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ABCB5 maintains melanoma-initiating cells through a pro-inflammatory cytokine signaling circuit

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Abstract

The drug efflux transporter ABCB5 identifies cancer stem-like cells (CSC) in diverse human malignancies, where its expression is associated with clinical disease progression and tumor recurrence. ABCB5 confers therapeutic resistance but other functions in tumorigenesis independent of drug efflux have not been described that might help explain why it is so broadly overexpressed in human cancer. Here we show that in melanoma-initiating cells ABCB5 controls IL-1 β secretion which serves to maintain slow-cycling, chemoresistant cells through an IL-1 β /IL8/CXCR1 cytokine signaling circuit. This CSC maintenance circuit involved reciprocal paracrine interactions with ABCB5-negative cancer cell populations. ABCB5 blockade induced cellular differentiation, reversed resistance to multiple chemotherapeutic agents, and impaired tumor growth in vivo. Together, our results defined a novel function for ABCB5 in CSC maintenance and tumor growth.

Keywords

Melanoma; ABCB5; Interleukin-1 β ; Interleukin-8; CXCR1

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Introduction

ABCB5 (ATP-binding cassette, sub-family B (MDR/TAP), member 5) is a plasma membrane protein and human P-glycoprotein family member, shown to be highly overexpressed by cancer stem cells (CSC) in diverse human malignancies (1–7). ABCB5 is associated with clinical tumor progression, therapeutic resistance and recurrence in cancer patients (1, 4, 6–12), including in malignant melanoma (1, 4, 8–12). Melanoma stem cells, also known as malignant melanoma-initiating cells (MMIC) (1), are malignant subpopulations in which clinical virulence resides as a consequence of unlimited self-renewal capacity, resulting in inexorable tumor progression and metastasis (1, 2, 4, 5, 9, 13–18). Our laboratory and others recently identified MMIC (1, 2, 4, 5, 13, 14, 18) and showed them to express the targetable biomarker (1, 4, 6, 7, 9–12, 16) and drug-resistance mediator (6, 7, 19), ABCB5.

While successful MMIC isolation techniques have been shown to require trypsin-free tumor dissociation protocols (reviewed in (20, 21)) that have as of yet not been adopted by all investigators in the field (22, 23), the existence of clinically relevant MMIC that express ABCB5 (1, 15, 17, 19) has been broadly confirmed in diverse experimental model systems (2–5, 16) and across multiple patient cohorts (1, 4, 9–12, 24). Moreover, consistent with a potential functional role in MMIC, ABCB5 genetic variation is associated with melanoma risk (25). Nevertheless, despite the established relationship between ABCB5 expression and MMIC (1–5, 15–17, 19) or CSC in other cancers (6, 7), the intrinsic molecular function of ABCB5 in tumorigenic and therapy-refractory cancer subpopulations is currently unknown (26).

Here, we hypothesized that ABCB5 might provide a novel functional link between MMIC maintenance, multidrug-resistance and tumor growth in human malignant melanoma. Our data reveal that ABCB5 drives secretion of IL-1 β to maintain an IL-1 β /IL8/CXCR1 cytokine signaling circuit controlling these properties of aggressive melanomas.

Materials and Methods

Melanoma cell lines and clinical specimens

Authenticated human melanoma cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA). MUM-2B and MUM-2C were a gift of Dr. Mary Hendrix (Northwestern University) and are identical with cutaneous C8161 melanoma cells and OCM-1 uveal melanoma cells, respectively, based on STR analysis (E. Seftor/M. Hendrix, personal communication). RNA from 8 additional melanoma cell lines of the NCI-60 panel (19) (Fig. 2) was obtained from the NCI/NIH. Stable shRNA pSUPER-retro-puro-ABCB5-KD cell populations and the respective vector- or shRNA-control cell populations used the previously validated ABCB5 shRNA targeting sequence (7) or control shRNA sequence 5'-AATCTCTCCGAACGAGTCAGT-3'. A375 and G3361 WFDC1-myc, or vector, populations used a pCMV6 plasmid (Origene, Rockville, MD). A375/DTIC cells were generated by incubation of wildtype A375 cells with increasing concentrations of DTIC (Sigma, St. Louis, MO) (up to 900 μ M) over a period of 4 weeks. Clinical cutaneous melanoma cells and melanoma tissue sections were derived from surgical specimen

according to Institutional Review Board–approved human subjects research protocols as described previously (1, 15).

Xenotransplantation

NOD/SCID IL2 $\gamma^{-/-}$ (NSG) mice were purchased from The Jackson Laboratory. Mice were maintained in accordance with the institutional guidelines of Children's Hospital Boston and Harvard Medical School and experiments were carried out according to approved experimental protocols. Human xenografts were established by subcutaneous injection (10^6 cells per recipient) as described (1, 15, 17). Differences in tumor volume (TV), determined as described (1), were statistically compared by the nonparametric Mann–Whitney test, with a 2-sided value of $P < 0.05$ considered significant. G3361 tumor xenografts were harvested for pigmentation measurements after collagenase treatment, as described (25). In additional experiments aimed at the assessment of *in vivo* tumor cell proliferation, fluorescent membrane dye DiO (27)-labeled G3361 melanoma cells were xenografted at 5×10^6 cells/recipient, and established tumors harvested at 3 weeks post xenotransplantation for flow cytometric assessment of label retention.

Drugs and cell viability assays

For ABCB5-KD and control melanoma cells, 2.5×10^2 cells/well were seeded into 96-well plates with drug or vehicle, for up to 7 days. For antibody-mediated ABCB5 inhibition, 1×10^4 cells/well were seeded for up to 3 days drug exposure in the presence of 50 μ g/ml anti-ABCB5 mAb 3C2-1D12 or MOPC31C isotype control mAb (Sigma, St Louis, MO). Treated cultures were subjected to the MTT assay as described (19). For examination of the effects of ABCB5 blockade on IL8 and WFDC1 gene expression in A375 and G3361 cells and clinical melanoma specimens, statistical differences in mRNA expression ($n=5$ /group) were determined using the student t-test.

Flow cytometry

ABCB5/CXCR1 or ABCB5/CXCR2 stains used APC-conjugated anti-ABCB5 mAb (clone 3C2-1D12) or isotype control mAb and FITC-conjugated anti-CXCR1 or anti-CXCR2 mAbs (R&D Systems, Minneapolis, MN) or FITC-conjugated isotype controls (BD Pharmingen). Analysis of ABCB5 expression by A375/DTIC cells and A375/WT controls was performed following fixation and permeabilization of cells to detect total ABCB5 protein. BrdU incorporation: 2×10^5 purified ABCB5(+) G3361 cells were cultured for 72h in the presence of 10 μ M BrdU and 5 μ g/ml inhibitory CXCR1 antibody or isotype control antibody (R&D Systems). Subsequently, cells were first surface-stained for ABCB5, then permeabilized and subjected to DNase digestion, followed by counterstaining with FITC-conjugated anti-BrdU antibody and 7-AAD using a BrdU/7-AAD flow cytometry kit (BD Pharmingen, San Diego, CA). 7-AAD gated ABCB5(+) cells and derived ABCB5(–) cells were then analyzed for BrdU incorporation. DiO (Invitrogen Molecular Probes, Grand Island, NY) *in vivo* label retention: G3361 cells were labeled *in vitro* as described (27), xenografted as described above, followed by preparation of single tumor cell suspensions (1), and DiO label-retention by ABCB5(+) vs. ABCB5(–) cells in each tumor was then

measured by flow cytometry (n=4). Statistical differences between marker expression levels were determined by the Mann–Whitney test.

Cell isolation

ABCB5(+) or CXCR1(+) cells were isolated by magnetic bead sorting as described (1).

Correlation of ABCB5 mRNA expression and cell doubling times

Melanoma cell cDNA was prepared from RNA extracts using SuperScript First-Strand Synthesis System (Invitrogen). ABCB5 qPCR was performed as described (19). ABCB5 expression was assessed by the ratio of the expression level in the sample against mean expression in all samples, in $n = 3$ independent experiments. Culture doubling times for the 8 NCI-60 cell lines were obtained from the National Cancer Institute. Growth kinetics for the remaining cell-lines were established according to the formula:

$$\text{doubling time (h)} = T2 - T1 / (\log_2 (\text{cell count}_{T2} / \text{cell count}_{T1})),$$
 where T2 and T1 represent two distinct time points (h) in the logarithmic culture growth phase. Upon correlation of ABCB5 mRNA expression with culture doubling times, a Pearson coefficient was calculated and the criteria of $P < 0.05$ and $r > 0.3$ or $r < -0.3$ were used to identify significance (19).

Gene expression microarray analyses

Microarray analyses were carried out on ABCB5-KD or control shRNA G3361 or A375 RNA (n=3 replicates per group). Microarrays (HG-U133A 2.0; Affymetrix, Santa Clara, CA) were performed by the Dana-Farber Cancer Institute (Boston, MA) core facility. CEL files were normalized using Robust Multi-chip Averaging (RMA) available at <http://www.babelomics.org>. Probe-sets detected on fewer than 4 of the 6 chips for each cell-line (P as determined by MAS5) were discarded. Differentially expressed probe-sets were identified using one-way ANOVA. Probesets with $P < 0.05$ (estimated false discovery rate of 6.1%) were subjected to Tukey's post-hoc test and those with significant differences in ABCB5-KD cells vs. controls were ranked by fold change. The expression data set in its entirety is available through GEO (Gene Expression Omnibus) DataSets (accession number GSE38290).

Quantitative real-time PCR analysis

qPCR (SYBR green) used 18s as a normalizing control. IL8 and WFDC1 primers were from Origene (Rockville, MD). Primers for 18s and ABCB5 were as described (7), and for CXCR1, F: 5'-TATTCATCAAGTGCCCTCTAGCTGT-3' and R: 5'-TTAGATCATCAAATCCCACATCTG-3'. Quantitative WNT expression analysis utilized WNT real-time qPCR arrays (PAHS-043) from SA Biosciences (Qiagen, Frederick, MD).

Western blots and ELISA

RIPA cell lysates were incubated with IL8 antibody (Abcam clone 6217), WFDC1 antibody (Sigma Prestige rabbit polyclonal, HPA031411), or α Tubulin antibody (Abcam rabbit polyclonal ab24246). IL8 ELISA used 50 μ l of debris-cleared supernatants of ABCB5(+) or ABCB5(-) G3361 cultures (72h cultures of 10^5 cells/well in 24-well plates containing 0.5ml/well of serum-free media) compared to medium-only negative controls and rhIL8

standard curves (Quantikine ELISA, R&D Systems, Minneapolis, MN). For *in vivo* ELISA analysis of hIL8 production from human G3361 xenografts, mice were sacrificed 3 weeks post-xenotransplantation. Subsequently, 50µl of cell-cleared serum (cardiac puncture then spun 5min/5000rpm) was assayed in triplicate wells for duplicate mice/xenograft cell type, using internal negative serum controls. For ABCB5 blockade, purified CXCR1(+) or CXCR1(-) cells were seeded in 96-well plates with addition of 100µg/ml ABCB5 mAb or MOPC31C isotype mAb on ice for 30 min. After 30 min, cells were placed at 37°C with 5% CO₂ and stimulated with 5µg/ml LPS for 1h, then 5mM ATP+1µM nigericin for 3h. Media was extracted from all samples, spun to remove cells, and hIL-1β ELISAs were then performed according to the manufacturer's protocol (Quantikine-HS, R&D Systems).

Immunohistochemistry and immunofluorescence staining

Cryostat sections (5µm) from snap-frozen specimens obtained from metastatic melanoma tumors were fixed in acetone for 10 minutes and then dried and blocked with 0.3% peroxidase for 5 minutes. MAbs to CXCR1 (clone LS-A806), CXCR2 (clone LS-C90306) and IL8 (clone LS-C8034) were purchased from LS Biosciences (Seattle, WA). MAbs to TLR4 (clone ab13556) and IL-1β (clone ab2105) were purchased from Abcam (Cambridge, MA). The ABCB5 antibody (clone 3C2-1D12) was used as described (15).

Immunohistochemistry: After primary antibody incubation samples were treated with secondary horseradish peroxidase (HRP)-conjugated antibody or secondary AP-conjugated antibody (Dako) followed by DAB (3,3 Diaminobenzidin-Tetrahydrochlorid) (Bio Genex, The Hague, NL) or Fast Red (5361) (Dako) and nuclear staining (Mayer's hemalaun, Merck, Darmstadt, Germany). Immunofluorescence staining of paraffin-embedded clinical melanoma involved TLR4 antibody (Novus Biologicals) followed by Alexa Fluor-594 secondary antibody, and ABCB5 primary antibody (clone 3C2-1D12) followed by addition of biotinylated horse anti-mouse IgG and Alexa Fluor-488-labeled streptavidin.

Results

ABCB5 regulates melanoma growth and multidrug-resistance

In order to initially address the functional role of ABCB5 in melanoma growth, we generated ABCB5 shRNA stably transfected knockdown (KD) melanoma cell populations (termed ABCB5-KD herein, KD efficiency >80% at mRNA level and validated by IP Western blotting), using the well-studied non-pigmented melanoma cell-line model A375 and the pigmented melanoma cell-line G3361, which had previously been utilized in MMIC characterization studies and confirmed to express representative levels of ABCB5 (1, 15, 17, 27). G3361 or A375 ABCB5-KD cells or respective control populations were subcutaneously injected (1×10^6 cells/mouse) into highly immunocompromised NSG mice (G3361 n=5 and A375 n=12 animals/group) and tumor progression was followed for up to 9 weeks (G3361) or until experimental protocol stipulations necessitated animal sacrifice due to excessive tumor burden (A375: 5 weeks), by serial determination of tumor volumes (TV). We observed significant impairment of *in vivo* tumor growth upon ABCB5 inhibition at serial time points throughout the course of the experiment (Fig. 1A), with final TV measurements showing 2.9-fold inhibition for G3361 (TV week 9 ABCB5-KD: 126 ± 43 mm³ (mean±SEM) vs. TV week 9 control: 364 ± 37 mm³, $P=0.0030$) and 3.1-fold inhibition

for A375 (TV week 5 ABCB5-KD: $638.5 \pm 78 \text{ mm}^3$ vs. TV week 5 control: $1801 \pm 168 \text{ mm}^3$, $P=2.32 \times 10^{-5}$) (Fig. 1A), demonstrating a novel intrinsic functional role of ABCB5 in melanoma growth. Furthermore, for G3361 (a pigmented melanoma line as opposed to non-pigmented A375 cells), we also noted significantly enhanced pigmentation of ABCB5-KD tumors vs. controls (2.7-fold enhancement detected spectrophotometrically; $\text{OD}^{490\text{nm}}$ ABCB5-KD: 0.0626 ± 0.0136 (mean \pm SEM) vs. $\text{OD}^{490\text{nm}}$ control: 0.0234 ± 0.0095 , $n=5$ respectively, $P=0.044$) (Fig. 1B), demonstrating ABCB5 inhibition-mediated *in vivo* differentiation of at baseline lesser-differentiated and lower-pigmented (1) ABCB5(+) MMIC.

We next performed an in-depth analysis to assess the functional role of ABCB5 as a multidrug-resistance mediator in melanoma with respect to chemotherapeutic agents either approved for clinical use in melanoma (dacarbazine, DTIC), or previously shown to exhibit significant strong positive correlations between drug resistance and ABCB5 expression (paclitaxel, teniposide, docetaxel, etoposide, doxorubicin and vincristine) in melanoma and other malignancies (19, 20), using both shRNA-mediated or monoclonal antibody (mAb)-mediated ABCB5 inhibition strategies. Targeted shRNA-mediated ABCB5 inhibition resulted in significant reversal of chemoresistance to all agents (IC_{90} , $n=6$ each: DTIC: 15-fold, $P=3 \times 10^{-7}$; paclitaxel: 9.4-fold, $P=8.7 \times 10^{-4}$; teniposide: 7.3-fold, $P=3.4 \times 10^{-4}$; docetaxel: 6-fold, $P=2 \times 10^{-5}$; etoposide: 3.9-fold, $P=2 \times 10^{-7}$; doxorubicin: 3-fold, $P=2 \times 10^{-6}$; vincristine: 2.6-fold, $P=2 \times 10^{-5}$) (Fig. 1C), demonstrating for the first time that ABCB5 serves as a *bona fide* multidrug-resistance mediator in human melanoma. MAb-mediated ABCB5 inhibition, using the extracellular loop-specific antibody clone 3C2-1D12 (1, 2, 8), also significantly sensitized melanoma cells to killing by this set of agents, thus confirming the results obtained with shRNA-mediated ABCB5 inhibition (IC_{50} , $n=6$ each: Vincristine: 8.4-fold, $P=0.038$; doxorubicin: 4.9-fold, $P=8.2 \times 10^{-3}$; teniposide: 2.9-fold, $P=2 \times 10^{-5}$; paclitaxel: 1.9-fold, $P=0.012$; etoposide: 1.9-fold, $P=3 \times 10^{-5}$; DTIC: 1.5-fold, $P=9 \times 10^{-5}$; docetaxel: 1.5-fold, $P=0.011$) (Fig. 1C). Results of mAb-mediated ABCB5 inhibition with respect to chemosensitization to the agent approved for clinical use in melanoma (dacarbazine, DTIC) also revealed significant sensitization in A375 melanoma cells (IC_{50} : 1.6-fold, $P<0.001$), similar to the effect observed in G3361 cells. These results demonstrated two novel important functional roles of ABCB5 in melanoma: First, that ABCB5 is required for more efficient melanoma growth, with inhibition resulting in smaller, more differentiated tumors; and second, that ABCB5 functions as a melanoma multidrug-resistance mechanism for diverse chemotherapeutic compounds, including the mainstay clinical melanoma chemotherapeutic DTIC, to which ABCB5(+) melanoma subpopulations have been reported to be therapy-refractory in patients (8).

ABCB5 identifies and regulates slow-cycling melanoma cells

Recent studies suggested that CSC can be slow-cycling, potentially causing resistance to cancer treatments that target more rapidly-dividing cells (16, 28). Based on the known role of ABCB5 as a marker of MMIC and its herein-defined functions in DTIC and multidrug-resistance, we hypothesized that ABCB5(+) MMIC are slow-cycling and that ABCB5 might functionally regulate cellular quiescence, a potential mechanism of multidrug-resistance independent of drug transport function. Using an experimental model system whereby

established G3361 melanoma cells were labeled with the fluorescent cell-membrane dye DiO (27) and subsequently xenografted s.c. to immunodeficient mice in order to assess cellular dye retention as a measure of cellular quiescence in growing tumors, we found that ABCB5(+) MMIC retained significantly higher amounts of DiO compared to ABCB5(-) melanoma bulk populations (5-fold, $P<0.05$, $n=4$), providing initial *in vivo* evidence that ABCB5(+) MMIC represent a slow-cycling tumor cell subpopulation (Fig. 2A). Furthermore, separation of unlabeled ABCB5(+) and ABCB5(-) G3361 melanoma cells by magnetic cell sorting and subsequent comparison of their relative growth rates by serial daily MTT assays for 1 week confirmed *in vitro* that ABCB5(+) cells grow more slowly than ABCB5(-) melanoma bulk populations (log OD/h, $n=5$: ABCB5(+) cells: $11.5\pm0.4\times10^{-3}$ (mean \pm SD) vs. ABCB5(-) cells: $9.4\pm0.6\times10^{-3}$, $P=0.0158$) (Fig. 2B), consistent with a previously demonstrated cell cycle profile of ABCB5(+) cells with enhanced G2 arrest compared to ABCB5(-) cells (19). To assess a possible functional role of ABCB5 in this slow-cycling phenotype, we compared the growth rates of ABCB5-KD G3361 cells to control G3361 cells, demonstrating a significantly increased growth rate upon ABCB5 inhibition (day 5 MTT OD⁵⁹⁵: ABCB5-KD cells= 1.317 ± 0.025 (mean \pm SEM) vs. control cells= 0.931 ± 0.024 , $P<0.01$) (Fig. 2C) and hence a functional role of ABCB5 in regulating melanoma cell quiescence. Moreover, analysis of a panel of 12 melanoma cell-lines demonstrated a significant correlation of relative ABCB5 expression with culture doubling times (Pearson correlation coefficient $r=0.655$, $P<0.0001$) (Fig. 2C), consistent with the newly discovered role of ABCB5 in melanoma cellular quiescence. These results showed that ABCB5 identifies a slow-cycling melanoma cell subpopulation and indicated a functional role of ABCB5 in the maintenance of this slow-cycling MMIC subset.

ABCB5 represses the tumor suppressor WFDC1 and induces the melanoma tumor promoter IL8 through the WNT pathway

To obtain mechanistic insight to explain why ABCB5 ablation led to impaired tumorigenesis, chemosensitization and reversal of melanoma quiescence we first compared triplicate samples of G3361 ABCB5-KD and A375 ABCB5-KD melanoma cells versus their respective controls for differentially expressed genes using global gene expression microarray analysis, revealing a list of 31 distinct genes with stringent P values (Supplemental Data Table 1). We restricted our focus to whey acidic protein 4-disulphide core domain 1 (WFDC1/ps20), the most highly up-regulated gene in ABCB5-KD cells (G3361 4.1-fold, A375 13.2-fold, microarray $P=6.3\times10^{-9}$), and IL8, the most significantly down-regulated gene in ABCB5-KD cells (G3361: -4.2-fold, A375: -1.8-fold; microarray $P=1.9\times10^{-7}$), the altered expression levels of which were further validated at both the mRNA and protein levels by qPCR and western blot analysis, respectively (Figs.3A&B). Levels of human melanoma-derived circulating hIL8 determined by human-specific ELISA were also found to be significantly decreased *in vivo* in the serum of murine recipients of ABCB5-KD vs. control xenografts, 3 weeks following xenotransplantation when tumors were established (4.4-fold, ABCB5-KD: 31 ± 5 pg/ml (mean \pm SEM) vs. control: 141 ± 16 pg/ml, $P=0.0023$) (Fig. 3C). Because WFDC1 is a tumor suppressor protein expressed in differentiated epidermal melanocytes and down-regulated by gene methylation in melanoma cells (29), and because overexpression of WFDC1 in A375 melanoma cells has been demonstrated to inhibit tumor growth *in vivo* (30), induction of WFDC1 following ABCB5

inhibition provided one explanation for ABCB5-KD-induced tumor inhibition. Moreover, because IL8 is a known mediator of clinical melanoma aggressiveness acting to functionally promote carcinogenesis (31, 32) and DTIC resistance (33, 34), reduced IL8 production following ABCB5 inhibition provided an additional explanation for ABCB5 inhibition-induced impairment of melanoma growth and sensitization to DTIC-induced cell killing. Importantly, mAb-mediated ABCB5 inhibition at the protein level in G3361 and A375 cells, as well as in a panel of three patient-derived melanoma specimens, confirmed the specificity of shRNA-mediated ABCB5-KD on WFDC1 and IL8 expression (WFDC1: 2.54-fold increased expression in ABCB5 mAb- vs. isotype control mAb-treated samples, $n=5$, $P=0.0079$; IL8: 0.57-fold expression in ABCB5 mAb- vs. isotype control mAb-treated samples, $n=5$, $P=0.0019$), and demonstrated that these results can be extrapolated to clinical melanoma specimens (Fig. 3D).

WFDC1 has recently been hypothesized to act as a repressor of WNT signaling in melanoma, while IL8 has been demonstrated to be a downstream WNT target gene (30, 35, 36). In light of the findings of the present study, this suggested a potential functional link between WFDC1 and IL8 via WNT signaling in an ABCB5-regulated pathway. In support of this hypothesis, qPCR-based WNT pathway array analysis demonstrated down-regulated expression levels of several WNT members, i.e. WNT1, WNT5A, WNT6, WNT7B, WNT9A, WNT10A and WNT11 in ABCB5-KD G3361 and A375 cells compared to controls (Fig. 3E). In order to establish if relative WFDC1 overexpression in ABCB5-KD cells might be partly responsible for WNT down-regulation, we next overexpressed WFDC1-myc in G3361 or A375 cells and remeasured expression of WNT pathway members by qPCR array (Fig. 3F). Similar to ABCB5 inhibition, WFDC1-myc overexpression resulted in significant inhibition of WNT1, WNT7B, WNT9A, WNT10A and WNT11 in WFDC1-myc cells compared to vector controls (Fig. 3F), indicating that ABCB5 regulates WNT signaling at least in part through inhibition of the WNT repressor WFDC1. Moreover, like ABCB5 inhibition, WFDC1-myc overexpression also significantly inhibited IL8 production compared to vector controls (Fig. 3F), identifying IL8 as a downstream target of inhibitory WFDC1 signaling. The role of intermediate WNT signaling in ABCB5(-) and WFDC1-regulated IL8 production in human melanoma was further confirmed utilizing drugs mimicking either canonical or non-canonical (calcium) WNT signaling (i.e. lithium chloride or ionomycin/thapsigargin, respectively), which resulted in significant enhancement of IL8 expression through either activation of the canonical signaling pathway (1.75-fold and 2.32-fold in treated G3361 and A375 melanoma cells vs. controls, respectively) or the non-canonical pathway (6.6-fold and 13.7-fold in treated A375 and G3361 cells, respectively) (Fig. 3G). Together, these data revealed a signaling pathway whereby ABCB5 leads to melanoma expression of IL8 through WNT signaling, in part through repression of WFDC1 (Fig. 3H).

We tested applicability of this novel signaling pathway further in a clinically relevant experimental model system of ABCB5 overexpression in DTIC-resistant melanoma cells, based on recent demonstrations that DTIC treatment induces ABCB5 *in vitro* and *in vivo* in tumor xenotransplantation models and human patients (8, 28). Following culture of human A375 melanoma cells in gradually increasing doses of DTIC over the course of several

weeks, A375/DTIC cells were resistant to 900 μ M DTIC (a concentration capable of killing A375/WT cells) and significantly overexpressed ABCB5 at both the mRNA level (18-fold) and the protein level (19.8-fold) (Fig. 3I). Moreover, ABCB5-overexpressing A375/DTIC cells displayed a significantly slower rate of growth compared to A375/WT cells (Fig. 3J), in line with the newly demonstrated role for ABCB5 in cellular quiescence (Fig. 2C). Consistent with the newly identified ABCB5-regulated signaling axis (Fig. 3H), WFDC1 levels were inhibited in ABCB5-overexpressing A375/DTIC melanoma cells compared to controls (Fig. 3K), IL8 levels were significantly enhanced (2.5-fold at the mRNA level, 2.6-fold at the level of secreted protein) (Fig. 3K), consistent with their reciprocal down-regulation in WFDC1-myc-overexpressing cells (Fig. 3F) and their known association with melanoma DTIC resistance (33, 34), and several WNT members, including WNT1, WNT7B, WNT9A, WNT10A and WNT11, were significantly induced (Fig. 3L). In aggregate, these results delineated a novel ABCB5-regulated signaling axis culminating in expression of the oncogenic mediator and DTIC resistance-conferring cytokine IL8, which provided mechanistic insight into the newly discovered functional role of ABCB5 in regulating melanoma growth and DTIC resistance.

ABCB5(+) MMIC express the IL8 receptor CXCR1, and IL8/CXCR1 signaling is required to maintain MMIC quiescence

Both receptors for the IL8 cytokine, i.e. the IL8-specific CXCR1 (IL8RA) and the more promiscuous CXCR2 (IL8RB), have been shown to be important to melanoma progression (37, 38). In breast cancer CXCR1 is also considered important for maintenance of the CSC (39). Since ABCB5 marks the MMIC subpopulation in melanoma and, as shown herein, regulates the CXCR1/2 ligand IL8, we examined expression of CXCR1/2 on MMIC. Co-staining and FACS analysis of G3361 melanoma cells for ABCB5 and CXCR1 or CXCR2 co-expression revealed that ABCB5(+) cells (7% of all analyzed cells) specifically co-expressed CXCR1 but not CXCR2, which was overall less abundantly expressed (Fig. 4A). Moreover, live-sorting of melanoma cells into ABCB5(+) and ABCB5(−) or CXCR1(+) and CXCR1(−) populations followed by CXCR1 mRNA determination by qPCR showed relative overexpression of CXCR1 in either ABCB5(+) cells (3.7-fold enhanced) or CXCR1(+) cells (4.3-fold enhanced) compared to their respective marker-negative controls, consistent with the findings at the protein level that ABCB5 and CXCR1 are preferentially co-expressed on the MMIC subpopulation (Fig. 4B). IL8 determinations performed by ELISA in supernatants of sorted ABCB5(+) and ABCB5(−) or CXCR1(+) and CXCR1(−) cells confirmed that IL8 was secreted by ABCB5(+) or CXCR1(+) melanoma subpopulations, and furthermore showed that ABCB5(−) or CXCR1(−) melanoma cells also secrete IL8, at enhanced levels (ABCB5(+) cells: 204ng/ml vs. ABCB5(−) cells: 1780ng/ml; CXCR1(+) cells: 399ng/ml vs. CXCR1(−) cells: 2143ng/ml), indicating the potential for either autocrine IL8 signaling by ABCB5(+)/CXCR1(+) MMIC, or paracrine IL8 signaling from melanoma bulk populations to the ABCB5(+) MMIC, to stimulate the CXCR1 receptor (Fig. 4C). Consistent with these results obtained in established G3361 melanoma cells, *in situ* analysis of clinical melanoma specimens also demonstrated exclusive co-expression of ABCB5 with CXCR1 (Fig. 4D, left panel), whereas IL8 expression was associated with either ABCB5(+) MMIC (Fig. 4D, right panel, black arrows) or, more often,

ABCB5(–) melanoma cells in spatial proximity to ABCB5(+) MMIC (Fig. 4D, right panel, red arrows).

Based on our demonstration that ABCB5(+) MMIC represent a slow-cycling melanoma subpopulation and the finding that shRNA-mediated ABCB5 inhibition inhibited IL8 levels and reduced *in vitro* melanoma cell doubling time, we hypothesized that the IL8 receptor CXCR1 expressed on MMIC functions to maintain the cellular quiescence of MMIC. Consistent with this hypothesis, specific mAb-mediated inhibition of CXCR1 on purified ABCB5(+) MMIC, cultured in the presence of BrdU DNA label in order to assess BrdU incorporation as a correlate of actively dividing cells, resulted in a 5-fold increase in BrdU^{high} cells (CXCR1 mAb treatment: 21.43% vs. isotype control mAb treatment: 4.17%), demonstrating that CXCR1 signaling in ABCB5(+) MMIC is required to maintain MMIC quiescence (Fig. 4E, left panels). We did not observe enhanced BrdU incorporation by ABCB5(–) cells derived from purified ABCB5(+) MMIC, in the same cultures, which were discernible by absence of co-staining for ABCB5 (we have previously demonstrated that ABCB5(+) melanoma cells have differentiation capacity and generate ABCB5(–) cells (1)) (Fig. 4E, right panels), a finding consistent with negativity of ABCB5(–) melanoma cells for CXCR1 (Fig. 4A&D). These results showed that ABCB5(+) MMIC selectively express the IL8 receptor CXCR1 and that ABCB5-regulated IL8 signaling through CXCR1 is functionally required to maintain MMIC quiescence.

ABCB5 controls secretion of IL-1 β , an activator of IL8

In view of the newly discovered role of ABCB5 in regulating IL8 production, including by ABCB5(–) melanoma bulk populations, we hypothesized that ABCB5 controls secretion of an extracellular signaling molecule that activates IL8. Based on the considerations that IL-1 β is a known secreted mediator of IL8 activation (40) and that IL-1 β might represent a substrate for ABC transporter-mediated cellular efflux (41), we investigated whether ABCB5 controls melanoma IL-1 β secretion. First, consistent with ABCB5 and IL8 overexpression in DTIC-resistant vs. wildtype melanoma cells, DTIC-resistant tumor cells exhibited higher (3.2-fold) levels of IL-1 β secretion compared to their wildtype counterparts (Fig. 5A). Furthermore, exogenous rIL-1 β treatment significantly induced IL8 and downregulated WFDC1 expression ($P < 0.001$, respectively) (Fig. 5B), consistent with the demonstrated effects of ABCB5 modulation. Moreover, we found toll-like receptor 4 (TLR4), a principal regulator of IL-1 β expression, to be selectively expressed by ABCB5(+) MMIC (Fig. 5C–E) and that ABCB5(+) MMIC preferentially express and secrete IL-1 β (Fig. 5E&F). In order to directly demonstrate a role of ABCB5 in IL-1 β secretion, we subjected purified ABCB5(+) MMIC (isolated using co-expressed CXCR1 as a surrogate selection marker) to mAb-mediated ABCB5 blockade or isotype control mAb treatment and assessed IL-1 β secretion (Fig. 5F). ABCB5 blockade abrogated IL-1 β release into culture supernatants by ABCB5(+) MMIC, demonstrating that ABCB5 controls IL-1 β secretion (Fig. 5F). Thus, our results reveal that ABCB5 controls MMIC maintenance and tumor growth through a cytokine-dependent signaling circuit. Specifically, our findings support a model in which ABCB5(+) MMIC-produced IL-1 β activates bulk tumor cell populations to produce pro-tumorigenic IL8, which, in a rheostat mechanism, subsequently induces MMIC maintenance via CXCR1 signaling (Fig. 5G).

Discussion

Our results provide initial evidence that ABCB5 represents not only a marker for MMIC in human melanomas as demonstrated previously (1, 2, 4, 16), but that it also serves as a functional driver of melanoma aggressiveness features through a common molecular role in MMIC maintenance, tumor growth and drug resistance. Mechanistically, our study reveals a novel ABCB5-regulated signaling pathway in human melanoma, whereby ABCB5 controls MMIC IL-1 β secretion, a function that serves to maintain this slow-cycling, chemoresistant melanoma subpopulation through an IL-1 β /IL8/CXCR1 cytokine signaling circuit involving reciprocal paracrine interactions with ABCB5-negative cancer bulk populations (Fig. 5G). Our results hereby show that ABCB5, through its role in IL-1 β secretion, functionally links three distinct features of human melanoma through this newly identified signaling pathway, i.e. (1) maintenance of the slow-cycling MMIC subpopulation via IL8 signaling through MMIC-expressed CXCR1; (2) MMIC-driven tumorigenic growth and maintenance of undifferentiated tumor phenotype associated with IL8 production; and (3) IL8-driven melanoma resistance to the clinical anti-melanoma agent DTIC.

Previously, we demonstrated the existence of a tumor hierarchy in human melanoma, in which ABCB5(+) cells, enriched for MMIC, self-renew and give rise to more-differentiated ABCB5(-) tumor progeny (1). Moreover, we showed that highly tumorigenic ABCB5(+) MMIC can drive more differentiated and otherwise non-tumorigenic ABCB5(-) melanoma bulk populations to contribute, albeit less efficiently, to a growing tumor mass (1). Our current study now reveals that ABCB5 represents a key molecular mechanism governing such CSC/tumor bulk interactions through an IL-1 β -dependent signaling circuit and that this mechanism also controls MMIC rheostasis under chemotherapeutic selection pressure. Our results hereby indicate that IL-1 β stimulates IL8 production predominantly by ABCB5(-) melanoma bulk populations, facilitating *paracrine* IL8-mediated activation of MMIC-expressed CXCR1 (Fig. 5G). However, IL-1 β also significantly induced IL8 production by purified MMIC, suggesting additional relevance of the identified ABCB5-driven cytokine circuit to *autocrine* IL8-mediated CXCR1 activation. Such autocrine signaling might be particularly relevant under conditions of relatively low frequencies or absence of ABCB5(-) tumor cells within a cancer, for example in early phases of metastasis formation by disseminated circulating ABCB5(+) tumor cells (9, 24), or following chemotherapeutic depletion of more proliferative, chemosensitive ABCB5(-) cancer cells with persistence of relatively quiescent, chemoresistant ABCB5(+) subpopulations (8).

Slow-cycling cancer cells have previously been associated with more aggressive tumorigenic phenotypes, and have been proposed to coincide with CSC in melanoma and other cancers. Our results provide evidence for this hypothesis, because ABCB5 has previously been shown to mark the MMIC (1, 2, 4, 5, 9, 15, 17). Furthermore, because both direct ABCB5 blockade or CXCR1-targeted inhibition of ABCB5-regulated IL8 signaling abrogated cellular quiescence, and because direct ABCB5 blockade induced melanoma differentiation and inhibited tumor growth *in vivo*, our results provide initial evidence that ABCB5 functionally controls slow-cycling MMIC maintenance and MMIC-driven tumor growth. These results are consistent with recent demonstrations that slow-cycling melanoma cells are required for melanoma maintenance and progression (16, 42). Additionally, the

newly identified role of ABCB5 as a functional negative regulator of melanoma differentiation is also in line with previous studies demonstrating that terminal melanoma differentiation is associated with ABCB5 down-regulation (43), and conversely, that Oct4-induced melanoma de-differentiation associated with enhanced tumorigenicity and chemoresistance is associated with ABCB5 up-regulation (3).

Our finding that ABCB5 functionally regulates IL8/CXCR1 signaling in human melanoma might be relevant to additional human malignancies in which ABCB5 is expressed and in which critical functions of IL8/CXCR1 signaling have been identified. For example, ABCB5 is expressed by doxorubicin-resistant human breast cancer cells and has been shown to be down-regulated following differentiation therapy in patients afflicted by breast cancer (44), a malignancy where IL8/CXCR1 signaling was originally shown to critically regulate CSC maintenance (39, 45). Similarly, ABCB5 is known to maintain CD133+ CSC phenotype and to mediate doxorubicin resistance in hepatocellular carcinoma (6), a further malignancy in which IL8/CXCR1 signaling has been implicated in CSC function (46). Additionally, ABCB5 is overexpressed and mediates 5-FU resistance in CD133+ CSC in human colorectal cancer (7), where IL8/CXCR1 signaling also regulates drug resistance and tumor aggressiveness features (47). Thus, our study raises the possibility that ABCB5 represents a broadly relevant mechanism of IL8/CXCR1 regulation in diverse human cancers, in addition to its role in human melanoma.

Our results place ABCB5 and ABCB5-dependent IL-1 β signaling downstream of MMIC-expressed TLR4, a receptor for members of the S100 protein family of endogenous inflammatory damage-associated molecular patterns (DAMPs) mediators with key roles in the initiation and amplification of cancer development and tumor spread (48). This finding, in light of the observation that ultraviolet (UV) radiation-induced melanoma carcinogenesis is associated with increased S100 expression (49), is consistent with the herein newly identified role of ABCB5 in melanomagenesis and raises, furthermore, the possibility that ABCB5 might also be functionally involved in additional instances of inflammation-driven carcinogenesis and TLR4-dependent immunoevasion (50).

In aggregate, the herein identified novel functional role of ABCB5 in controlling MMIC quiescence and maintenance, tumor growth and multidrug-resistance in human melanoma implicates ABCB5 for the first time as a key driver of several features of cancer aggressiveness. Moreover, this role provides a potential explanation for its broadly observed preferential expression by tumor-initiating and therapy-refractory cancer subpopulations in many human malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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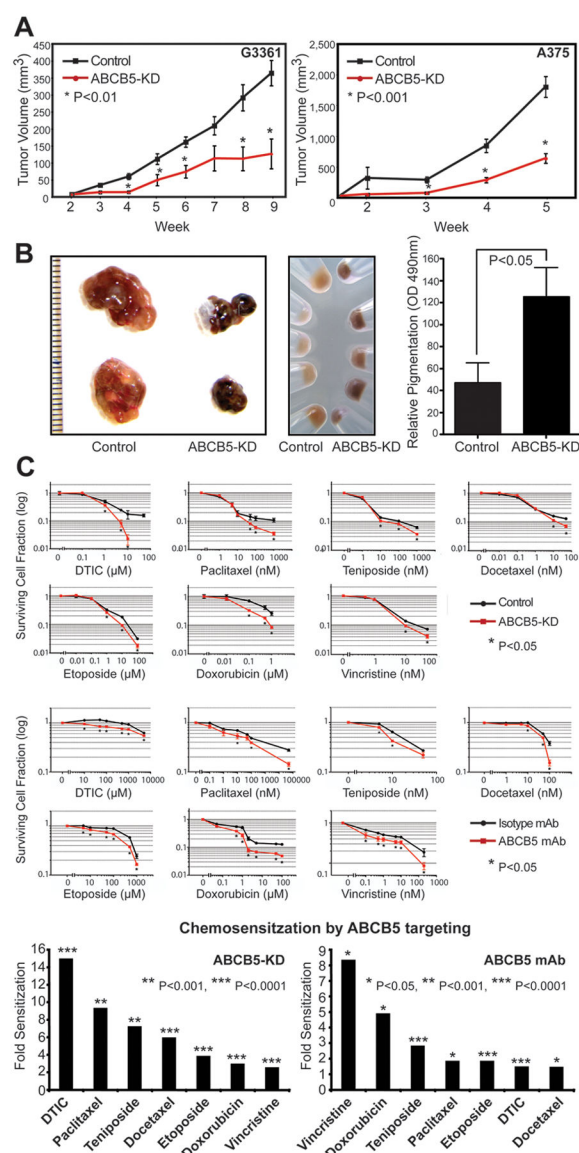


Figure 1. ABCB5 regulates melanoma growth and multidrug-resistance

Tumor growth kinetics of stable G3361 ABCB5-KD (red line) versus control (black line) xenografts (left), or A375 ABCB5-KD (red line) versus control (black line) xenografts (right). **B**, Macroscopic appearance of representative resected G3361 ABCB5-KD or control tumors (left), and of cell-pellets prepared from resected G3361 ABCB5-KD or control tumors (n=5 each) (middle). Quantitative relative pigmentation of G3361 ABCB5-KD or control tumors (n=5 each, right). **C**, Drug-dependent cell killing for G3361 ABCB5-KD cells (red) vs. G3361 control cells (black) (top), and for ABCB5 mAb-treated (red) vs. control mAb-treated (black) G3361 cells (middle). Illustrated are surviving cell fractions as a function of drug concentrations for n=6 replicate samples, respectively (*, $P < 0.05$). Bottom: Summaries of fold-sensitizations to drugs are shown for ABCB5 inhibition by shRNA targeting (left) or mAb blockade (right) (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).

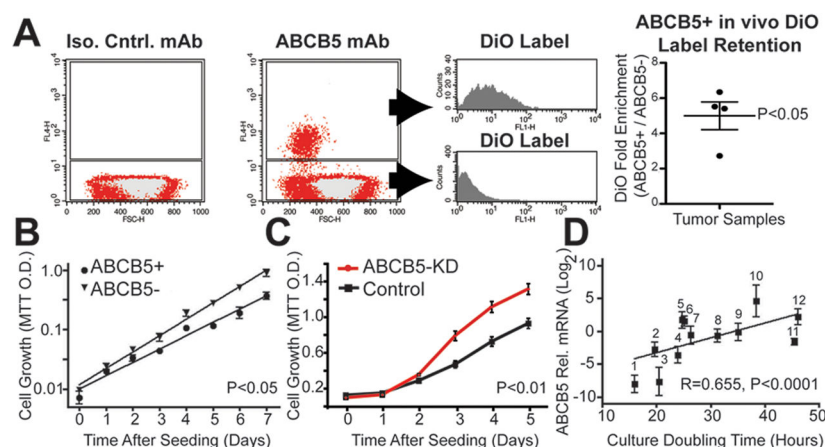


Figure 2. ABCB5 identifies and regulates slow-cycling melanoma cells

A, ABCB5 expression and DiO fluorescent membrane dye retention by melanoma cells derived from xenograft tumors established by DiO-labeled G3361 melanoma cells. A representative flow cytometry result is shown in the left three panels. Right: Aggregate analysis for DiO fold enrichment in ABCB5(+) vs. ABCB5(-) cells ($n=4$). **B**, Cell growth kinetics of purified ABCB5(+) vs. ABCB5(-) G3361 melanoma cell subsets. Optical density of MTT assay (mean \pm SD, $n = 5$ independent experiments) shown as a function of time after seeding. **C**, *In vitro* growth kinetics of G3361 ABCB5-KD (red line) versus control cells (black line) as determined by MTT assay. **D**, Pearson correlation of relative ABCB5 gene expression (mean \pm SD, $n = 3$ independent experiments) and doubling times of 8 NCI-60* and 4 additional melanoma cell lines (1, MUM-2B; 2, A375; 3, LOX IMVI*; 4, MUM-2C; 5, G3361; 6, SK-MEL-5*; 7, M14*; 8, UACC-62*; 9, SK-MEL-28*; 10, UACC-257*; 11, SK-MEL-2*; 12, MALME-3M*); r , Pearson correlation coefficient.

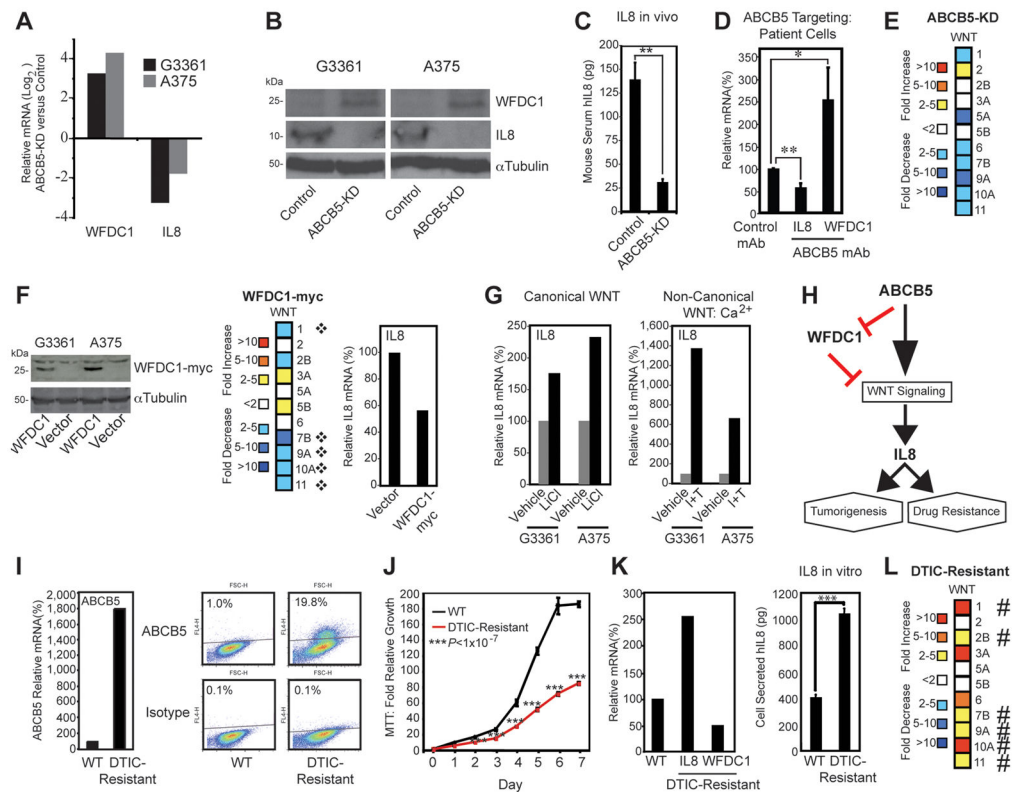


Figure 3. ABCB5 represses the tumor suppressor WFDC1 and induces the melanoma tumor promoter IL8 through the WNT pathway

A, Relative WFDC1 and IL8 mRNA expression by ABCB5-KD vs. control G3361 or A375 melanoma cells. **B**, Western blots for WFDC1 (~25kDa, indicated by arrow) and IL8 (~11kDa) protein expression by ABCB5-KD vs. control melanoma cells (Tubulin - loading control). **C**, Human IL8 in sera of murine recipients of ABCB5-KD vs. control G3361 melanoma xenografts determined by human-specific hIL8 ELISA (**, $P=0.0023$). **D**, WFDC1 and IL8 mRNA expression by ABCB5 mAb-treated vs. control mAb-treated melanoma cells derived from 3 clinical specimens or the G3361 and A375 cell-lines (n=5; *, $P<0.05$, **, $P=0.0052$). **E**, mRNA expression of WNT members by ABCB5-KD vs. control melanoma cells. **F**, G3361 and A375 stably transfected cells expressing pCMV6-WFDC1-myc, or pCMV6 vector (left), mRNA expression of WNT members by WFDC1-myc vs. control G3361 or A375 melanoma cells (♦, common regulated genes in WFDC1-myc and ABCB5-KD cells) (middle), and of IL8 (right). **G**, IL8 mRNA expression by melanoma cells treated with LiCl or ethanol vehicle for 4h or ionomycin (I) and thapsigargin (T) or ethanol vehicle control for 16h. **H**, Model pathway arising from results. **I**, ABCB5 expression by wildtype vs. DTIC-resistant A375 melanoma cells at the mRNA (left) and the protein level (right). **J**, Cell growth kinetics of DTIC-resistant (red) vs. wildtype (black) A375 melanoma cells cultured in the absence of DTIC. Fold relative growth (n=6 replicates) is shown as a function of time after seeding. **K**, WFDC1 and IL8 mRNA expression (left) and IL8 secretion (ELISA, right) by DTIC-resistant vs. wildtype A375 cells (***, $P<1 \times 10^{-5}$). **L**, mRNA expression of WNT members by DTIC-resistant vs. wildtype A375

cells (#, WNT gene level changes in DTIC-resistant cells reciprocal to those determined WFDC1-myc cells as shown in panel f).

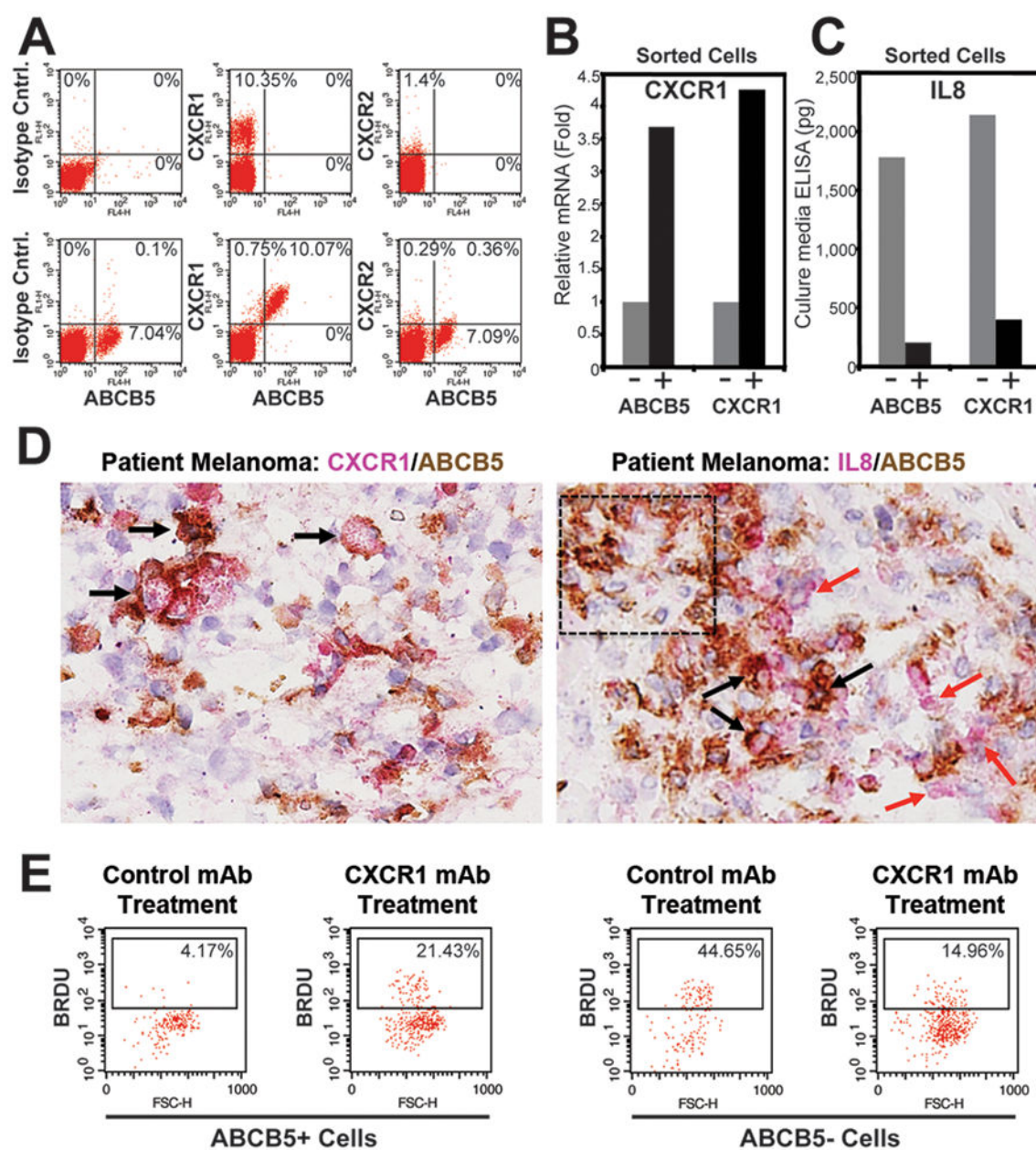


Figure 4. ABCB5(+) MMIC express the IL8 receptor CXCR1, and IL8-CXCR1 signaling is required to maintain MMIC quiescence

A, CXCR1 or CXCR2 expression (top) and CXCR1/ABCB5 or CXCR2/ABCB5 co-expression (bottom) on G3361 cells. **B**, CXCR1 mRNA expression and **C**, IL8 secretion (ELISA, right) by ABCB5(+) or CXCR1(+) vs. ABCB5(-) or CXCR1(-) G3361 cells. **D**, Left: Immunohistochemical staining of clinical melanoma for ABCB5 (HRP, brown) and CXCR1 (AP, red), showing the majority of cells to exhibit co-localization of variable admixture of both chromagens (arrows represent cells showing prominent red and brown staining). Right: Immunohistochemical staining of clinical melanoma for ABCB5 (HRP, brown) and IL8 (AP, red), showing greater staining heterogeneity consisting of exclusively

ABCB5-positive cells (brown, enclosed within broken square), exclusively Il-8-positive cells (red arrows), and scattered cells showing brown and red co-localization (black arrows). **E**, BrdU incorporation in the presence of CXCR1-inhibitory or control mAb by purified ABCB5(+) melanoma cells (left), or by ABCB5(–) cells derived from ABCB5(+) cells through differentiation (right).

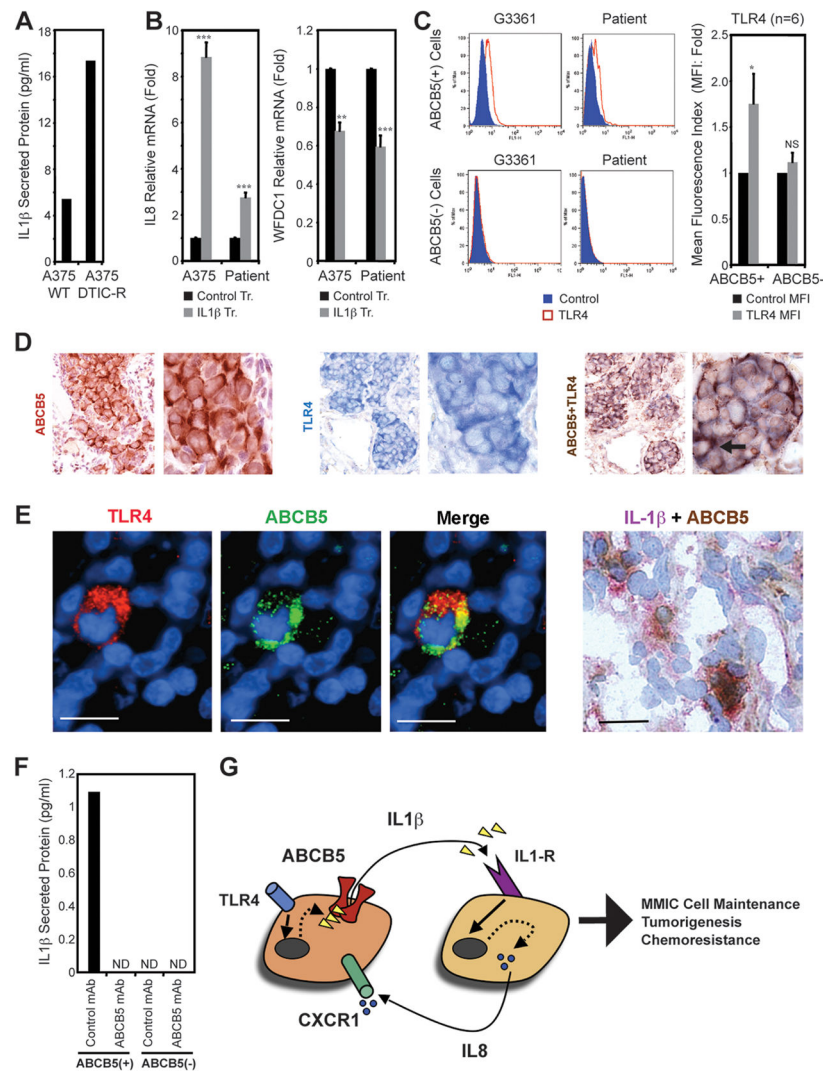


Figure 5. ABCB5 controls secretion of IL-1 β , an activator of IL8, to stimulate IL8
A, IL-1 β secretion by wildtype or DTIC-resistant A375 cells (ELISA). **B**, IL8 (left) or WFDC1 (right) mRNA expression by A375 WT or patient-derived melanoma cells following exogenous IL-1 β treatment vs. control (means of n=3 independent experiments with 3 replicates each per datapoint; ** P <0.005, *** P <0.0005). **C**, TLR4 expression on ABCB5(+) cells (top) and ABCB5(-) cells (bottom) in G3361 (left) and patient-derived melanoma cells (middle). Aggregate analysis for n=6 distinct melanoma specimens (G3361, A375, and SK-MEL-28 cell-lines, 3 patient-derived specimens) is shown on the right (* P <0.05, NS=not significant). **D**, Immunohistochemistry staining for ABCB5 (left, brown), TLR4 (middle, blue) and co-staining for ABCB5 and TLR4 (right, purple shows costaining) of a human C8161 melanoma xenograft lung metastasis (left 400x, right 1000x magnification). **E**, Left: Immunofluorescence co-staining of clinical melanoma for TLR4 (red) and ABCB5 (green). TLR4 and ABCB5 are seen in single channels and in a merged image (first three panels), with staining localized to a single cell among a majority of negative cells (nuclei blue [DAPI], paraffin section). Right: Immunohistochemical dual

labeling for IL-1 β (red) and ABCB5 (brown) of clinical melanoma. Note several cells among negative populations that reveal co-expression of both epitopes (bars 25 microns). **F**, IL-1 β secretion (ELISA) by ABCB5(+) or ABCB5(–) G3361 cells in the presence or absence of ABCB5 blockade; ND – not detectable. **G**, Functional model consistent with the overall datasets of this study.