Supplementary Data for:

ABCC Multidrug Transporters in Childhood Neuroblastoma: Clinical and Biological Effects Independent of Cytotoxic Drug Efflux

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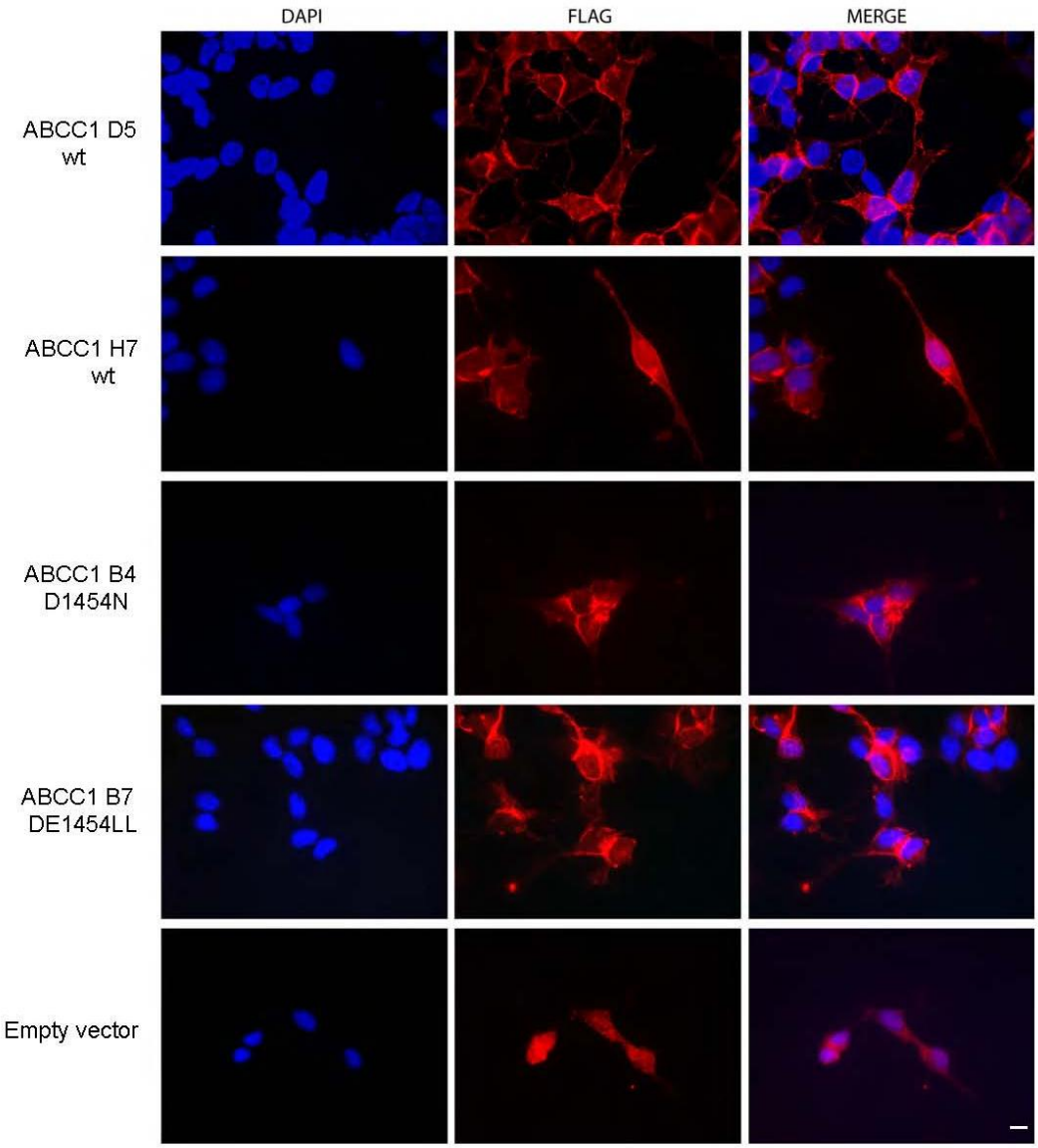
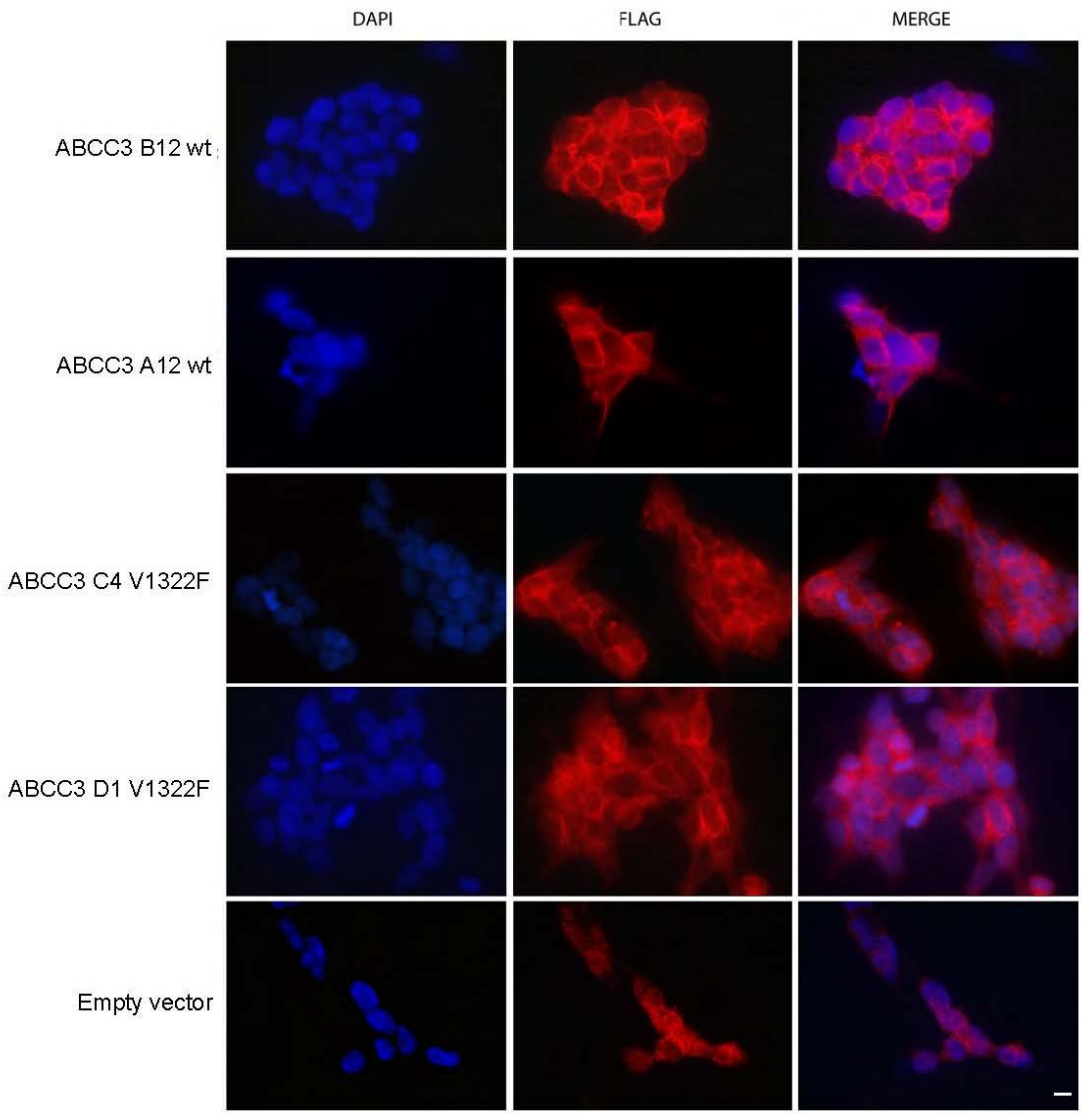
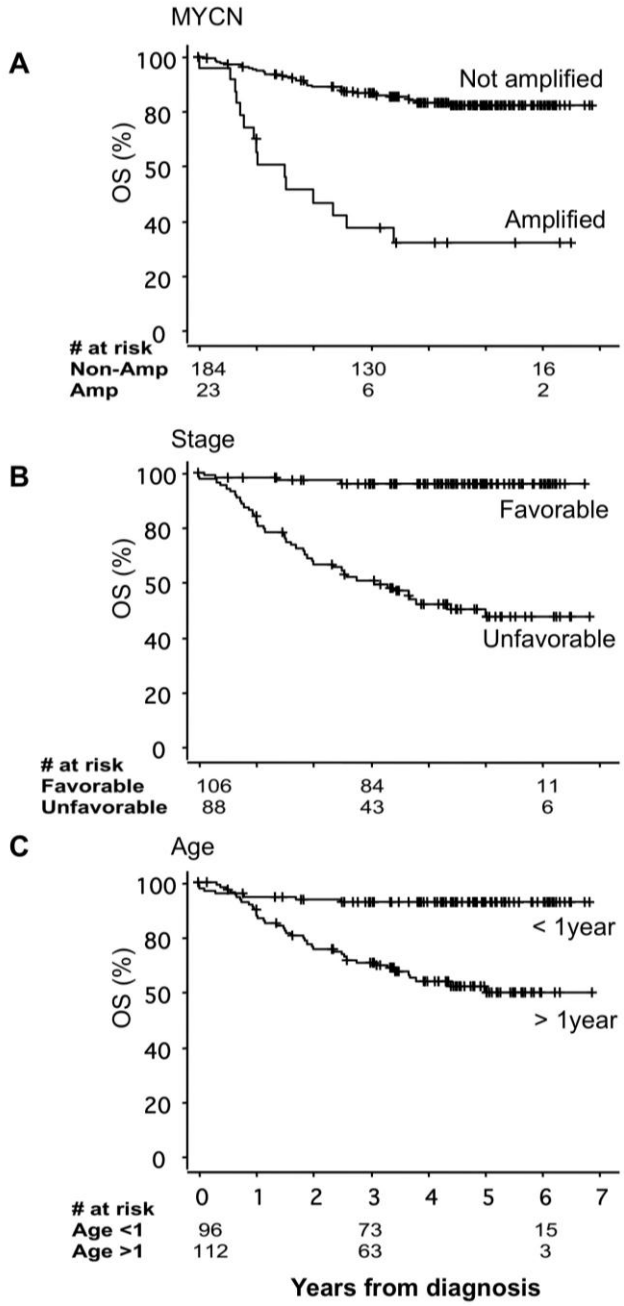


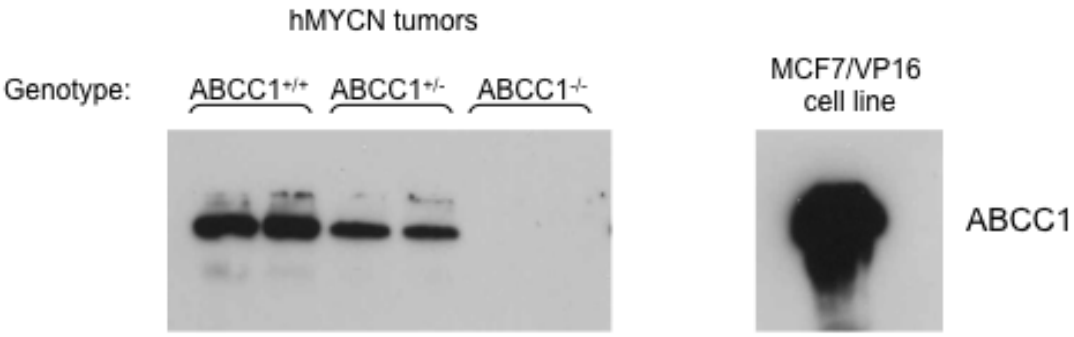
Supplementary Figure 1. Expression of FLAG-ABCC1 in SH-SY5Y neuroblastoma cells. Immunofluorescent detection of FLAG-tagged ABCC1 in SH-SY5Y neuroblastoma cell clones stably expressing either ABCC1-wt (clones D5, H7), ABCC1 D1454N single mutant (clone B4) or ABCC1 DD1454LL double mutant (clone B7). Cell preparations were stained with either monoclonal antibody M2 anti-FLAG (FLAG), or DAPI to visualize nuclei. Strong membranous staining of FLAG-ABCC1 was observed in the majority of cells transfected with all ABCC1 constructs (MERGE). Scale bar = 15μm. ABCC1= ATP-binding cassette, sub-family C, member 1; DAPI= 4'-6-Diamidino-2-phenylindole.



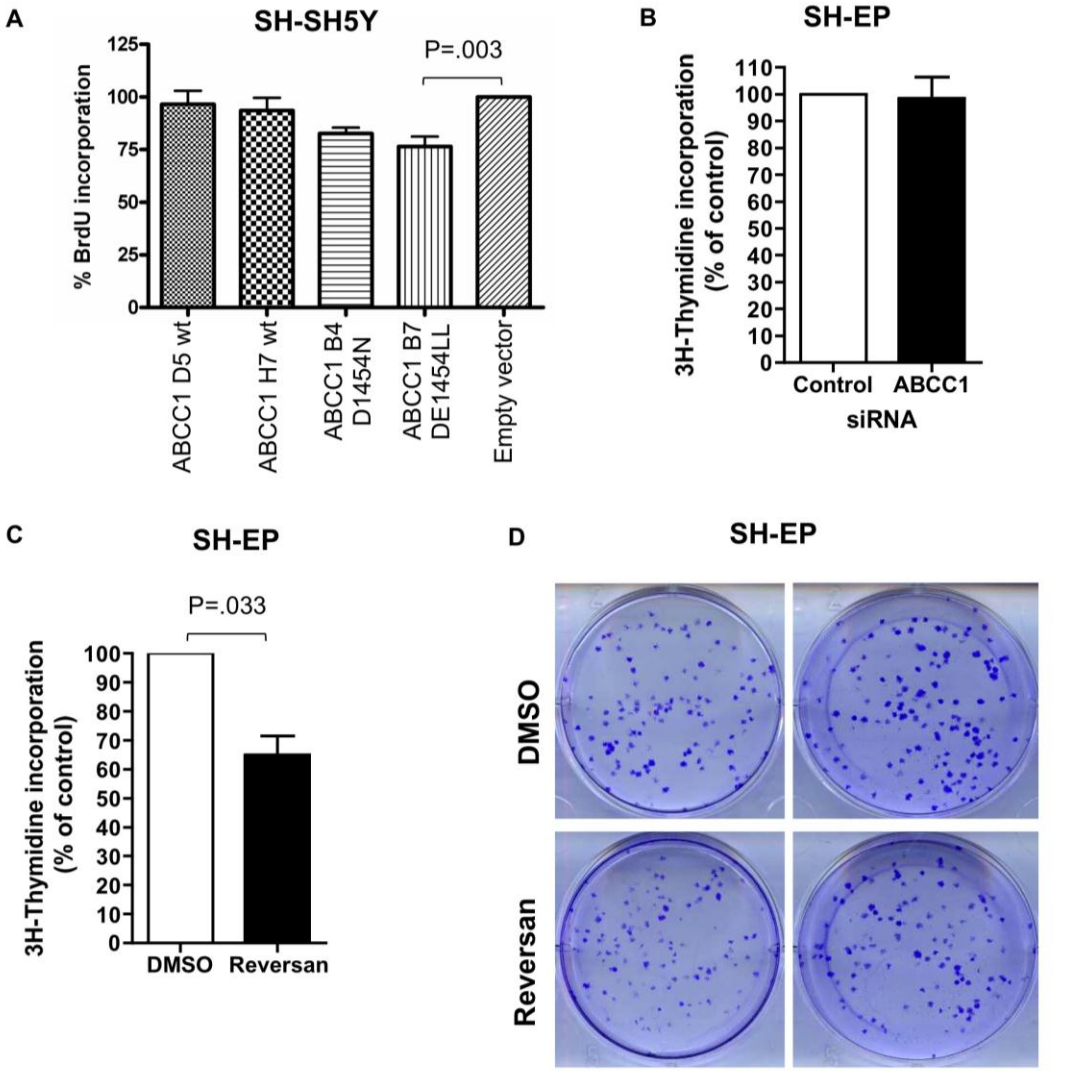
Supplementary Figure 2. Expression of FLAG-ABCC3 in BE(2)-C neuroblastoma cells. Immunofluorescent detection of FLAG-tagged ABCC3 in BE(2)-C neuroblastoma cell clones stably expressing either ABCC3-wt (clones A12, B12) or ABCC3 V1322F mutant (clones C4, D1). Cell preparations were stained with either monoclonal antibody M2 anti-FLAG (FLAG), or DAPI to visualize nuclei. Strong membranous staining of FLAG-ABCC3 was observed in the majority of cells transfected with both ABCC3 constructs (MERGE). Scale bar = 15μm. ABCC3= ATP-binding cassette, sub-family C, member 3; DAPI= 4'-6-Diamidino-2-phenylindole.



Supplementary Figure 3. Cumulative survival of 209 patients with neuroblastoma. The Kaplan-Meier curves show the probability of overall survival (OS) with respect to **A)** the number of copies of *MYCN* found by fluorescence *in situ* hybridization (5-year overall survival rate ± SEM: *MYCN* amplified, 32±10 % vs not amplified, 82±3 %, *P* <.001); **B)** tumor stage, categorized as favorable (INSS stages 1, 2A/2B or 4S) or unfavorable (INSS stages 3 or 4) (5-year overall survival rate ± SEM: favorable stage, 96±2 % vs unfavorable stage, 50±6 %, *P* <.001); and **C)** the patient's age at diagnosis (5-year overall survival rate ± SEM: age < 1 year, 93± 3 % vs age > 1 year, 62±5 %, P<.001). *P* values were determined by the log-rank test. Tick marks indicate the length of follow-up of individual patients who survived. The median follow-up after diagnosis among the surviving patients was 55 months (range, 0-196). At 0, 3 and 6 years from diagnosis, the number of patients at risk of death are shown. SEM = standard error of the mean. *MYCN = v-myc myelocytomatosis viral related oncogene, neuroblastoma derived.* INSS = International Neuroblastoma Staging System.

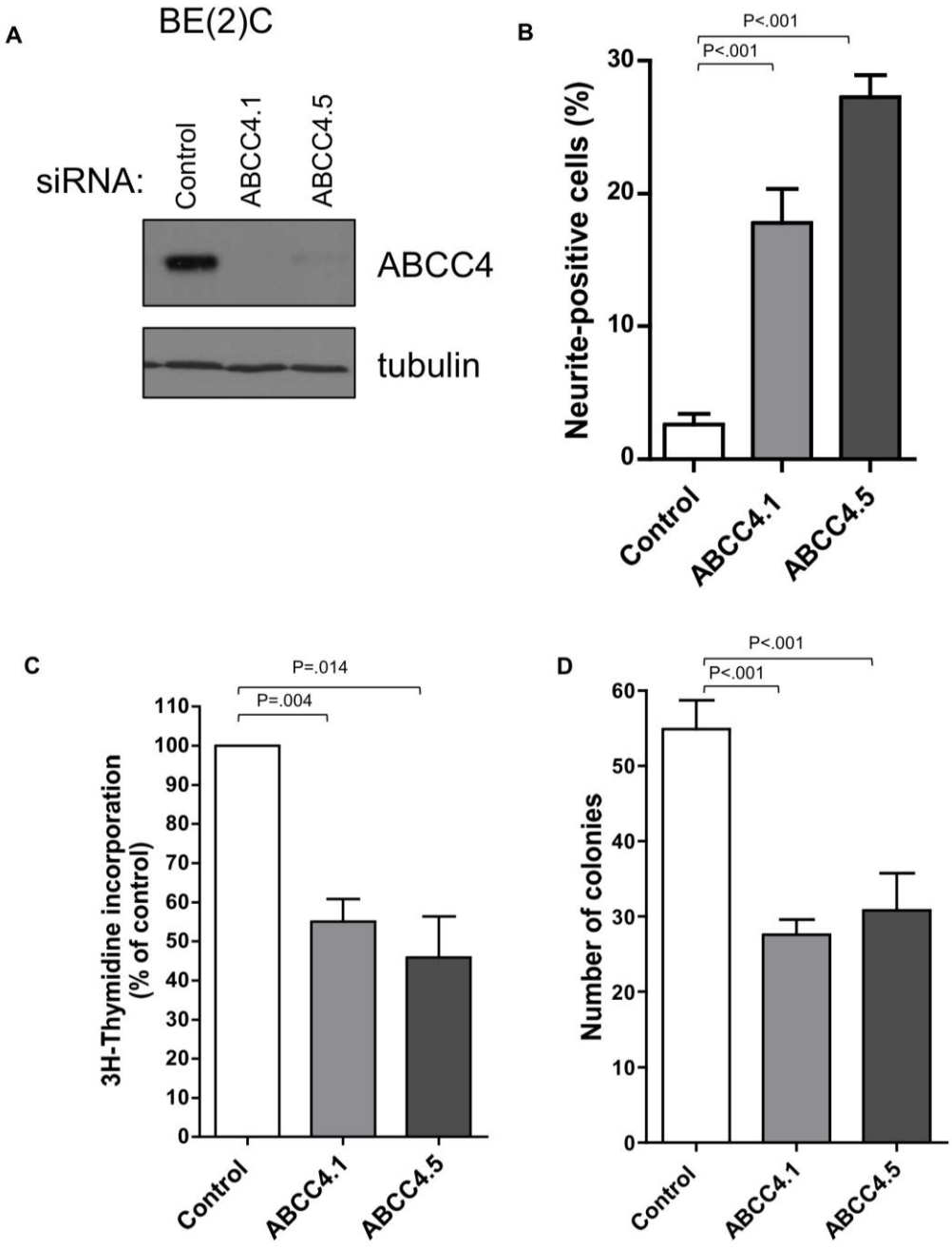


Supplementary Figure 4. ABCC1 protein expression in tumors from *Abcc1*$^{+/+}$*/hMYCN*, *Abcc1*$^{+/-}$*/hMYCN* and *Abcc1*$^{-/-}$*/hMYCN* mice. Neuroblastoma tumors were harvested from individual *hMYCN* transgenic mice that were of genotype *Abcc1*$^{+/+}$ (lanes 1-2), *Abcc1*$^{+/-}$ (lanes 3-4) or *Abcc1*$^{-/-}$ (lanes 5-6). Membrane proteins were purified and ABCC1 levels determined by western blot as previously described (10). Even loading of samples was confirmed by Ponceau staining (not shown). MCF7/VP cells, which express high levels of ABCC1, were used as a positive control. *hMYCN = human v-myc myelocytomatosis viral related oncogene, neuroblastoma derived.* ABCC1= ATP-binding cassette, sub-family C, member 1.

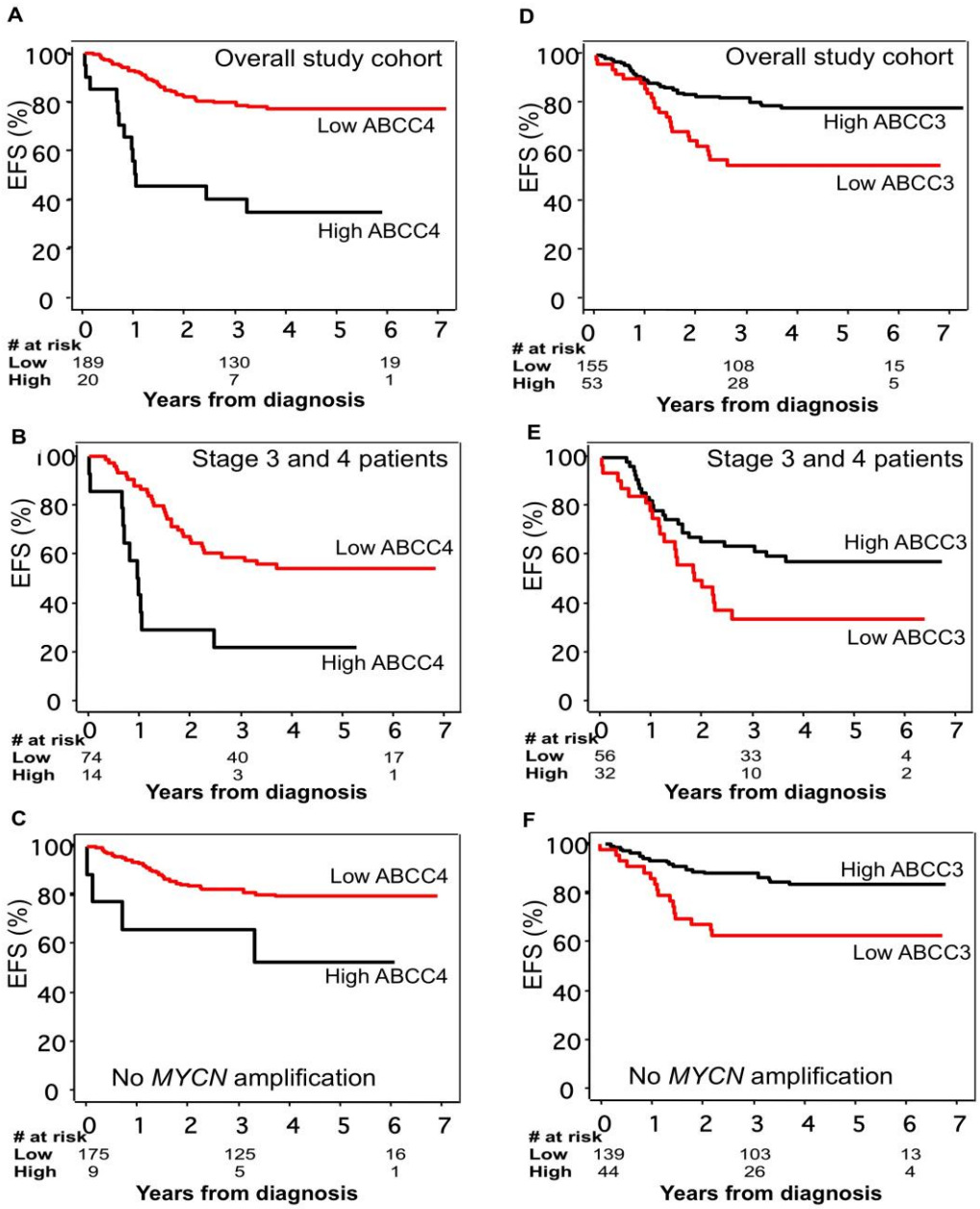


Treatment	No. of colonies >300 cells	P
DMSO	99 ± 15	
Reversan	33 ± 3.7	0.01

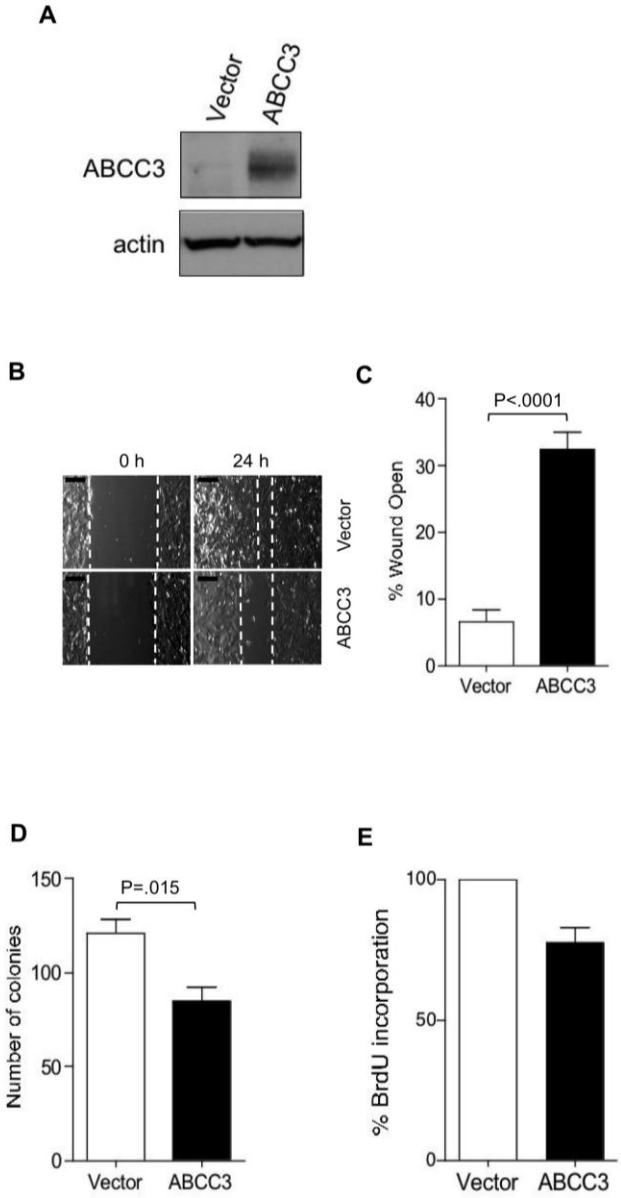
Supplementary Figure 5. Impact of *ABCC1* overexpression or suppression on proliferation in human neuroblastoma cells. **A)** Quantification of 5-bromo-2-deoxyuridine (BrdU) incorporation in SH-SY5Y cells expressing high levels of ABCC1-wt (clones D5, H7) or catalytically inactive mutants: ABCC1 D1454N single mutant (clone B4) or ABCC1 DD1454LL double mutant (clone B7). **B)** ^{3}H-Thymidine incorporation in SH-EP cells upon siRNA-mediated suppression of ABCC1. **C)** ^{3}H-Thymidine incorporation in SH-EP cells upon pharmacological inhibition of ABCC1 using 5µM Reversan. **D)** Representative images and quantification showing colony size distribution in SH-EP cells exposed to 5µM Reversan compared to cells exposed to vehicle control (DMSO, dimethyl sulfoxide). Means displayed in all panels are derived from at least 3 independent experiments. P values were derived from one-sample *t* test vs control (H_0, µ=100%), except for panel **D**, for which two-sided Student's *t* test was used. ABCC1= ATP-binding cassette, sub-family C, member 1.



Supplementary Figure 6. Impact of *ABCC4* suppression by individual siRNA duplexes in BE(2)-C neuroblastoma cells. **A)** Western blot analysis of ABCC4 protein expression following exposure of BE(2)-C cells to SMARTpool siRNA duplex 1 (ABCC4.1), independent siRNA duplex 5 (ABCC4.5) or to control siRNA. **B)** Enhanced neurite extension in SH-SY5Y cells upon depletion of ABCC4. **C)** ^{3}H-thymidine incorporation was reduced in SH-SY5Y cells depleted of ABCC4. **D)** Clonogenic capacity upon depletion of ABCC4 was assayed after 12 days. Means displayed in all panels are derived from at least 3 independent experiments. In panels **B** and **D**, *P* values were derived from one-way analysis of variance followed by two-sided *t* test vs control, while one-sample *t* test was used in panel C (H_0, μ=100%). ABCC4 = ATP-binding cassette, sub-family C, member 4; siRNA = short interfering RNA.



Supplementary Figure 7. Prognostic significance of *ABCC3* and *ABCC4* gene expression in neuroblastoma. **A)** Expression of *ABCC4* predicts cumulative event-free survival (EFS) in 209 patients with neuroblastoma (relative hazard = 4.7, 95% CI = 2.5 to 8.9, $P < .001$). Similar associations between high *ABCC4* gene expression and increasingly poor outcome were also observed in subgroups of patients **B)** with Stage 3 or 4 disease (five-year event-free survival rate ± SE: high *ABCC4*, 21±22 % vs low *ABCC4*, 54±12 %, $P < .001$) or **C)** whose tumors lacked *MYCN* amplification (five-year event-free survival rate ± SE: high *ABCC4*, 53±34 % vs low *ABCC4*, P=.02). **D)** Expression of *ABCC3* predicts cumulative event-free survival in 209 patients with neuroblastoma (relative hazard = 2.4, 95% CI = 1.4 to 4.2, P=.001). Similar associations between low *ABCC3* gene expression and increasingly poor outcome were also observed in subgroups of patients **E)** with Stage 3 or 4 disease (five-year event-free survival rate ± SE: low *ABCC3*, 34±17 % vs high *ABCC3*, 57±13 %, $P = .03$) or **F)** whose tumors lacked *MYCN* amplification (five-year event-free survival rate ± SE: low *ABCC3*, 63±14 % vs high *ABCC3*, 84±7 %, $P = .0016$). At 0, 3 and 6 years from diagnosis, the number of patients at risk of relapse are shown. SE = standard error; *MYCN = v-myc myelocytomatosis viral related oncogene, neuroblastoma derived. ABCC*= ATP-binding cassette, sub-family C.



Supplementary Figure 8. Impact of stable ABCC3 expression in BE(2)-C cells. **A)** Western blot analysis of ABCC3 protein expression following stable retroviral transduction and mass-culturing of BE(2)-C cells with either empty vector or pCMV-ABCC3 (kindly provided by P. Borst, Netherlands Cancer Institute, (1)). **B)** Representative images of wound closure assay. Scale bar = 100μm. **C)** Quantification depicting impaired motility of BE(2)-C cells stably overexpressing ABCC3. **D)** Clonogenic capacity was significantly reduced in BE(2)-C cells overexpressing ABCC3. **E)** Effect of ABCC3 overexpression on cell proliferation in BE(2)-C cells was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation. One-way analysis of variance followed by two-sided *t* tests vs control were used to generate *P* values for wound closure and colony forming assays whereas one-sample *t* test was used for BrdU incorporation (H_0, μ=100%). In all panels means are derived from 3 replicate experiments and error bars represent standard error of the mean. ABCC3 = ATP-binding cassette, sub-family C, member 3.

Supplementary Table 1. DNA sequence of primers and TaqMan assay codes of assays used for real-time quantitative PCR*

Gene Primer or Probe	Sequence
ABCC1 F	TCCTCTATCTCTCCCGACATGAC
ABCC1 R	CCCGACTTCTTTCCCAGAAAG
ABCC1 PROBE	AGGTCTGCCCAGCAGACGATCCA
ABCC2 F	ACAGCTTTCGTCGAACACTTAGC
ABCC2 R	CATTCCGAGTTTTCAAGGAGTTTC
ABCC2 PROBE	AGGCATCTGAAGTCC
ABCC3 F	AGCTGTGGCCGTGAAGATG
ABCC3 R	TGCGCGAGTCCTTCAATTTC
ABCC3 PROBE	CCTTCCAGGTAAAGCA
ABCC4 F	CGAGTAGCCATGTGCCATATGA
ABCC4 R	TGACTATCTGGCCTGTGGTTGTCT
ABCC4 PROBE	CGGAAGGCACTTCGTCTTAGTAACATGGC
ABCC5 F	CATTCGAGGAGTTGTCTTTGTCAA
ABCC5 R	CCTTCGGAAAAGCTCGTCAT
ABCC5 PROBE	CTGCGAGCTTCCTCCCGGCTG
B2M F	ACTGGTCTTTCTATCTCTTGTACTACACTGA
B2M R	TGATGCTGCTTACATGTCTCGAT
B2M PROBE	TGCCTGCCGTGTGAACCATGTGAC
Gene	**TaqMan assay code**
ABCC6	Hs00184566_m1
ABCC7	Hs00357011_m1
ABCC8	Hs00165861_m1
ABCC9	Hs00245832_m1
ABCC10	Hs00375716_m1
ABCC11	Hs00261567_m1
ABCC12	Hs00264354_m1

***** PCR, polymerase chain reaction; ABCC = ATP-binding cassette, sub-family C; B2M, beta-2 microglobulin.

Supplementary Table 2. Relation of age and tumor stage at diagnosis to ABCC gene expression in the tumors in the 209 study patients with neuroblastoma

Clinical characteristics	No. of patients											
	ABCC2 expression *			*ABCC5* expression *			*ABCC8* expression *			*ABCC9* expression *		
	High	Low	*P*	High	Low	*P*	High	Low	*P*	High	Low	*P*
Age												
< 1 yr	57	38	.008	64	31	< .001	54	41	.09	64	31	< .001
≥ 1 yr	46	66		38	73		49	62		40	71	
Tumor stage †												
Favorable	48	57	.15	49	56	.56	54	51	.77	58	47	.19
Unfavorable	50	38		45	43		42	45		39	48	

* The level of expression was considered high or low in relation to the median of ΔΔCt values for all tumors analyzed. *P* values are derived using two-sided Fisher's exact tests.

† Tumor stage was categorized as favorable (INSS stages 1, 2A/2B or 4S) or unfavorable (INSS stages 3 or 4), and was unknown in 15 cases.

ABCC = ATP-binding cassette, sub-family C; ΔΔCt method is the comparative threshold cycle method of transcript quantification. INSS = International Neuroblastoma Staging System.

Supplementary Table 3. Univariate analysis of ABCC gene expression and outcome in neuroblastoma

***ABCC* gene expression**	**Event-free survival**		**Overall survival**	
	Relative Hazard * (95% CI)	**P †**	**Relative Hazard * (95% CI)**	**P †**
ABCC2 (n=208)	1.4 (0.8 to 2.4)	.22	1.5 (0.8 to 2.7)	.17
ABCC5 (n=207)	1.4 (0.8 to 2.4)	.25	1.3 (0.7 to 2.3)	.37
ABCC8 (n=207)	1.0 (0.6 to 1.8)	.88	1.15 (0.5 to 1.6)	.63
ABCC9 (n=207)	1.3 (0.7 to 2.2)	.37	1.3 (0.7 to 2.3)	.45

*Relative hazards were calculated as the antilogs of the regression co-efficients in the proportional hazards regression. CI = confidence interval; *ABCC = ATP-binding cassette, sub-family C*.

†Log-rank test

References to Supporting Information

1. Kool, M., van der Linden, M., de Haas, M., et al. 1999. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci U S A* 96(12):6914-6919.