SUPPLEMENTARY MATERIALS AND METHODS

Microarray analysis

Total RNA (5 ng) was purified and amplified using NuGEN WT-Ovation™ Pico system (NuGEN Technologies). Biotinylated cRNAs were hybridized to Human HT-12 v3 Expression Beadchips (Illumina). After normalization, significantly changed probes were calculated using significance analysis of microarrays. GeneSpringGX Version 10.0 (Agilent Technologies) was used to analyze microarray data. Expression of the genes reported to be responsible for temozolomide resistance was displayed in a heatmap generated using the Treeview software.

RNA extraction and real-time quantitative reverse transcription-PCR (qRT-PCR) analysis

RNA was extracted from SP and non-SP cells from F1 tumors and HS294T cell line using the RNAqueous-Micro kit (Ambion), and subsequently reverse transcribed. qRT-PCR was performed with *Power* SYBR Green PCR Master Mix (Applied Biosystems) on the MX3000P PCR system (Strategene). GAPDH was used as an internal control. Primer sets are listed below: *ABCB1* forward: 5'-CTCATGATGCTGGTGTTTGG-3', reverse: 5'-TGGTCATGTCTTCCTCCAGA-3'; *ABCB5* forward: 5'-CACAAAAGGCCATTCAGGCT-3', reverse: 5'-GCTGAGGAATCCACCCAATCT-3' (Frank *et al.*, 2005); *FUK* forward: 5'-TACCATGGCCTATGTCTCCA-3', reverse: 5'-AGGCTGAGCAGGAACTCACT-3'; *GAPDH* forward: 5'-TGCACCACCAACTGCTTAGC-3'; reverse: 5'-GGCATGGACTGTGGTCATGAG-3'; *PCDHB11* forward: 5'-GCACTCAGCAGATAAGGCAA-3', reverse: CTTTGCCAGATTGCCTACAA-3'; *TBX2*

forward: 5'- GCTGACCAACAACATCTCTGA-3', reverse:

5'-GCTGGTACTTGTGCATGGAG-3'; *IL-8* forward:

5'-ACTGAGAGTGATTGAGAGTGGAC-3', reverse: 5'-AACCCTCTGCACCCAGTTTTC-3'.

Flow cytometry

Sorted SP or non-SP cells were stained with isotype control (rabbit IgG) or rabbit antibodies against human ABCB1 or ABCB5 (Lifespan Biosciences or Abgent, respectively), followed by secondary labeling with PE-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), and subjected to flow cytometry analysis with Summit software (DakoCytomation).

***in vitro* drug treatment**

Single cell suspensions from F1 xenografted patient tumors were seeded in 2 ml of culture medium in six-well culture plates. After 24 hours, medium was changed and the cells were treated with vehicle control, 20 nM paclitaxel (LC Laboratories) or 100 μM temozolomide (Sigma-Aldrich). Drugs were only added at the beginning. Ten days after the treatment, cells were collected and washed, followed by SP staining and flow cytometric analysis. For HS294T cells, sorted SP and non-SP cells were cultured in 96-well plates at a density of 300 cells/well. For IL-8 experiments, HS294T cells were treated with vehicle or 50 μM temozolomide in the presence or absence of anti-human CXCR1 (10 μg/ml) (R&D). For drug resistance experiments, HS294T cells were treated with varying doses of paclitaxel (10 nM, 20 nM and 100 nM) or temozolomide (50 μM, 100 μM and 200 μM).

Cell viability assay

Sorted melanoma cells from human melanoma tissues or HS294T cells were subcultured in 96-well plates at a density of 300 cells/well. Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). 100 µl of the substrate were added to each well, and cell lysate was measured by luminescence plate reader to determine cell viability.

***in vivo* drug treatment**

F1 tumors were excised after reaching 1,500 mm^3. They were cut into small 3 × 3 × 3 mm fragments and then implanted subcutaneously in F2 mice, with two small fragments in each mouse. F2 tumors were allowed to grow to 200 mm^3, then randomized to three groups with four mice / eight tumors in each group: (a) vehicle control, (b) temozolomide (Sigma-Aldrich), 30 mg/kg dissolved in 10% DMSO and 90% PBS, once daily for 5 days by i.p. injection and (c) paclitaxel (LC Laboratories), 10 mg/kg dissolved in 200 µl of 5% EtOH, 5% tween80 and 90% saline, once a week for 2 weeks by i.v. injection. Tumor size was evaluated twice a week by caliper measurements using the following formula: tumor volume = [length × $width^2$] / 2. Relative tumor growth was calculated by tumor volume of treated mice divided by tumor volume at the initiation of therapy. Experiments were terminated on day 14.

ABCB1 and ABCB5 immunostaining

Immunohistochemical studies were carried out as previously described (Okamoto *et al.*, 2010). Heat-induced antigen retrieval was performed in 0.01 mol/L citrate buffer (Antigen retrieval citra solution; BioGenex Laboratories). Rabbit antibodies against human ABCB1 and ABCB5 were obtained from Lifespan Biosciences and Abgent, respectively. Biotinylated goat anti-rabbit IgG and ABComplex/horseradish peroxidase avidin-biotinylated peroxidase complex were purchased form Vector Laboratories. The immunoreactants were visualized with 3,3'-Diaminobenzidine (Sigma-Aldrich) as chromogen. Hematoxylin (Sigma-Aldrich) was used for counter staining. Immuno-stained cells and tumor cells (with coarse chromatin and large nucleoli) were counted from nine high-power microscopic fields per each section and three sections were examined.

siRNA transfection

siRNA duplexes targeting ABCB1, ABCB5 and IL-8 were from Sigma-Aldrich (ABCB1, SASI_Hs01_00087519; ABCB5, SASI_Hs01_00087520; IL-8, SASI_Hs01_00222387). Scrambled control siRNA for nonspecific gene silencing was obtained from Qiagen. siRNA transfection was performed using Lipofectamine^{TM} 2000 (invitrogen) according to the manufacturer's instructions. siRNA transfected cells were cultured at 37°C, harvested for confirmation of transfection by RNA at 18 hours and by protein at 72 hours after transfection, and subjected to analyses of SP numbers and cell viability.

Western blotting

Western blot analysis was carried out as previously described (Okamoto *et al.*, 2010). Primary antibodies were rabbit anti-human ABCB1 or ABCB5 antibodies and mouse anti-human β-Actin antibody (Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG and anti-rabbit IgG) were purchased from Sigma-Aldrich. β-actin was used as an internal control.

IL-8 ELISA

Supernatants were analyzed by ELISA for IL-8 (eBioscience), according to the manufacturer's instructions. The limit of sensitivity for IL-8 was 7.8 pg/ml.

Statistical analysis

The data in this report are represented as mean ± S.E. The figures in this study are representatives of more than two separate experiments. The difference between two samples was analyzed with Student's test. $P<0.05$ was considered statistically significant.

Frank NY, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M*, et al.* (2005) ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res* 65:4320-33.

Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA*, et al.* (2010) Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem* 285:6477-88.

Supplementary Figure S1. SP flow cytometry profile from the HS294T cell line. Hoechst staining of HS294T cells in the presence (*left panel*) and absence (*right panel*) of verapamil. SP cells are shown in the lower left gated areas.

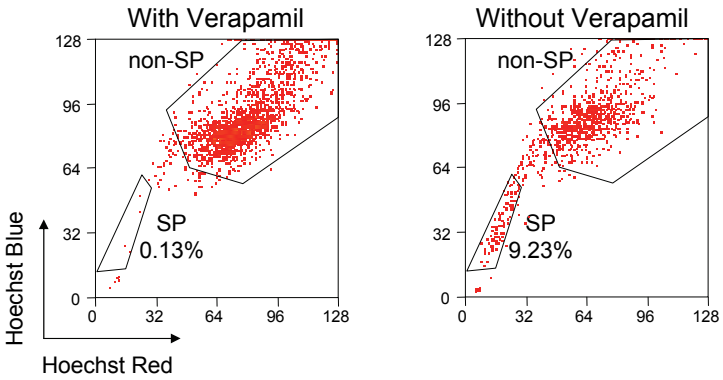
Supplementary Figure S2. The effect of siRNA transfection of ABC transporters on IL-8 expression and that of IL-8 on ABC transporters. (**a**) qRT-PCR of ABCB1 and ABCB5 at 18 hours after siRNA transfection of scrambled (si-ctrl) or IL-8 (si-IL-8) in HS294T cells. (**b**) qRT-PCR (*left panel*) and ELISA (*right panel*) of IL-8 at 18 hours after siRNA transfection of scrambled (si-ctrl), ABCB1 (si-ABCB1) or ABCB5 (si-ABCB5) in HS294T cells.

Supplementary Figure S3. Gene profiling of SP and non-SP cells in melanomas (from MB952-F1 and MB1009-F1 tumors). (**a**) Supervised hierarchical clustering of differentially expressed genes, showing clustering of 3617 transcripts which exhibited more than 2-fold differences between melanoma SP and non-SP cells with a P value < 0.05. Colors represent significant up- (red) or down- (blue) regulations. Sample tree originated from the clustering of values with Euclidean distance analyzed by the GeneSpringGX 10.0. (**b**) Gene ontology analysis from the 3617 differentially expressed transcripts using the GeneSpringGX 10.0.

Supplementary Figure S4. Pathway analysis from the microarray data. The analyses using the 3617 differentially expressed transcripts predict NF-κB as one of the differentially expressed signaling pathways between SP and non-SP cells. Analysis was performed using the

GeneSpringGX 10.0. Thirty two genes (highlighted with black circles) out of 121 selected genes in the NF-κB pathway were differentially expressed between SP and non-SP cells.

Figure S1


With Verapamil
non-SP
SP
0.13%
Without Verapamil
non-SP
SP
9.23%
Hoechst Blue
Hoechst Red
0
32
64
96
128

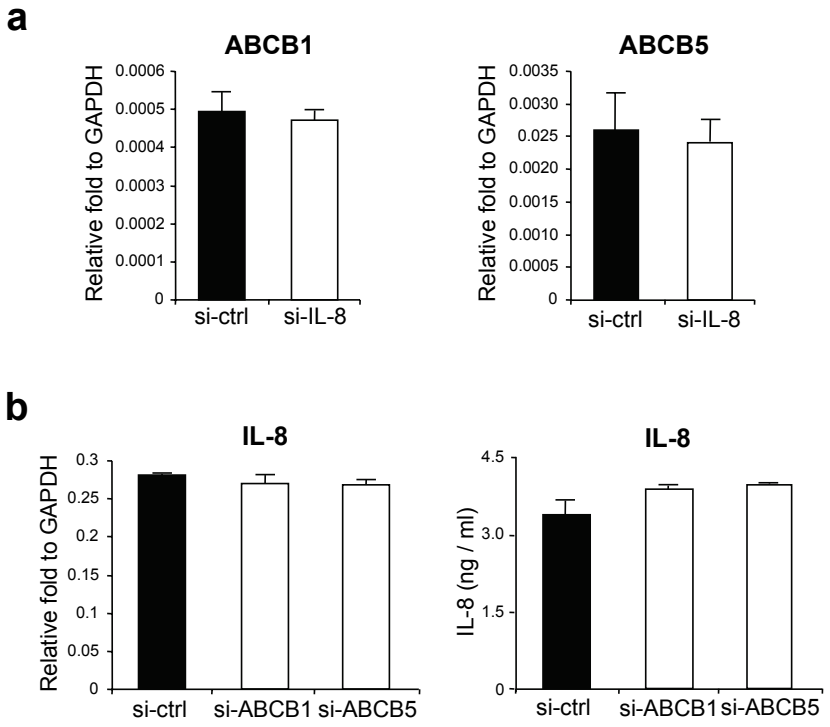
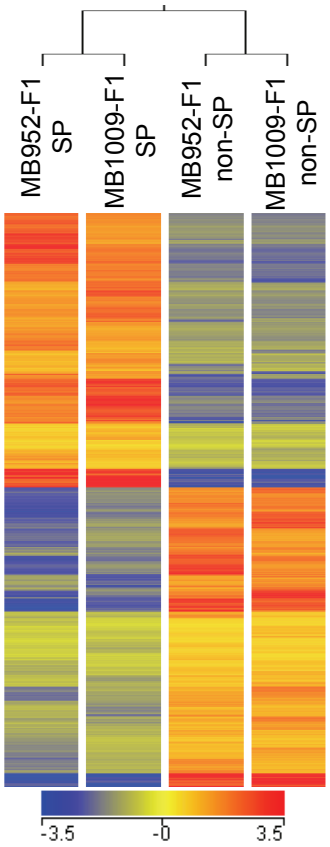

a
ABCB1
Relative fold to GAPDH
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
si-ctrl
si-IL-8
ABCB5
Relative fold to GAPDH
0.0035
0.0030
0.025
0.0020
0.0015
0.0010
0.0005
0
si-ctrl
si-IL-8
b
IL-8
Relative fold to GAPDH
0.3
0.25
0.2
0.15
0.1
0.05
0
si-ctrl
si-ABCB1
si-ABCB5
IL-8
IL-8 (ng / ml)
4.5
3.0
1.5
0
si-ctrl
si-ABCB1
si-ABCB5

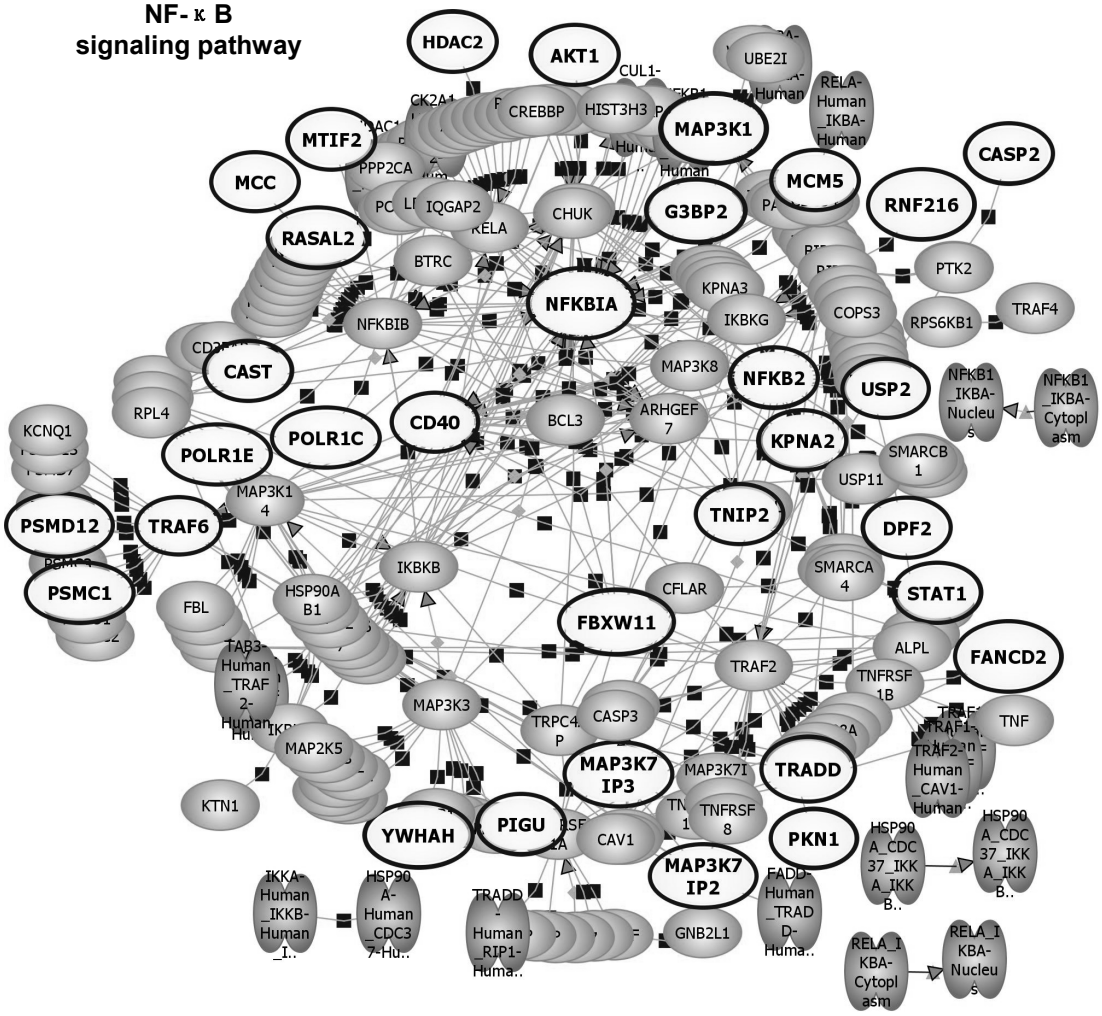
Figure S3

a



b

Gene Ontology	Count	Percentage
Cellular process	1092	28.54%
Metabolic process	670	17.51%
Biological regulation	665	17.38%
Localization	279	7.29%
Establishment of localization	256	6.69%
Developmental process	205	5.36%
Multicellular organismal process	203	5.31%
Response to stimulus	194	5.07%
Biological adhesion	83	2.17%
Immune system process	59	1.54%
Macromolecular complex subunit organization	45	1.18%
Reproduction	24	0.63%
Multi-organism process	23	0.60%
Locomotion	16	0.16%
Reproductive process	12	0.31%
Growth	6	0.16%
Rhythmic process	2	0.05%
Cell killing	1	0.03%
Viral reproduction	1	0.03%


NF-κB
signaling pathway
HDAC2
AKT1
UBE2I
CREBBP
HIST3H3
MAP3K1
MTIF2
MCC
MCM5
CASP2
RNF216
RASAL2
IQGAP2
CHUK
G3BP2
BTRC
PTK2
NFKBIA
KPNA3
IKBKG
COPS3
RPS6KB1
TRAF4
NFKBIB
CAST
MAP3K8
NFKB2
USP2
NFKB1_IKBA-Nucleus
NFKB1_IKBA-Cytoplasm
RPL4
CD40
BCL3
ARHGEF7
KPNA2
SMARCB1
KCNQ1
POLR1E
POLR1C
USP11
MAP3K14
PSMD12
TRAF6
TNIP2
DPF2
IKBKB
SMARCA4
STAT1
PSMC1
FBL
HSP90AB1
CFLAR
FBXW11
ALPL
FANCD2
TRAF2
TNFRSF1B
MAP3K3
CASP3
TNF
MAP2K5
MAP3K7IP3
MAP3K7I
TRADD
KTN1
YWHAH
PIGU
CAV1
TNFRSF8
PKN1
MAP3K7IP2
HSP90A_CDC37_IKKA_IKKB..
IKKA-Human_IKKB-Human_I..
HSP90A-Human_CDC37-Hu..
TRADD-Human_RIP1-Huma..
GNB2L1
FADD-Human_TRADD-Huma..
RELA_IKBA-Cytoplasm
RELA_IKBA-Nucleus