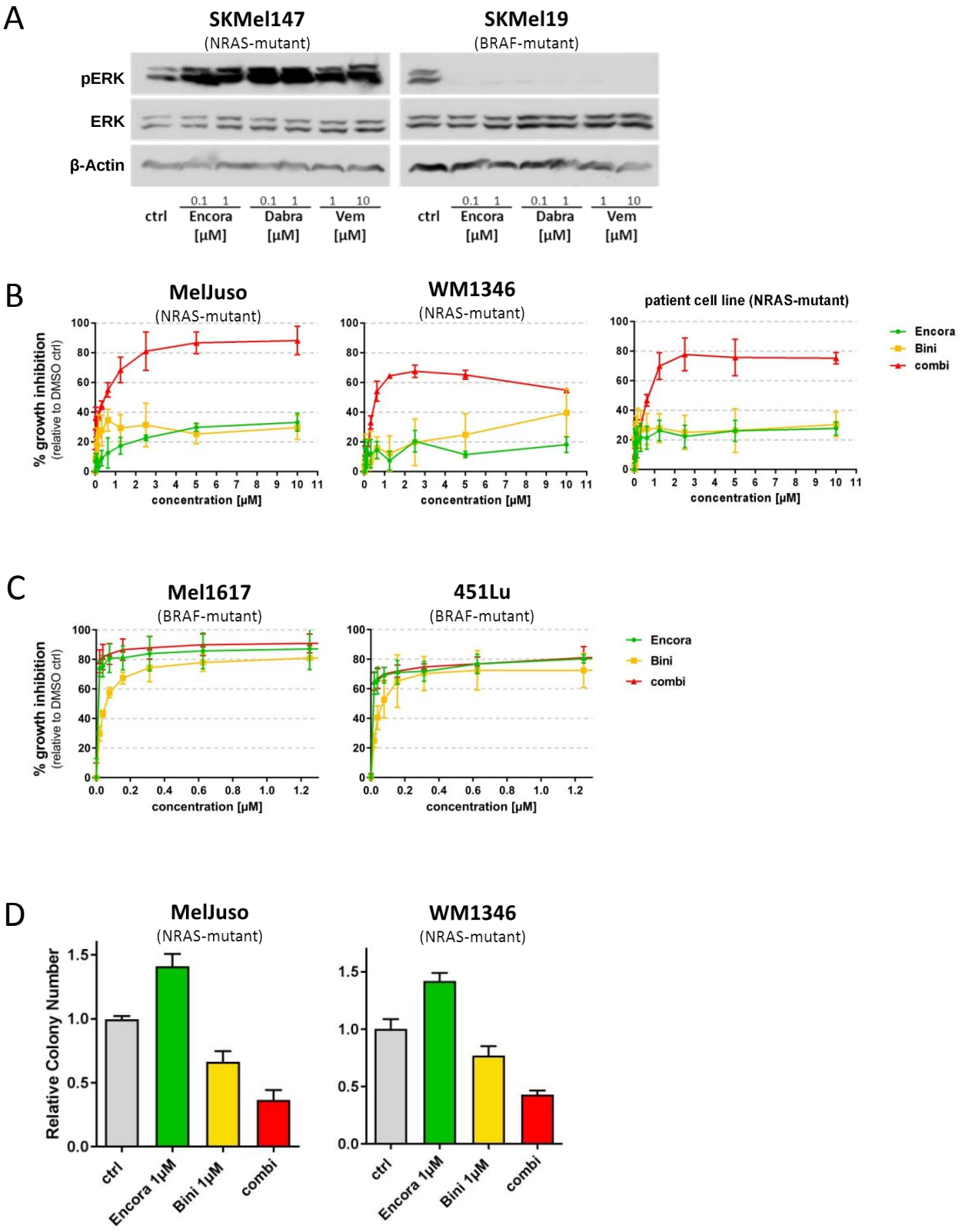
Supplementary Information

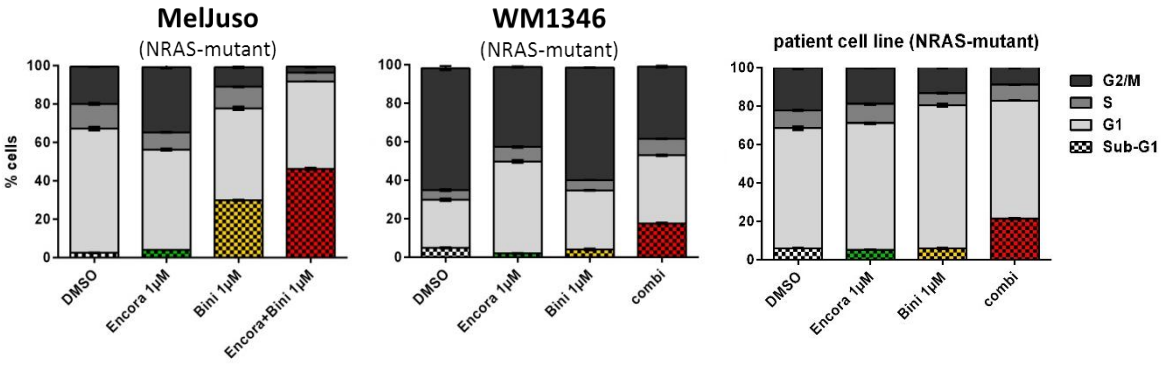
Supplementary Figures S1 to S8
Supplementary Tables 1 to 3

Supplementary Figure 1

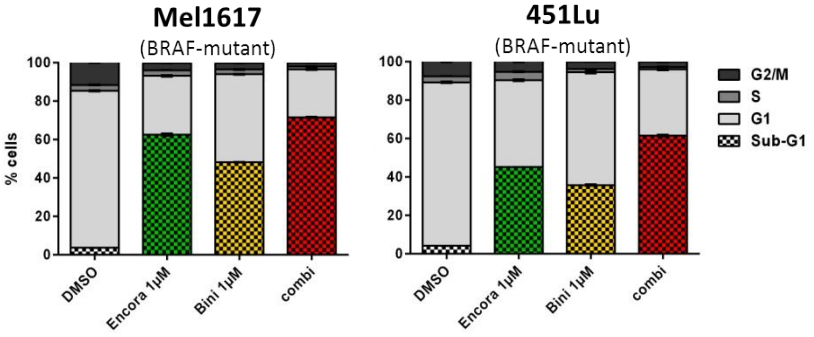


Supplementary Figure 1

E



F

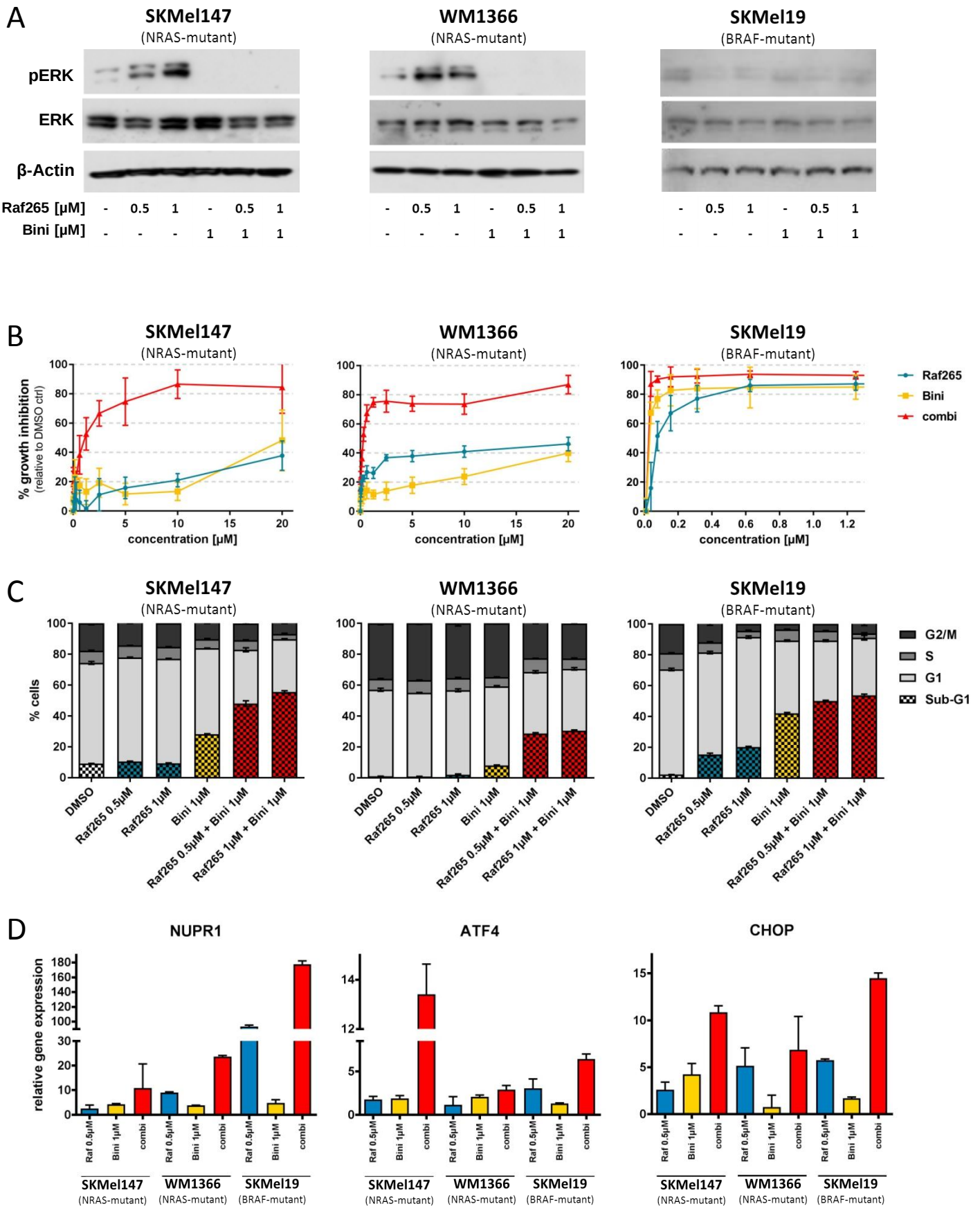


Suppl. Figure 1

Encorafenib combined with binimetinib leads to increased growth inhibition and increased induction of apoptosis in NRAS-mutant melanoma cells

(A) Whole cell lysates from melanoma cells with NRAS (SKMel147) or BRAF (SKMel19) mutation treated with encorafenib (0.1 and 1 µM), dabrafenib (0.1 and 1 µM) and vemurafenib (1 and 10 µM) or DMSO (control) for 24 h were subjected to Western blot to detect pERK and ERK levels; β-Actin served as a loading control. Experiment shown is a representative of two independent experiments. (B;C) Growth assessment (4-methylumbelliferyl heptanoate of indicated NRAS-mutant (B) or BRAF-mutant (C) melanoma cells treated with encorafenib, binimetinib or both for 72 h. The percentage of growth inhibition was calculated, normalized to the DMSO-treated control. One representative experiment of two is shown (mean ± SD of quadruplicates). (D) Anchorage-independent growth assays of NRAS-mutant cell lines treated with encorafenib, binimetinib or both for 10 d. Colonies were visualized with crystal violet, counted and normalized to the untreated control. One representative experiment of two is shown (mean ± SD triplicates). (E;F) FACS cell cycle analysis (propidium iodide; right) of indicated NRAS-mutant (E) or BRAF-mutant (F) melanoma cells treated with encorafenib, binimetinib or both for 72 h. The size of the sub-G1 fraction was calculated from FACS cell cycle analysis using propidium iodide (mean ± SD of duplicates from three independent experiments).

Supplementary Figure 2

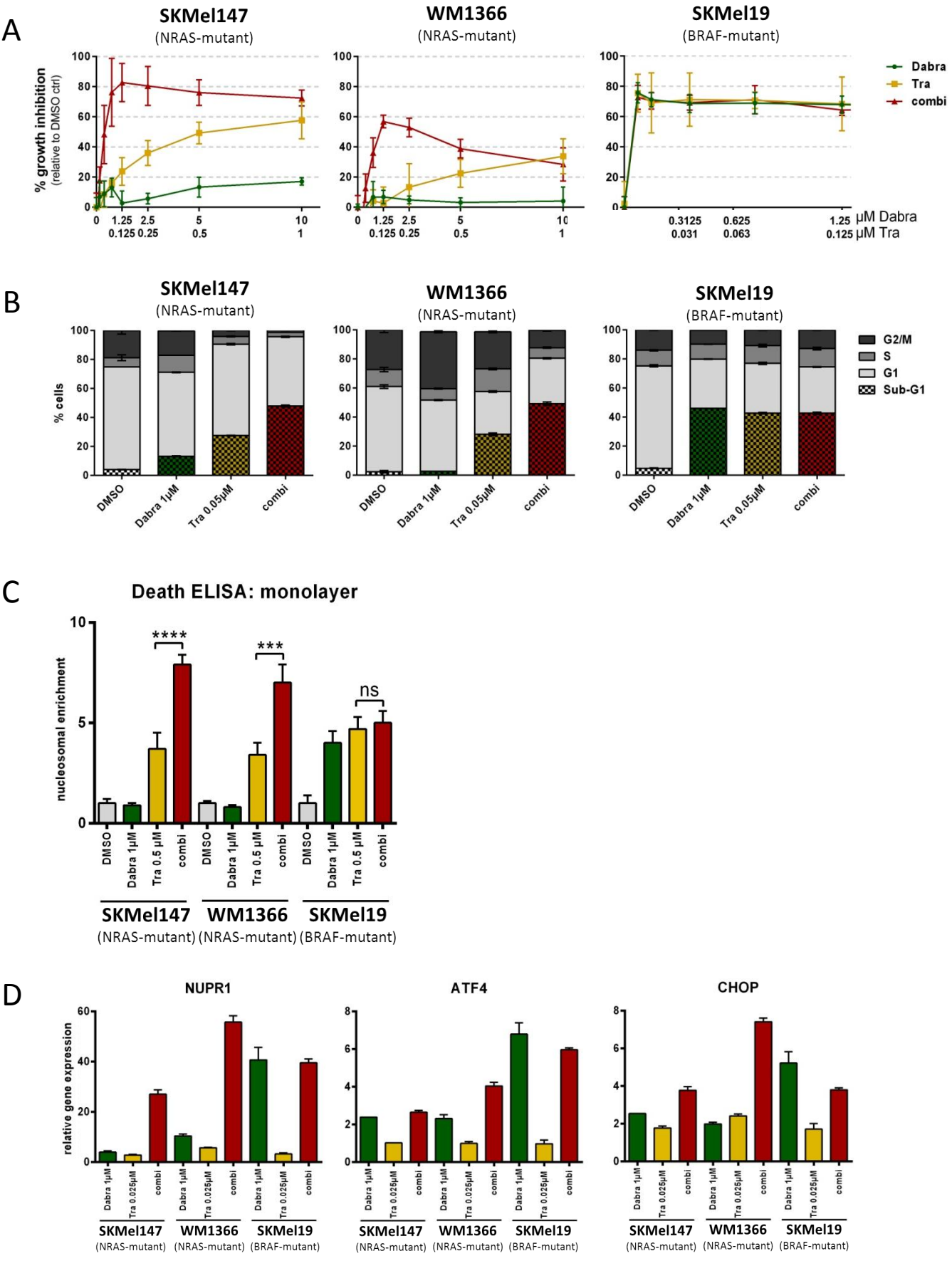


Suppl. Figure 2

The panRAF inhibitor Raf265 combined with binimetinib leads to growth inhibition, induction of apoptosis and ER stress genes in NRAS-mutant melanoma cells

(A) Whole cell lysates from NRAS-mutant (SKMel147, WM1366) or BRAF-mutant (SKMel19) melanoma cells treated with Raf265 or/and binimetinib or DMSO as a control for 24 h were subjected to Western blot analysis to detect pERK, ERK and β-Actin. Experiment shown is a representative of three independent experiments. (B) Growth assessment (4-methylumbelliferyl heptanoate (MUH)) of NRAS-mutant (SKMel147, WM1366) or BRAF-mutant (SKMel19) melanoma cells treated with Raf265, binimetinib (Bini), their combination or DMSO as control for 72 h. The percentage of growth inhibition was calculated, normalized to the DMSO-treated control. One representative experiment of two is shown (mean ± SD of quadruplicates). (C) Melanoma cells were treated with Raf265, binimetinib, Raf265 plus binimetinib or DMSO as control for 72 h. Apoptotic cells (sub-G1 fraction) were quantified by FACS cell cycle analysis (propidium iodide staining) (mean ± SD of duplicates from three independent experiments). (D) Melanoma cells were treated with Raf265 or/and binimetinib for 18 h. Real-time PCR results show levels of NUPR1, ATF4 and CHOP mRNA expression in comparison with DMSO treated cells. All samples were normalized to TBP mRNA (mean ± SD of triplicates from three independent experiments).

Supplementary Figure 3

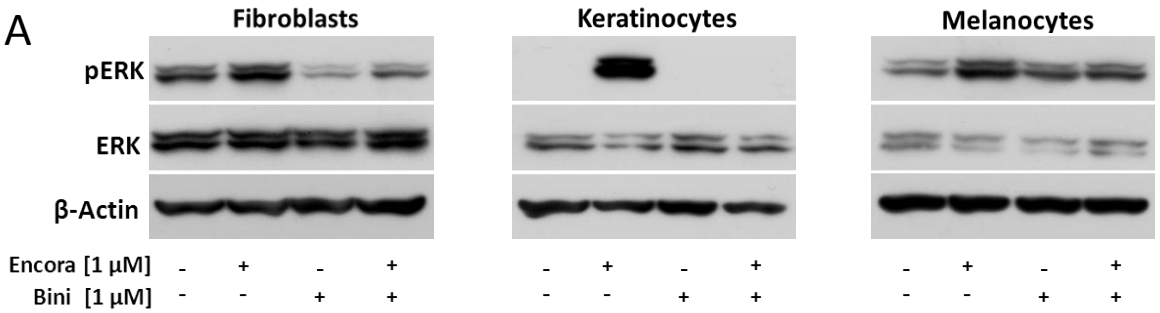


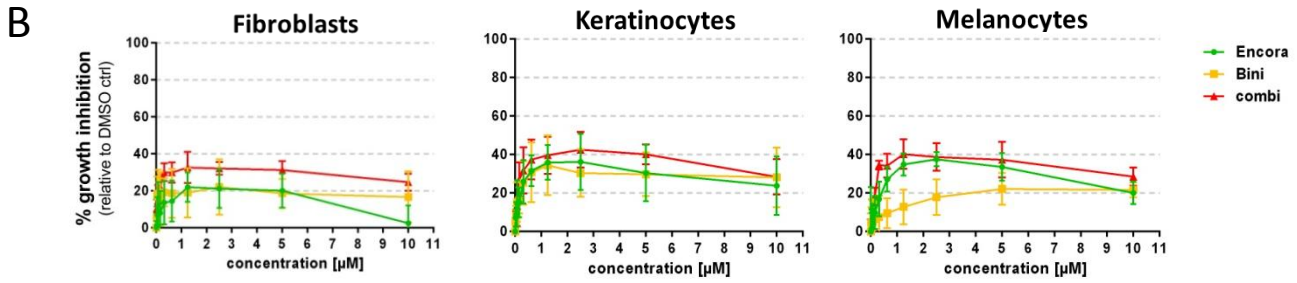
Suppl. Figure 3

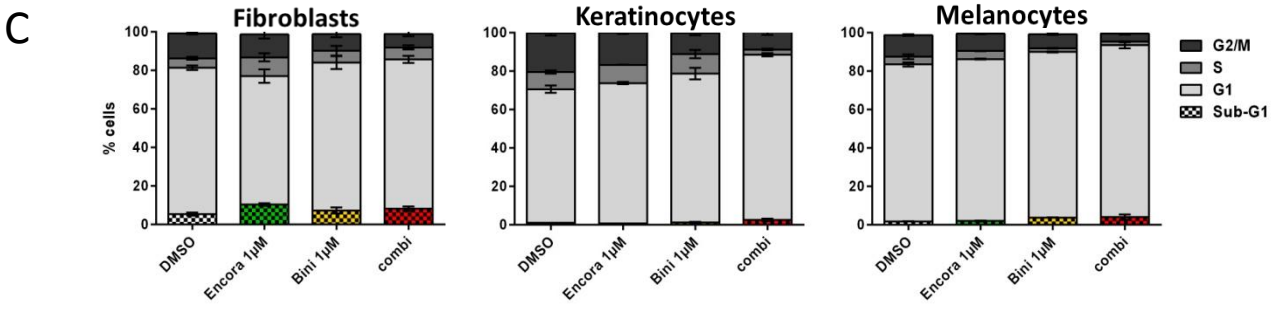
Dabrafenib combined with trametinib leads to strong growth inhibition, induction of apoptosis and ER stress genes in NRAS-mutant melanoma cells

(A) Growth assessment (4-methylumbelliferyl heptanoate (MUH)) of NRAS-mutant (SKMel147, WM1366) or BRAF-mutant (SKMel19) melanoma cells treated with dabrafenib (Dabra), trametinib (Tra), their combination or DMSO as control for 72 h. The percentage of growth inhibition was calculated, normalized to the DMSO-treated control. One representative experiment of two is shown (mean ± SD of quadruplicates). (B) Melanoma cells were treated with dabrafenib, trametinib, dabrafenib plus trametinb or DMSO as control for 72 h. Apoptotic cells (sub-G1 fraction) were quantified by FACS cell cycle analysis (propidium iodide staining) (mean ± SD of duplicates from three independent experiments). (C) Melanoma cells grown in monolayer were treated with dabrafenib or/and trametinib for 48 h. Nucleosomal enrichment in cytosolic fractions (apoptotic cell death) was calculated and displayed in a bar graph (mean ± SD of triplicates from three independent experiments). (D) Melanoma cells were treated with dabrafenib or/and trametinib for 18 h. Real-time PCR results show levels of NUPR1, ATF4 and CHOP mRNA expression in comparison with DMSO treated cells. All samples were normalized to TBP mRNA (mean ± SD of triplicates from three independent experiments).

Supplementary Figure 4





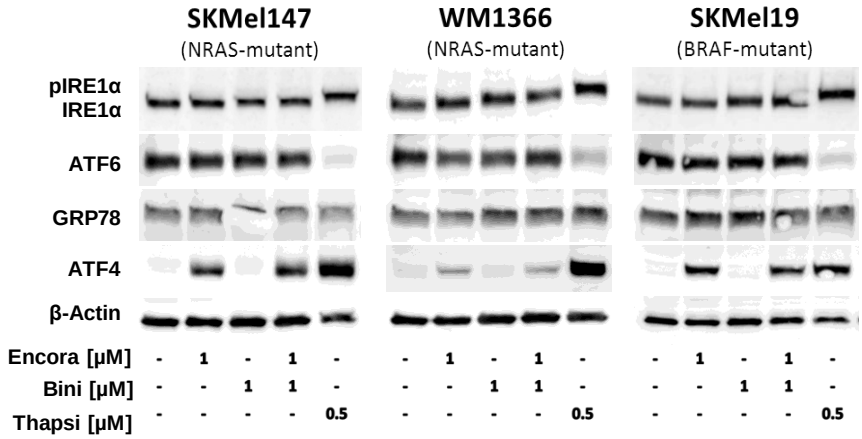


Suppl. Figure 4

In normal skin cells encorafenib and binimetinib induce only weak growth inhibition and no significant levels of apoptosis

Benign cells (Fibroblasts, keratinocytes, melanocytes) were treated with encorafenib, binimetinib, both and DMSO as control. (A) Western blot analysis of whole benign cell lysates was performed after 24 h of treatment with the indicated inhibitors or DMSO. pERK and ERK levels in comparison with β-Actin as loading control were determined. Experiment shown is a representative of two independent experiments. (B) Growth assessment (MUH; 4-methylumbelliferyl heptanoate) of indicated primary skin cells was measured after 72 h. The percentage of growth inhibition was calculated, normalized to the DMSO-treated control. One representative experiment of two is shown (mean ± SD of quadruplicates). (C) FACS cell cycle analysis (propidium iodide) treated with the indicated substances for 72 h (cell cycle). The size of the fractions was calculated from FACS cell cycle analysis using propidium iodide (mean ± SD of duplicates from three independent experiments).

Supplementary Figure S5

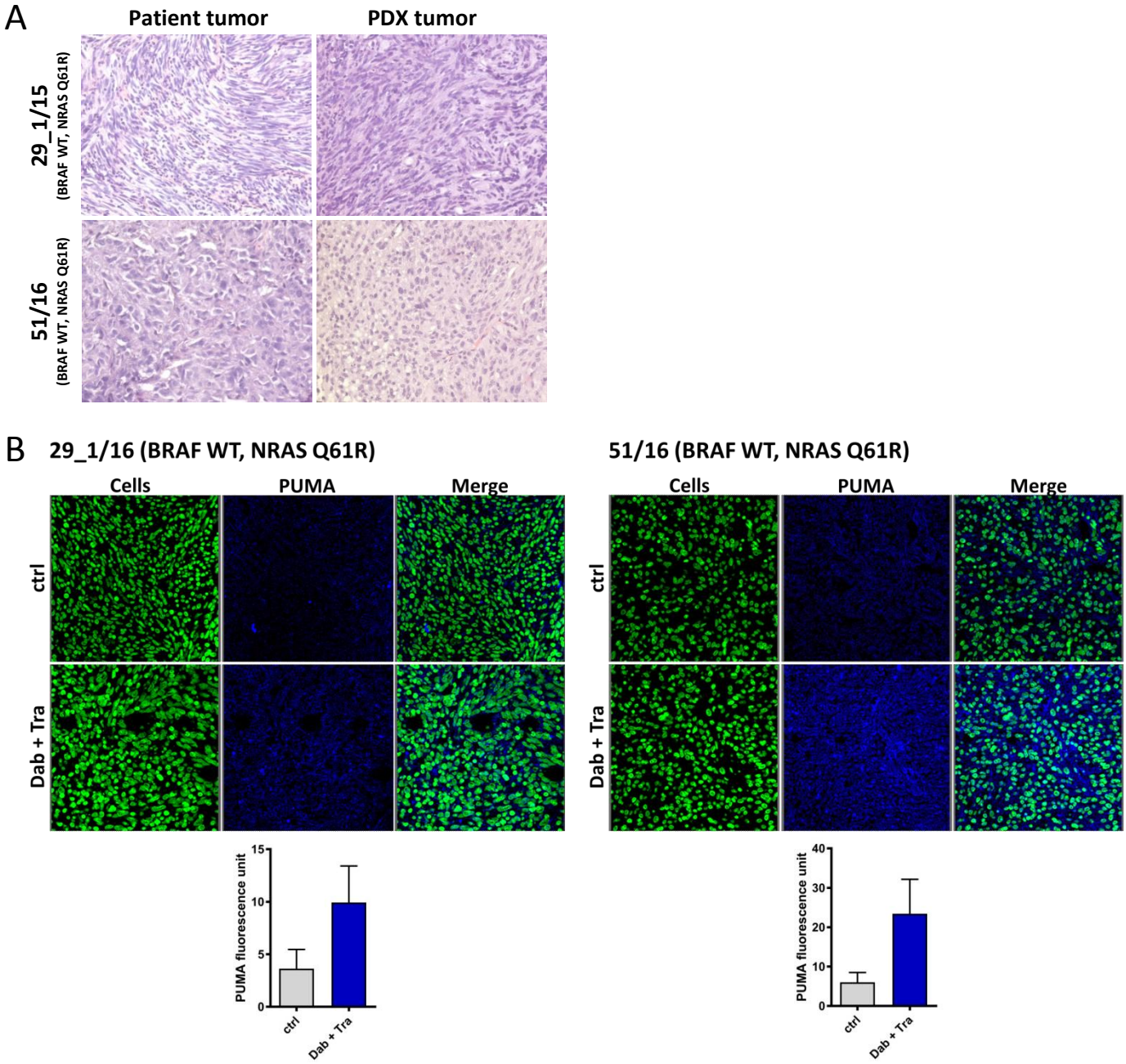


Suppl. Figure 5

BRAFi combined with MEKi leads to an ER stress response involving the PERK pathway?

Western blot analysis of whole cell lysates was performed after 4 h of treatment with the indicated inhibitors or DMSO. pIRE1α, IRE1α, ATF6, GRP78 and ATF4 levels in comparison with β-Actin as loading control were determined. Disappearance of the 90 kDa ATF6 band indicates ATF6 cleavage while a shift of the IRE1α band indicates its phosphorylation. Experiment shown is a representative of three independent experiments.

Supplementary Figure S6

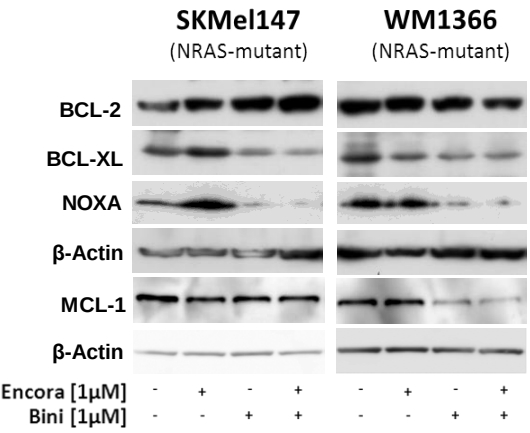


Suppl. Figure 6

BRAFi combined with MEKi leads to activation of PUMA in patient-derived tumor slice cultures.

(A) Tumor tissue from patients with NRAS-mutant melanoma and the corresponding PDX tumor were embedded in paraffin blocks and histologically analyzed by H&E staining. (B) Tumor tissue from patients with NRAS-mutant melanoma was expanded in a PDX model, sliced and then treated with the indicated inhibitor combinations. After 4 days the slices were stained with YOPRO (nuclei marker) or PUMA and analyzed by confocal microscopy. Images of one representative per treatment group are shown. Median Fluorescence intensities for PUMA were calculated and displayed in a bar graph (error bars represent SD of 4 random sites in each tumor per treatment group).

Supplementary Figure S7

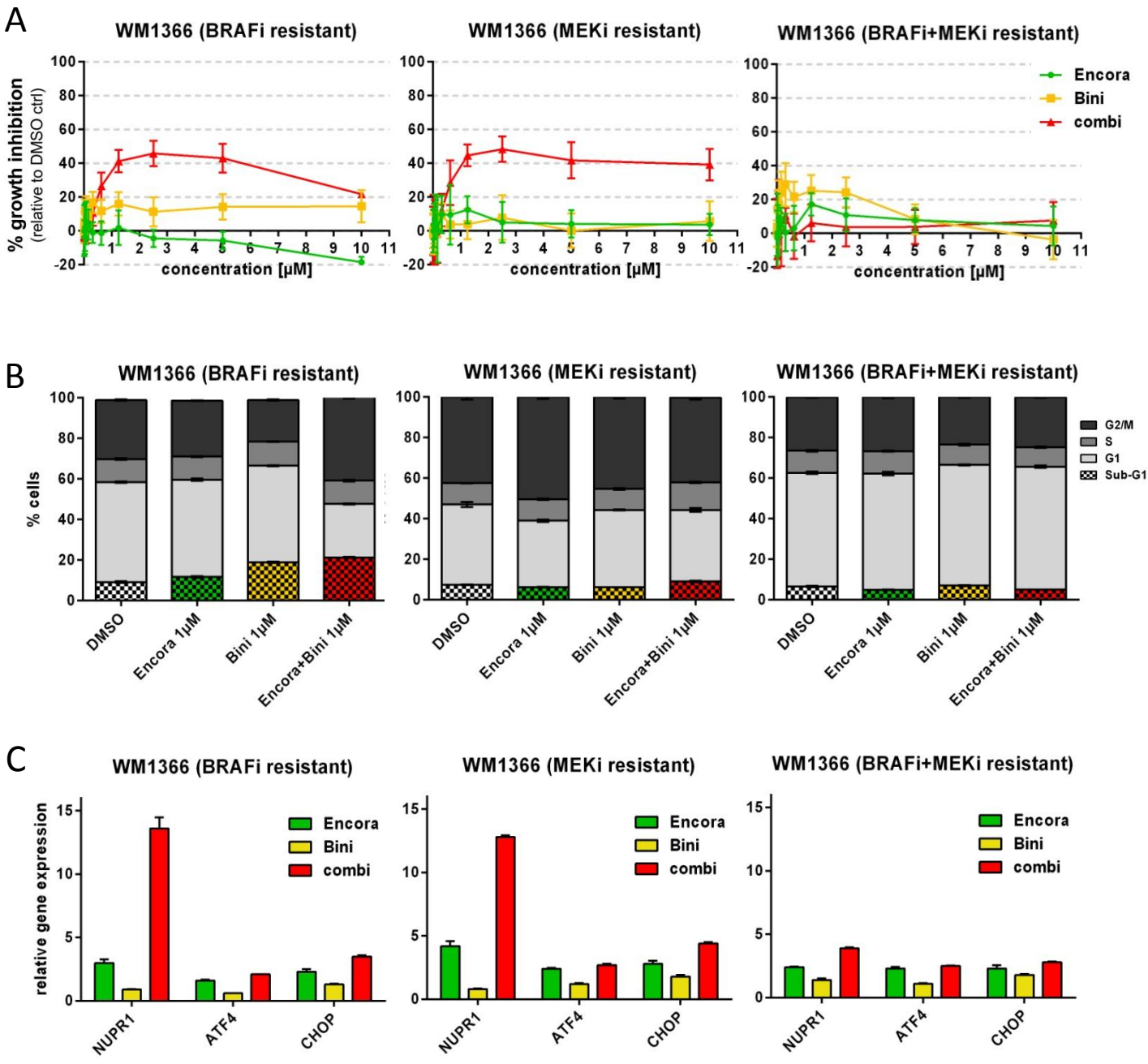


Suppl. Figure 7

Binimetinib and encorafenib induced apoptosis is partly mediated by the anti-apoptotic BCL-2 family members and by the pro-apoptotic BH3-only protein NOXA .

Melanoma cells were treated with encorafenib or/and binimetinib or DMSO for 24 h. Western Blot analysis of whole cell lysates was performed to detect BCL-2, BCL-XL, NOXA and MCL-1 compared to β-Actin as a loading control. Experiment shown is a representative of three independent experiments.

Supplementary Figure S8



Suppl. Figure 8

NRAS mutated cells resistant to encorafenib, binimetinib or the combination of encorafenib and binimetinib.

(A) Growth assessment (4-methylumbelliferyl heptanoate (MUH)) of melanoma cells (BRAFi resistant, MEKi resistant or BRAFi+MEKi resistant) treated with encorafenib (Encora), binimetinib (Bini), their combination or DMSO as control for 72 h. The percentage of growth inhibition was calculated, normalized to the DMSO-treated control. One representative experiment of two is shown (mean ± SD of quadruplicates). (B) Melanoma cells were treated with encorafenib, binimetinib, encorafenib plus binimetinib or DMSO as control for 72 h. Apoptotic cells (sub-G1 fraction) were quantified by FACS cell cycle analysis (propidium iodide staining) (mean ± SD of duplicates from three independent experiments). (C) Melanoma cells were treated with encorafenib (1 μM), binimetinib (1 μM) or both inhibitors for 18 h. Real-time PCR results show levels of NUPR1, ATF4 and CHOP mRNA expression in comparison with DMSO treated cells. All samples were normalized to TBP mRNA (mean ± SD of triplicates from three independent experiments).

Supplementary Table 1

Encora in µM	0.16	0.32	0.63	1.25	2.5	5	10
Bini in µM	0.16	0.32	0.63	1.25	2.5	5	10
SKMel147	0.78	0.03	0.01	0.01	0.02	0.05	0.17
WM1366	0.05	0.04	0.06	0.09	0.14	0.3	0.63
Dabra in µM	0.16	0.32	0.63	1.25	2.5	5	10
Tra in µM	0.02	0.03	0.06	0.13	0.25	0.5	1
SKMel147	0.08	0.08	0.09	0.15	0.33	0.73	161.3
WM1366	0.18	0.06	0.02	0.02	0.11	0.84	232.2

< 0.1 very strong
0.1 – 0.3 strong
0.3 – 0.7 moderate
0.7 – 0.85 slight
0.85 – 0.9 additive
> 0.9 antagonistic

Supplementary Table 2

Antibodies used for protein detection in Western Blot and Immunofluorescence		
Protein	Order No.	Source
pERK	#9101	Cell Signaling Technologies
ERK	#9102	Cell Signaling
NUPR1		Kindly provided by Juan Iovanna (INSERM, Marseille)
CHOP	#5554	Cell Signaling Technologies
ATF4	#1371	Abcam
BIM	#2933, #2819	Cell Signaling Technologies
PUMA	#4976	Cell Signaling Technologies
BCL-2	#2872	Cell Signaling Technologies
BCL-XL	#2762	Cell Signaling Technologies
MCL-1	#4572	Cell Signaling Technologies
NOXA	#14766	Cell Signaling Technologies
GRP78	#21685	Abcam
IRE1α	#3294	Cell Signaling Technologies
ATF6	#134561	Cell Signaling Technologies
peIF2α	#9721	Cell Signaling Technologies
eIF2α	#9722	Cell Signaling Technologies
Caspase 9/cl. Caspase9	#9502	Cell Signaling Technologies
Caspase 3	#9662	Cell Signaling Technologies
cl. Caspase 3	#9664	Cell Signaling Technologies
PARP/cl. PARP	#9542	Cell Signaling Technologies
Ki67	#M7240	DAKO
β-Actin	#4967	Cell Signaling Technologies

Supplementary Table 3

Forward (fo) and reverse (re) primer sequences for quantitative real-time PCR	
NUPR1_fo	5´-CCATTCCTACCTCGGGCCTCTCATC
NUPR1_re	5´-TCTTGGTGCGACCTTTCCGGC
CHOP_fo	5´-AAGGCACTGAGCGTATCATGT
CHOP_re	5´-TGAAGATACACTTCCTTCTTGAACAC
ATF4_fo	5´-TGGGGAAAGGGGAAGAGGTTGTAA
ATF4_re	5´-AGTCGGGTTTGGGGGCTGAAG
PUMA_fo	5´-GAAGAGCAAATGAGCCAAACG
PUMA_re	5´-GGAGCAACCGGCAAACG
TBP_fo	5´-TGCACAGGAGCCAAGAGTGAA
TBP_re	5´-CACATCACAGCTCCCCACCA