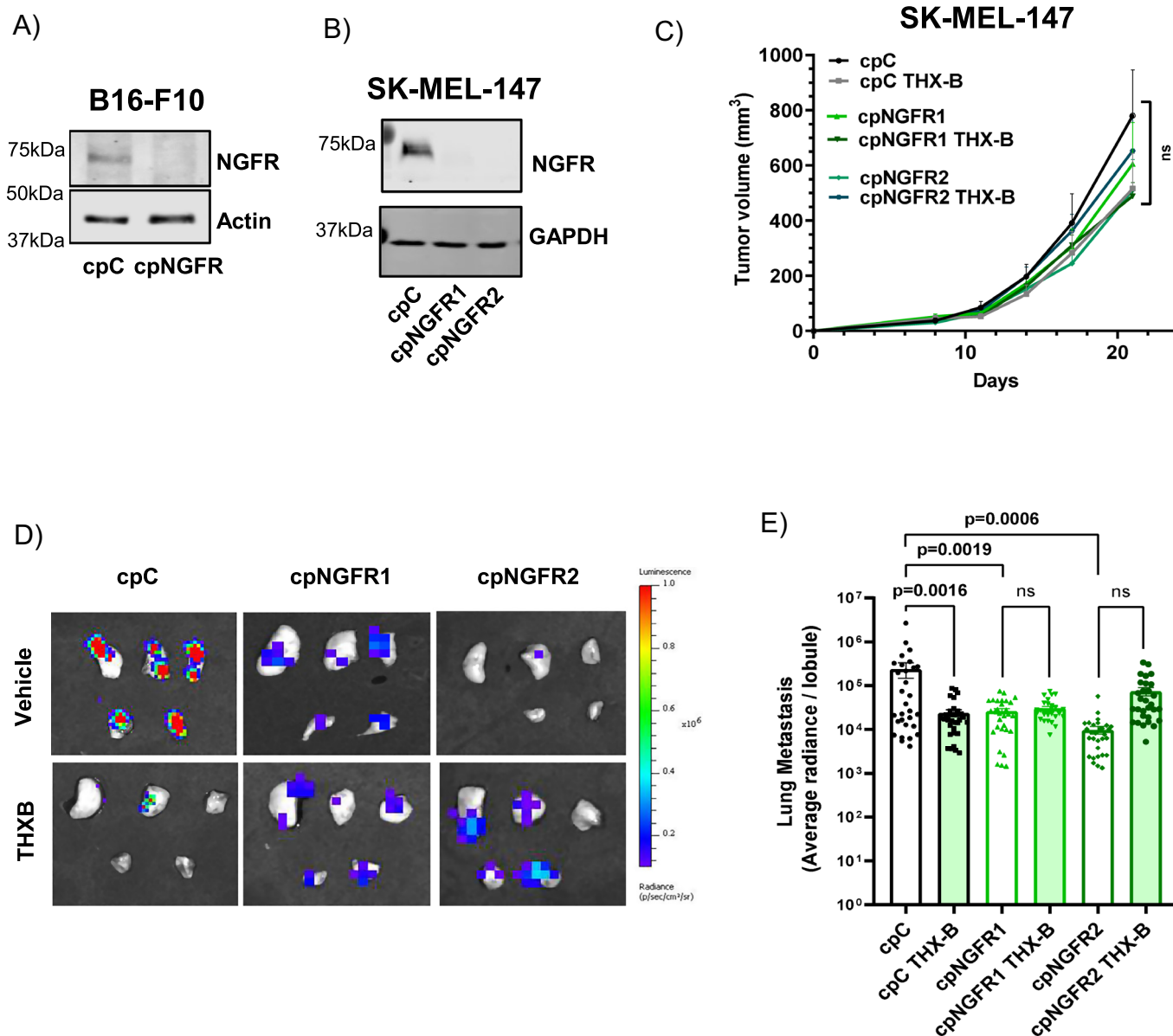


Figure EV1. THX-B decreases lung metastasis of human SK-MEL-147 melanoma tumors.

(A) Representative western blot showing NGFR expression in B16-F10 cpControl (cpC) and cpNGFR cells. (B) Representative western blot showing NGFR expression in SK-MEL-147, cpControl (cpC), and cpNGFR1/2 cells. (C) Tumor growth of SK-MEL-147 cpControl, cpNGFR1, or cpNGFR2 -GFPLuc tumors on Nude mice upon twice a week i.p. treatment with THX-B (5 mg/kg) or vehicle, starting at D7. Data were Mean + SEM of $n = 6$ mice/group. Two-way ANOVA with Tukey multi-comparison test was applied. ns, not significant. (D) Representative images of lung metastasis. (E) Quantification of the ex vivo SK-MEL-147 GFPLuc lung metastatic burden by measuring the average radiance per lobule by IVIS at endpoint. Two-way ANOVA were used to analyze tumor growth (C). Average radiance/lobule was calculated by one-way ANOVA with Tukey correction for multi-comparison (E). ns, not significant. Source data are available online for this figure.

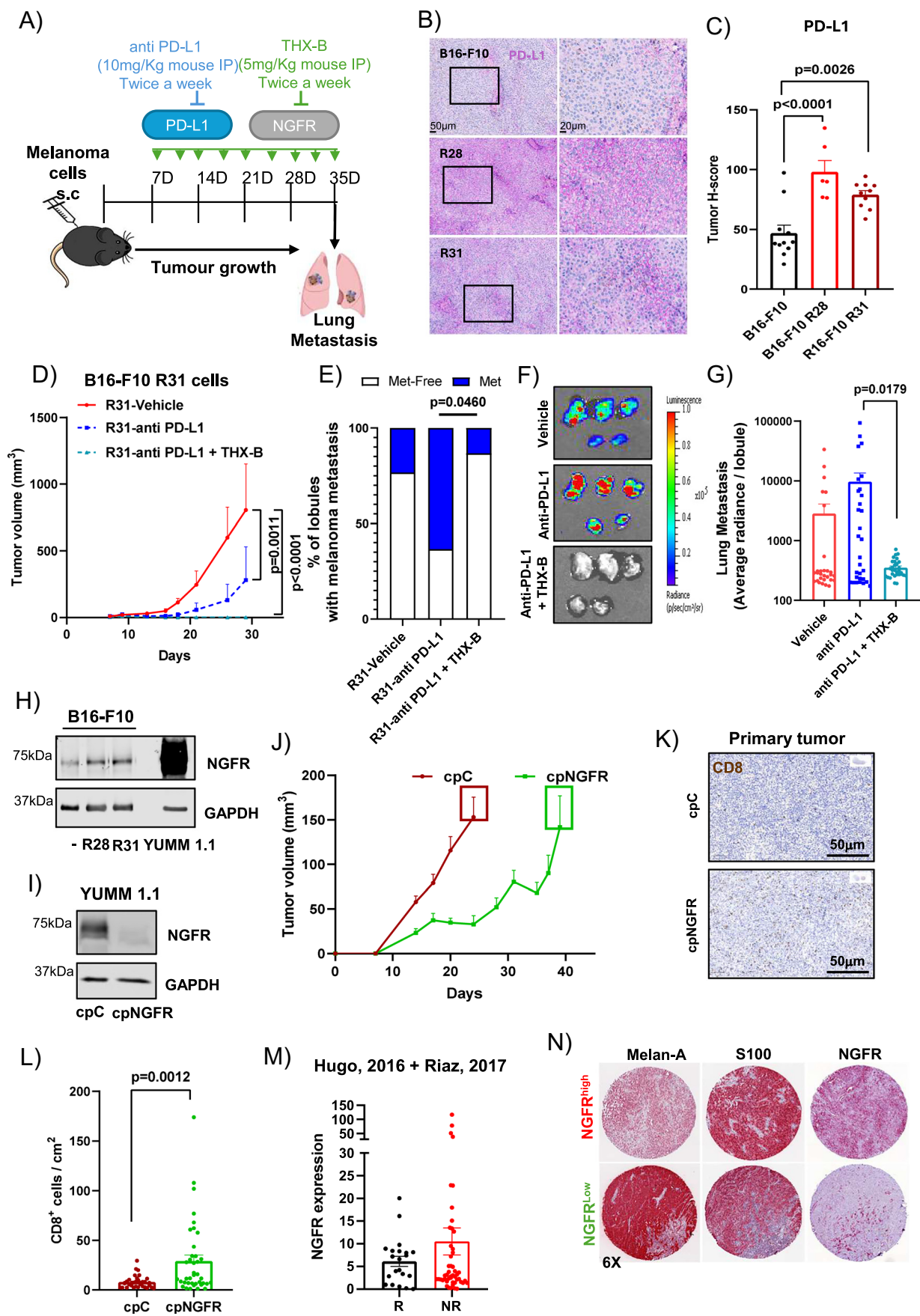
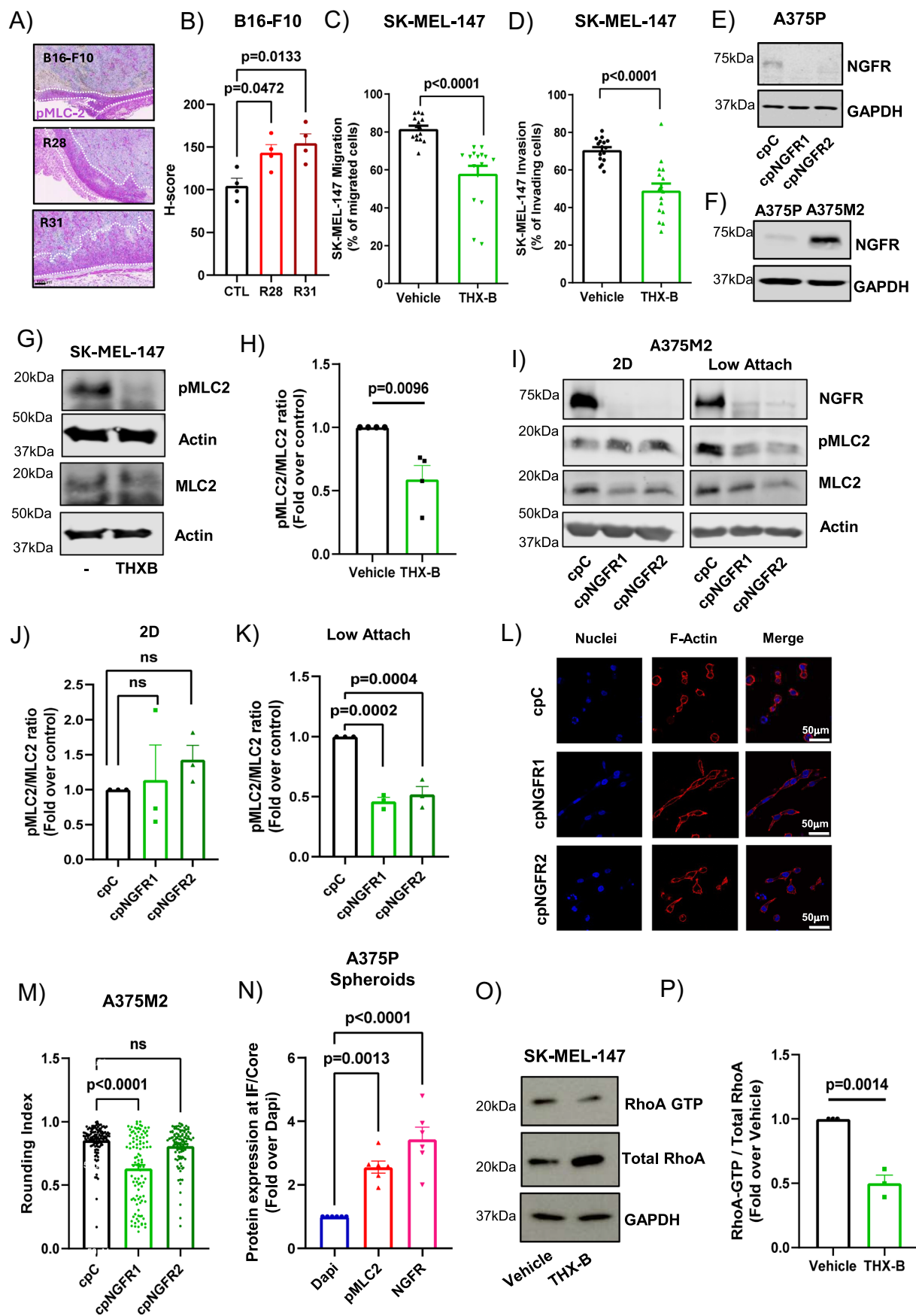


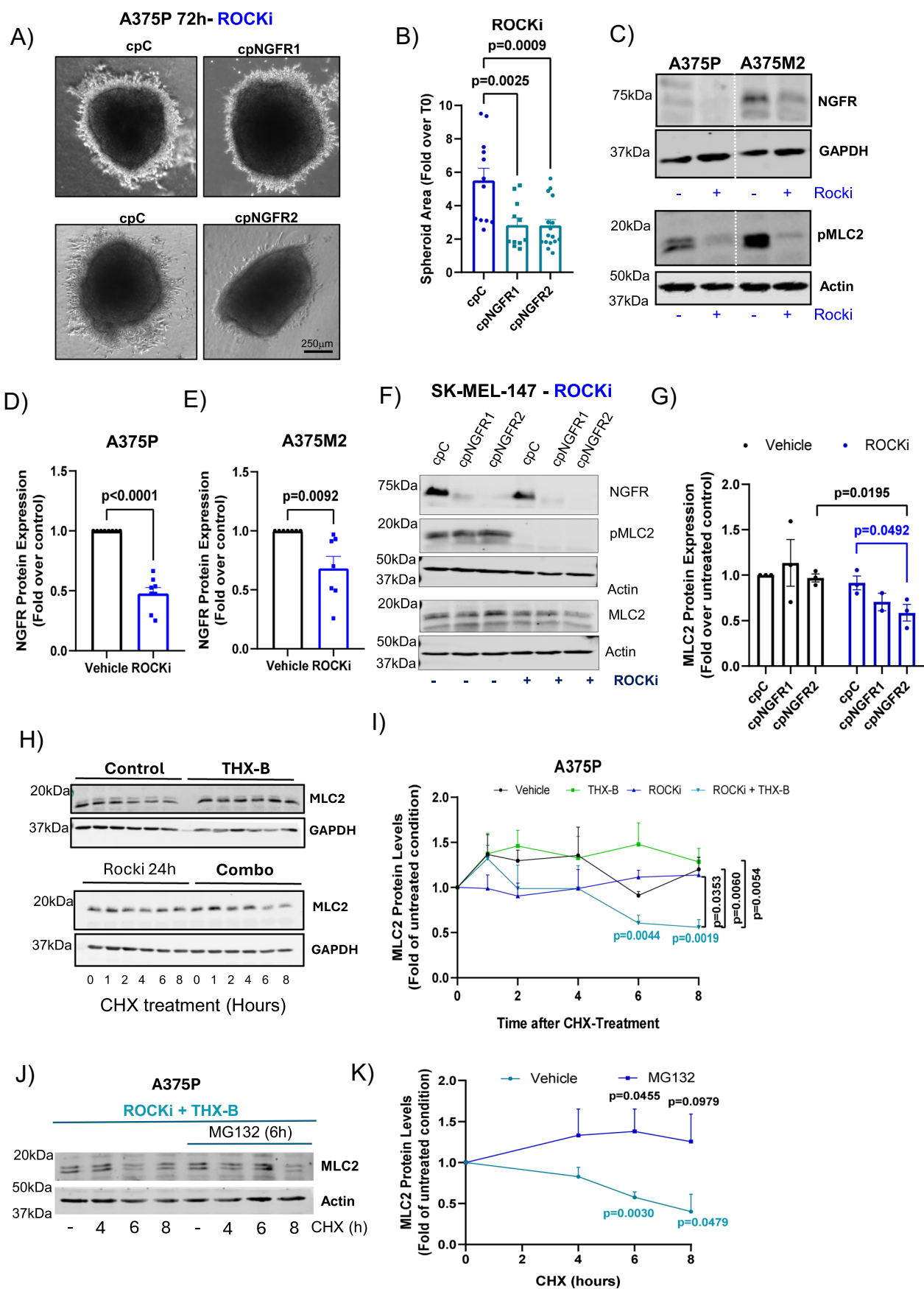
Figure EV2. Targeting NGFR restores immunotherapy responsiveness and promotes intratumoral CD8⁺ T-cell recruitment of IT-resistant tumors.

(A) Scheme of the experiment performed to study the effect of the THX-B and anti-PD-L1 combination therapy in melanoma subcutaneous immunocompetent models. Dosages of treatment are indicated. Treatments were administrated as monotherapy or in combination intraperitoneally twice a week beginning 7 days after tumor cell injection. (B, C) PD-L1 expression was measured by IHC of B16-F10 control and anti-PD-L1-resistant (R28 and R31) tumors. $n = 6-11$ different fields from 3 to 6 tumors /group. (B) Representative IHC image of PD-L1 staining in different groups. Black square indicates magnified area in the right. (C) Qupath quantification of intra-tumor PD-L1 expression. Data were mean + SEM. (D-G) B16-F10-R31 cells (200,000) were injected into the flank of B6 mice. On day 7, treatment started as indicated in (A) ($n = 6$ mice/group). (D) Tumor growth curves showing mean + SEM. (E) Percentage of lung lobules with melanoma metastases. (F) Representative IVIS image. (G) Quantification of the average radiance/lobule. Data were mean + SEM. ($n = 6$ mice/group). (H) NGFR expression in melanoma cell lines with acquired (B16-F10 R28 and R31) or intrinsic (Yumm1.1) immunotherapy resistance. A representative Western blot is shown. (I) Representative western blot of the efficient KO of NGFR in Yumm1.1 cells. (J) Tumor growth of cpC and cpNGFR YUMM1.1 tumors in B6 mice sacrificed at the same tumor size (100–300 mm³). The square indicates the mean size of the tumors analyzed by IHC. Data were mean + SEM of $n = 12$ tumors from 6 mice (injected in both flanks). (K) Representative image of CD8⁺ IHC staining in tumors from (J). Scale bar (50 μ m) is indicated. (L) The number of CD8⁺ T cells was calculated from 3 to 4 consecutive slides/tumor using QuPath-0.5.1 software and normalized to the area of the tumor. Data were mean + SEM. (M) NGFR expression in responders (R) or non-responders (NR) to anti-PD-1 therapies from datasets GSE78220 (Hugo et al, 2016) and GSE91061 (Riaz et al, 2017). Mean + SEM of $n = 70$ patients is represented. (N) Representative immunohistochemistry of TMAs from melanoma patients under immunotherapy. Serial sections stained for Melan-A, S100 and NGFR are shown. Upper panel: full TMA 258. Red and green boxes indicate representative cores with high or low NGFR expression, respectively. Objective magnification as indicated. (N) Statistical analyses performed were one-way ANOVA (C), Two-way ANOVA (D) and unpaired Student *t*-test (E, G, I). Tukey correction was used for multiple comparison. Source data are available online for this figure.



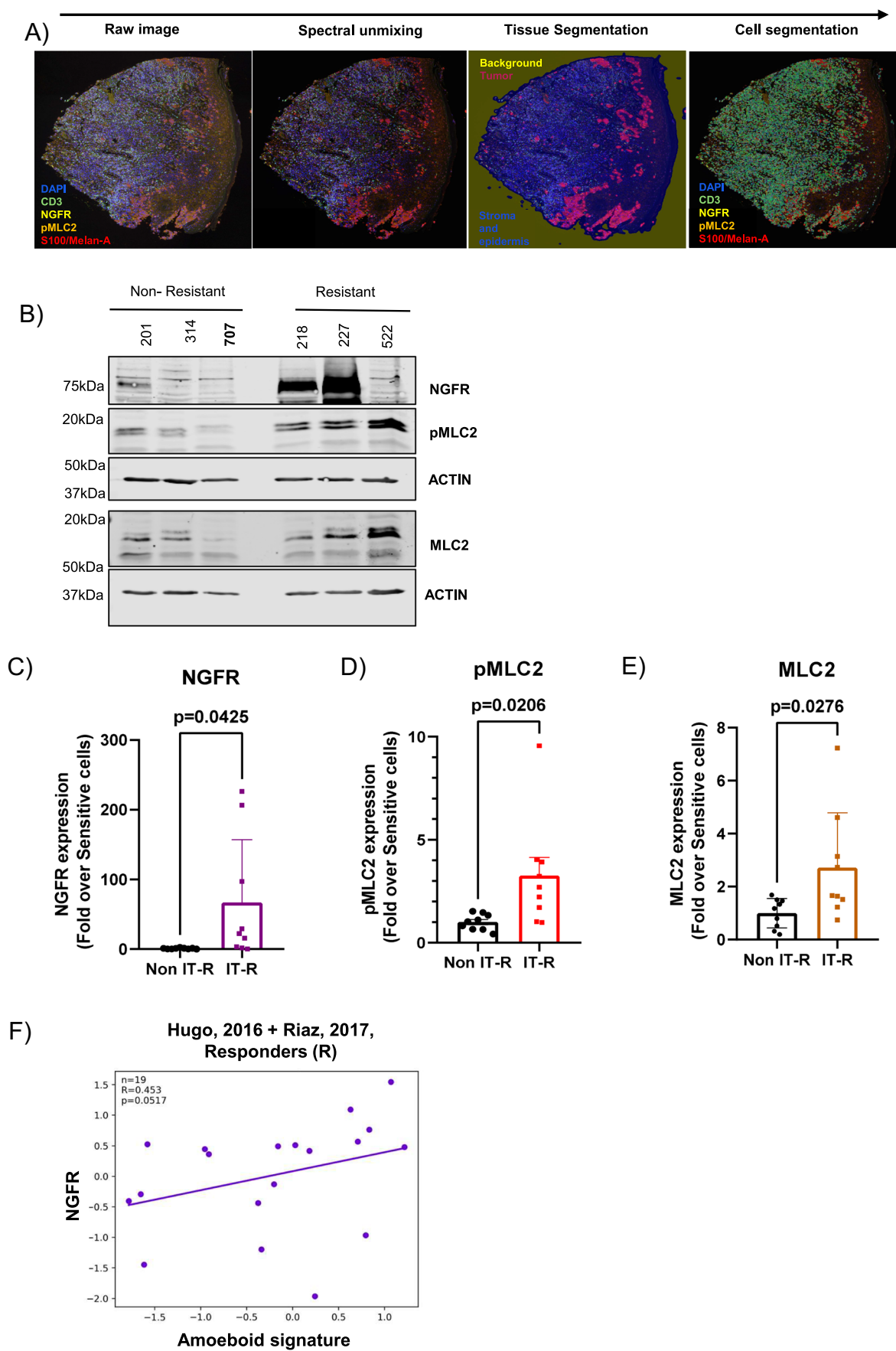
◀ **Figure EV3. NGFR is required for the ROCK-MLC2 ameboid metastasis of human melanoma tumors.**

(A) Representative IHC of pMLC2 activation at the invasive front of parental (CTL) or anti-PD-L1-resistant (R28 and R31) B16-F10 tumors. The invasive front (IF) was indicated by white dots. (B) Qu-Path quantification of pMLC2 expression at the IF (defined by white dots). One-way ANOVA with Tukey multi-comparison correction was performed. Representative data (Mean + SEM) from 1 experiment $n = 4$ mice replicated in 2 independent experiments. (C, D) Transwell SK-MEL-147 cell migration (C) and invasion (D) to FBS (% of total cells) after treatment with vehicle (DMSO 1.6%) or THX-B (20 μ M, ON). Data were mean + SEM of four fields per condition of two independent experiments performed in duplicate. Student's *t*-test. (E) Representative Western blot of NGFR knockdown in A375P cells. (F) Representative Western blot showing increased NGFR expression levels in ameboid A375M2 cells compared to parental A375P cells. (G) Representative Western blot showing reduced pMLC2 levels in SK-MEL-147 cells upon THX-B treatment (20 μ M, 24 h). (H) Quantification of the pMLC2/total MLC2 ratio and referred to SK-MEL-147 treated with vehicle. Data were mean + SEM of $n = 4$ independent experiments. Unpaired *t*-test was performed. (I) Representative Western blot of the effective CRISPR/Cas9 knockdown of NGFR in A375M2 cells and the activation of pMLC2 in 2D standard adhesion or low adhesion conditions. (J, K) Quantification of the pMLC2/total MLC2 ratio in A375M2 cpControl vs NGFR KO cells in 2D conditions (J) or low-attachment plates (K) Data were mean + SEM of $n = 3$ independent experiments. One-way ANOVA with Dunnett multi-comparison test was performed. ns not significant. (L) Confocal images of cpC, cpNGFR1 and cpNGFR2 A375M2 cells seeded on top of collagen for 24 h. (M) Quantification of rounded versus elongated cells. Data represent mean + SEM of 126, 102, and 108 cells from two independent experiments. One-way ANOVA with Dunnett correction was performed. ns, not significant. (N) Quantification of DAPI, NGFR, and pMLC2 protein expression at the invasive front of A375P-3D invasive spheroids upon 3 days of culture into collagen I matrix. Mean intensity was calculated as the ratio between the expression at the IF vs Core of the spheroid and represented as fold over the DAPI condition +SEM. $n = 6$ spheroids/group. One-way ANOVA analysis with Tukey multi-comparison was performed. (O, P) Pull down of RhoA-GTP in SK-MEL-147 cells treated with THX-B (20 μ M, 24 h). (O) Representative Western blot. (P) RhoA-GTP relative activation to total RhoA expression is represented as fold over the untreated condition. $n = 3$ independent experiments were performed and represented as mean + SEM. An unpaired *t*-test (P) statistics test was applied. Source data are available online for this figure.



◀ **Figure EV4. Depletion of NGFR in combination with ROCKi compromised MLC2 stability.**

(A) Multicellular spheroids of cpC, cpNGFR1 and cpNGFR2 A375P (3×10^3 cells) were prepared as in Fig. 4J–L in the presence of ROCKi (1 μ M). Representative bright-field images of T2 (72 h) invasive spheroids upon ROCKi are shown. (B) Invasion area at T2 was normalized to mean invasion area at T0 and plotted with error bars representing $\pm$ SEM ($n = 11$ –16 spheroids/group from two independent experiments). One-way ANOVA with Dunnett multiple comparison was performed. (C) Representative western blot of NGFR / GAPDH, pMLC2/Actin of A375P and A375M2 cells treated with ROCKi (1 μ M) 24 h. (D, E) NGFR protein expression represented as fold over vehicle-treated condition in A375P (D) and A375M2 (E) cells. Data were mean $\pm$ SEM of $n = 7$ and 8 independent experiments (A375M2 and A375P cells, respectively). Student's *t*-test was used as a statistical method. (F) Representative Western blot of NGFR, MLC2, and GAPDH of cpC, cpNGFR1, and cpNGFR2 SK-MEL-147 cells treated or not with ROCKi (1 μ M) 24 h. (G) MLC2 protein expression was quantified and represented as fold over untreated cpC cells. Data were mean $\pm$ SEM of 2–3 independent experiments. Student's *t*-test was used. (H) Representative immunoblots of A375P cells treated with vehicle, THX-B (20 μ M), ROCKi (1 μ M), and the combination of THX-B and ROCKi for 24 h. Cycloheximide (20 μ g/ml) was added to cells for the indicated periods before cell lysis. Total MLC2 and GAPDH levels were shown. (I) MLC2 protein levels were normalized with GAPDH expression and depicted as fold of expression over 0 h of cycloheximide treatment. Data (mean $\pm$ SEM.) from four independent experiments are shown. Unpaired *t*-test was performed comparing MLC2 expression at each time point vs time 0 of cycloheximide treatment. (J) Representative immunoblots of A375P cells treated with the combination of THX-B and ROCKi for 24 h and cycloheximide (20 μ g/ml) for the indicated times. MG132 (10 μ M) was added for 6 h to half of the wells to block proteasome-dependent degradation. Total MLC2 and GAPDH levels were shown. (K) MLC2 protein levels were normalized with GAPDH expression and depicted as a fold of expression over 0 h of cycloheximide treatment in the control group. Data (mean $\pm$ SEM.) from four independent experiments are shown. Unpaired *t*-test was performed comparing the protein decay at each time point vs T0 of cycloheximide treatment (*p* in blue) and comparing protein levels at each point of MG132-treated vs untreated A375P cells (*p* in black). Source data are available online for this figure.



◀ **Figure EV5. NGFR and pMLC2 are enriched in patient-derived immunotherapy-resistant cell lines.**

(A) Representative images of a core of TMAs from primary melanomas illustrating the analysis pipeline performed with the *inForm*[®] software. Different staining and tissue sections are indicated in colors. (B) Representative immunoblots of NGFR, pMLC2, MLC2, and Actin of non-immunotherapy resistant vs immunotherapy-resistant cell lines derived from metastatic samples from melanoma patients. (C-E) Quantification of the NGFR expression (B), pMLC2/MLC2 ratio (C), and MLC2 total levels (D) in cells indicated in (A). Data were mean + SEM of cell lysates grouped as non-IT-resistant and IT-resistant cells of three different lines in three independent experiments. Student's *t*-test was performed. (F) Associations between NGFR and the ameoboid signature in responder patients (*n* = 19) were quantified using Spearman's rank correlation (*R*) with corresponding *p* values. Results were visualized using scatter plots with a fitted linear trend line and annotation of *n*, *R*, and *p* value. Source data are available online for this figure.