Supplementary Figure 1. Epithelial to mesenchymal transition (EMT) from parental cells to resistant cells. 

a. The morphology of parental cells M225. Scale bar = 100 µm. b. The morphology of resistant cells M225R. M225R had a more elongated and fibroblast-like shape than parental cells M225 and resembled mesenchymal cells. Scale bar = 100 µm.
Supplementary Figure 2. Quantification of cell migration and invasion

a. Cell migration of M14R and M219R was significantly increased compared to parental cell M14 and M219 respectively (N=3).

b. Cell invasion of M14R and M219R was significantly increased compared to parental cell M14 and M219 respectively (N=3).

Error bars, s.d. (*p < 0.05).
Supplementary Figure 3. Migration and invasion of parental cells and resistant M225 cells

a. Migration analysis of parental cells M225 and resistant cells M225R. Scale bar = 100 µm.
b. Invasion analysis of parental cells M225 and resistant cells M225R. Scale bar = 100 µm.
c. Quantification of migration (left) and invasion (right) (N=3). Cell migration and invasion of M225R were increased significantly compared to parental cells M225. Error bars, s.d. (*p <0.05).
Supplementary Figure 4. **a.** Quantification of colony formation (N=3). **b.** M14 and M14R, M219 and M219R in BD matrigel growth medium (Day 1). Scale bar = 100 µm.
Supplementary Figure 5. Cell growth of parental and resistant cells

a. Cell growth curve of M14 and M14R, M219 and M219R. b. Cell growth curve of M225 and M225R. c. Immunoblots of Cyclin D1 expression. d. Quantification of Cyclin D1 expression. BRAF resistant cells grow faster than respective parental cells. Expression of Cyclin D1 was higher in BRAF resistant cells than in respective parental cells (N=3). Error bars, s.d. (*p <0.05).
Supplementary Figure 6. Xenograft tumor growth of melanoma cell lines in nude mice
Tumor volume of xenografts of M219 and M219R cell growth in nude mice (N=6 per cell line). Error bars, s.d.
RNA sequencing of EGFR was assessed in BRAFi-resistant melanoma cells versus parental cells. mRNA expression of EGFR in M14R was enhanced compared to that in M14 as assessed by RNA sequence analysis of M14 and M14R. Error bars, s.d. (*p <0.05).

**Supplementary Figure 7**

RNA sequencing of EGFR
Supplementary Figure 8. EGFR IHC staining of melanoma TMA, BRAFi pre-treatment and resistant melanoma

EGFR IHC intensity was assessed in pre-treatment and resistant melanoma with BRAFi, Stage III TMA and Stage IV TMA. EGFR expression in pre-treatment melanomas was lower than that in resistant melanomas (* p <0.05); EGFR expression in Stage III TMA and Stage IV TMA was significantly lower than that in resistant melanomas (**p <0.01). Error bars, s.d.
Supplementary Figure 9. Representative EGFR IHC staining of cutaneous melanomas in pre- and post-treated melanoma patients by BRAFi. **a.** Pre-treated melanomas. **b.** BRAFi resistant melanomas. EGFR expression was enhanced in BRAFi resistant melanomas compared to pre-treated melanomas. Scale bar = 100 µm.
Supplementary Figure 10. IHC staining of EGFR expression in paired pre- and BRAFi-resistant patients
Melanomas of patients before and after BRAFi treatment were assessed for EGFR expression. Comparison of EGFR expression in 12 paired pre-treated and BRAFi resistant melanomas were assessed. There was higher EGFR expression in BRAFi resistant melanomas than in respective pre-treated melanomas.
Supplementary Figure 11. EGFR mRNA expression of M14 and M219 treated by 5-Aza(N=3)

a. EGFR mRNA expression of M14.  b. EGFR mRNA expression of M219. EGFR mRNA expression of M14 and M219 was increased when M14 and M219 was treated by 5-Aza for 72h. Error bars, s.d. (*p <0.05 vs. Control)
Supplementary Figure 12. Quantitative MSP analysis of DNA methylation level of EGFR gene downstream enhancer

Methylation level of EGFR DNA downstream enhancer in M14R and M219R was shown to be much lower than that in respective parental M14 and M219 cells. Error bars, s.d. (*p <0.05)
Supplementary Figure 13. EGFR confers resistance to BRAFi in melanoma cell lines
Morphology of M14 (parental cells) and M14R (BRAFi-resistant cells) after treatment with BRAFi (SB590885), EGFRi (Erlotinib) and a combination of SB590885 and Erlotinib. Scale bar = 100 µm.
Supplementary Figure 14. Representative FACS scatter plots of control cells that were stained with Annexin V(y-axis) versus PI(x-axis). a. M14R treated by DMSO. B. M219 treated by DMSO.
Supplementary Figure 15. Quantification of EGFR, p-EGFR, p-AKT and p-p42/p44 expression in M225 treated by DMSO, AZD6244 alone, BRAFi alone and combination of AZD6244 and BRAFi(N=3)

a. Quantification of EGFR expression. b. Quantification of p-EGFR expression. c. Quantification of p-AKT expression. d. Quantification of p-p42/p44 expression. Expression of EGFR, p-EGFR and p-AKT in M225 treated by a BRAFi and AZD6244 combination was increased compared to control cells. Expression of p-p42/p44 in M225 treated by a BRAFi and AZD6244 combination was significantly decreased compared to control cells. Error bars, s.d. (*p < 0.05 vs. control).
Supplementary Figure 16. Cell growth of M219 and M219R treated EGFRi and BRAFi in 3D-matrix microenvironment

Parental cells M219 and resistant cells M219R were treated by DMSO, BRAFi SB590885, EGFRi Erlotinib alone, and a combination of SB590885 and Erlotinib in 3D-matrix microenvironment cultures. M219R was more sensitive to the combination of BRAFi and EGFRi treatment than BRAFi or EGFRi treatment alone. M219 was sensitive to BRAFi treatment and not sensitive to EGFRi treatment. Scale bar = 50 µm.
Supplementary Figure 17. M14 and M14R in BD matrigel growth medium with DMSO, BRAFi, EGFRi and combination of BRAFi and EGFRi (Day 1). Scale bar = 100 µm.
Supplementary Figure 18. M219 and M219R in BD matrigel growth medium with DMSO, BRAFi, EGFRi and combination of BRAFi and EGFRi (Day 1). Scale bar = 100 µm.
### Supplementary Table 1. Primers of Quantitative Reverse-Transcription PCR

| Forward Sequence | Reverse Sequence |
|------------------|------------------|
| EGFR(F) 5’-GGCAGTTTTGAAGATCATTTTCTC-3’ | EGFR(R) 5’-CTGTGTTGAGGGCAATGAG-3’ |

### Supplementary Table 2. Primers of Quantitative Methylation-specific PCR

**Unmethylated EGFR Primer**
- Forward: 5’-TGTGGGTTGTGGTGTTTTTGGTTTGTGT-3’
- Reverse: 5’-CATCCCAATCTAAACAAACAACACCCACCA-3’

**Methylation EGFR Primer**
- Forward: 5’-TGTGGGTTGTGGTGTTTTTGGTTTGTGC-3’
- Reverse: 5’-CGATCTAAGACGACGACCGACCGCCG-3’
### Supplementary Table 3. Primers of Quantitative Methylation-specific PCR

Methylation-allele sense primer: 5’-GGATTTGTTATAAAGTATAAAGTC-3’
Unmethylation-allele sense primer: 5’-GGATTTGTTATAAAGTATAAAGTTT-3’
Universal antisense primer: 5’-CTACCCCTAAACCAATAAACCAACAAA-3’

### Supplementary Table 4. Sequence of EGFR siRNA

EGFR siRNA1: 5’- TACGAATATTAAACACTTCAA-3’
EGFR siRNA2: 5’- ATAGGTATTTGATGTGAATTAAA-3’
Supplementary Material

Nude mice for xenograft assays

Male athymic nude mice (BALB/c background) (Harlan Laboratories, Jerusalem, Israel) (N=12) were housed in air conditioned, light controlled animal facilities of the Sackler Faculty of Medicine, Tel-Aviv University. Following experimental procedures mice were sacrificed using CO₂ chamber. Animal care and all experiments were in accordance with the institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the Sackler Faculty of Medicine, Tel-Aviv University in accordance with regulations of Institutional Animal Care and Use Committee. 1×10⁶ cells were inoculated subdermally into the right thigh. To test the tumorigenic properties of cells, parental and BRAFi-resistant cell lines (M219 and M219R) were assessed (6 mice for each cell line). Mice were weighed and subcutaneous tumors were measured using a caliper once a week; tumor volume was obtained by the ellipsoid volume calculation formula: 0.5 × (length × width²) (Jensen et al., 2008; Tomayko and Reynolds, 1989).

Cell growth and viability assays

Cell proliferation was assessed with the CellTiter 96® AQueous One Solution assay. According to the manufacturer’s instructions (Promega, Madison, WI), cells were seeded in triplicate in 96-well plates and treated with the BRAF inhibitor SB590885 (1 μM) and/or the EGFR inhibitor erlotinib (2 μM) (LCL Laboratories, Woburn, MA) in Matrigel (BD Biosciences, San Jose, CA). All drugs were dissolved in DMSO. Cells were placed in a 96-well microplate and cultured without or with EGF (20 ng/ml).

Cell migration and invasion assays
Migration and invasion assays were carried out according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Cells (2.5x10^4) were briefly placed in transwell migration (BD, Catalog Number: 354578) and invasion chambers (BD, Catalog # 354480). After 24 hrs, cells on the lower surface were fixed with methanol and stained with 2% crystal solution. The membrane was then mounted onto a microscope slide and the migrating cells were counted in five different areas using a light microscope. The experiments were performed three times in triplicate.

**Cell growth and soft agar colony assay formation**

Cell proliferation and viability were assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) obtained from Sigma-Aldrich (St. Louis, MO) in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibodies at 37°C in a humidified incubator with 5% CO₂. 3D cell culture was performed using Matrigel matrix (BD Biosciences, San Jose, CA) in a 96-well microplate according to the manufacturer’s instructions. A soft-agar colony formation assay was performed in six-well culture plates.

**Immunoblotting and antibody array analysis**

Whole cell extracts were prepared from cell lines M14, M14R, M219 and M219R. Membranes were immunoblotted for 24 hours with primary antibodies (Abs) (Qu et al., 2009). Abs assessed included mouse monoclonal anti-vimentin (clone: RV202), anti-fibronectin (clone: 10/Fibronectin), anti-N-cadherin (clone: 32/N-cadherin) (1: 1000, BD), anti-SMA (alpha smooth muscle actin) (clone: 1A4) (1: 1000, Sigma, St. Louis, MO) and primary rabbit polyclonal anti-EGFR Ab, anti-p-EGFR, anti-Akt, ERK1/2, anti-P85, anti-p-85, anti-P110 alpha, anti-P110 beta, anti-P110 gamma Ab, anti-p-4E-BP1 Ab (1: 1000, Cell Signaling), and anti-cyclin D1 Ab (1: 200, Santa Cruz, CA).
After immunoblotting, the membranes were washed three times with 1X phosphate buffered saline Tween-20 (PBST), followed by a 1-hour incubation with horseradish peroxidase-conjugated goat anti-rabbit Ab (1:5000) or horseradish peroxidase-conjugated rabbit anti-mouse Ab (1:5000, Santa Cruz). Immunoreactive bands were visualized with the SuperSignal West Dura extended substrate kit (Thermo Scientific, Rockford, MD) and the densities of protein bands were quantified using Alpha Ease FCTM software (Alpha Innotech, San Leandro, CA).

To determine whether EGFR expression was affected by methylation, M14 and M219 cells were treated by 2 μM 5-azacitidine or DMSO, respectively, for 24, 48 and 72 hours and then analyzed for EGFR expression by immunoblotting.

To identify the relative levels of phosphorylation of RTKs, we assessed extracted cell proteins using a human phosphor-RTK array kit (ARRY-001; R&D Systems Minneapolis, MN) according to the manufacturer’s instructions.

To determine whether activation of EGFR leads to signaling via the PI3K/AKT pathway, cells were pretreated with AKT inhibitor CCT128930 (10uM) for 1h followed by exposure to EGF (100ng/ml) or treated by EGF alone. Cells were lysed and cell lysate was used for immunoblotting.

**Quantitative reverse transcription-PCR**

Total extracted RNA (1μg) was used for cDNA synthesis, with Oligo(dT)20 primers (Invitrogen, Grand Island, NY). The cDNA was added to a quantitative reverse transcription-PCR mixture that contained 1X SYBR Green PCR master mix (Quanta Biosciences, Gaithersburg, MD) and 500 nmol/L gene-specific primers. Assays were performed in triplicate
on a CFX thermocycler (Bio-Rad, Hercules, CA). Specific gene plasmids for controls and β2MG were used in all PCR analyses. The primers are listed in Supplementary Table 1.

**Quantitative real-time methyl-specific PCR (qMSP)**

The qMSP assays were performed in triplicate and designed to amplify bisulfite-converted methylated DNA target sequences as previously described (Hoshimoto et al., 2012). The methylation-specific primers and unmethylated-specific primers are listed in Supplementary Table 2. For the qMSP, 2 μg of DNA was used for each reaction.

**Quantitative real-time DNA methylation-specific PCR for EGFR DNA enhancer**

Extracted DNA (2 μg) was treated with sodium bisulfite as described above. Methylated (M) and unmethylated (U) alleles for the EGFR downstream enhancer (chr7:55,177,159-55,177,216) were amplified using specific combination of primers as listed in Supplementary Table 3. The quantitative amplification of M and U alleles was performed in the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, CA) and the difference between the methylated and unmethylated cycle thresholds (ΔCt = CtM − CtU) was calculated for each promoter region. The relative copy number of methylated alleles was established using $2^{-\Delta C_t}$ method (Asaga et al., 2011). The percentage of DNA methylation was estimated by using a standard curve of serial dilutions of the universal methylated control in universal unmethylated control, as previously described by our group (Sunami et al., 2011; Umetani et al., 2005).

**Immunohistochemistry (IHC)**

Mouse anti-EGFR Ab clone 31G7 (Invitrogen, Camarillo, CA) was used for IHC staining. Clinically annotated melanoma TMAs (AJCC stage III and IV) with >5 yrs follow-up as well as individual tumor tissue blocks were assessed. IHC was carried out as previously
described (Nguyen et al., 2010). Photographs of each IHC-stained section were taken for analysis using a Nikon Eclipse Ti microscope and NIS elements software (Nikon, Melville, NY). Staining density was determined by Image J software (http://sbweb.nih.gov/ij/). After adjustment for background on each selected field, the density of the individual melanoma specimen was quantified and given a numerical value from 0–255. There were duplicates for each melanoma specimens, so the average of the two staining intensity values was used for statistical analysis.

**Overexpression and knockdown of EGFR in melanoma cells**

M14R and M219 cells were seeded at 2.5–3.0×10^5 cells/60-mm dish and then transfected for 72 hours with EGFR (1μg) expression plasmid (Origene Technologies, Rockville, MD) or 100 nmol/L (final concentration) EGFR siRNA(Qiagen, Valencia, CA) with controls. Cells were lysed and expression of EGFR in these transfected cells was assessed. Cells were treated with SB590883 (1μM) or erlotinib (2μM) for 48 hrs, after which apoptosis was measured using the annexin V apoptosis assay (BD Biosciences, San Jose, CA). Sequences of EGFR siRNA are given in Supplemental Table 4.

**Flow cytometry and apoptosis assays**

Flow cytometric analysis of EGFR expression was performed on a BD FACS Calibur System (BD Biosciences, San Jose, CA). Isotype-matched Ab was used as a negative control. Cells were plated at 1.5 × 10^6 per well in a six-well plates and were treated by SB590883 (1μM) and/or erlotinib (2μM) for 72 hours. Cells were harvested, washed with PBS, and incubated with Alexa Fluor 488-conjugated annexin V and propidium iodide (PI), and flow cytometry was performed. Cells were stained by PI (5 μg/mL; Invitrogen Grand Island, NY,) and SYTO-
13(1μmol/L, Invitrogen, Grand Island, NY). Apoptosis was measured using the Caspase-Glo 3/7 assay (Promega, Madison, WI).
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