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Drug Resistance Is Conferred on the Model Yeast *Saccharomyces cerevisiae* by Expression of Full-Length Melanoma-Associated Human ATP-Binding Cassette Transporter ABCB5

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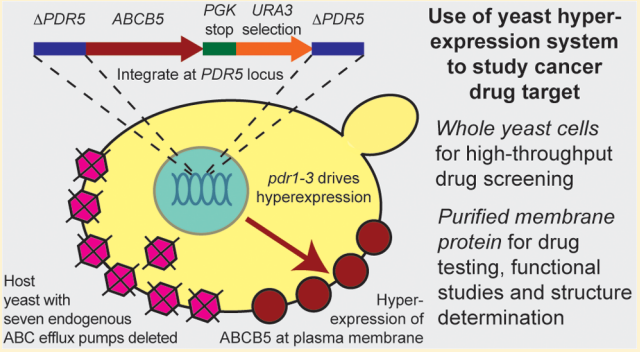
Supporting Information

ABSTRACT: ABCB5, an ATP-binding cassette (ABC) transporter, is highly expressed in melanoma cells, and may contribute to the extreme resistance of melanomas to chemotherapy by efflux of anti-cancer drugs. Our goal was to determine whether we could functionally express human ABCB5 in the model yeast *Saccharomyces cerevisiae*, in order to demonstrate an efflux function for ABCB5 in the absence of background pump activity from other human transporters. Heterologous expression would also facilitate drug discovery for this important target. DNAs encoding ABCB5 sequences were cloned into the chromosomal *PDR5* locus of a *S. cerevisiae* strain in which seven endogenous ABC transporters have been deleted. Protein expression in the yeast cells was monitored by immunodetection using both a specific anti-ABCB5 antibody and a cross-reactive anti-ABCB1 antibody. ABCB5 function in recombinant yeast cells was measured by determining whether the cells possessed increased resistance to known pump substrates, compared to the host yeast strain, in assays of yeast growth. Three ABCB5 constructs were made in yeast. One was derived from the ABCB5-β mRNA, which is highly expressed in human tissues but is a truncation of a canonical full-size ABC transporter. Two constructs contained full-length ABCB5 sequences: either a native sequence from cDNA or a synthetic sequence codon-harmonized for *S. cerevisiae*. Expression of all three constructs in yeast was confirmed by immunodetection. Expression of the codon-harmonized full-length ABCB5 DNA conferred increased resistance, relative to the host yeast strain, to the putative substrates rhodamine 123, daunorubicin, tetramethylrhodamine, FK506, or clorgyline. We conclude that full-length ABCB5 can be functionally expressed in *S. cerevisiae* and confers drug resistance.



KEYWORDS: *human ABC transporters, ABCB5, P-glycoprotein, ABCB1, drug resistance, melanoma*

INTRODUCTION

ATP-binding cassette (ABC) proteins constitute a superfamily of membrane transporters found in all kingdoms of life.[1] Eukaryotic ABC proteins usually consist of two homologous halves. Each half contains a nucleotide binding domain and a transmembrane domain with six transmembrane spans. ABC proteins are expressed in many human tissues and are responsible for the transport of substrates such as lipids and drugs across membranes. They are also implicated in many debilitating diseases, the resistance of multiple cancers to chemotherapy and the tissue distribution of many drugs.[2] Several ABC proteins, such as ABCB1 (P-glycoprotein, P-gp), ACBG2, and ABCC1, contribute to the resistance of tumors to chemotherapeutic agents by pumping anti-cancer drugs out of tumor cells.[3−6] Some are expressed in many different types of cancer cells, but some, such as ABCB5, are selectively expressed.[7] Expression of ABCB5, which has high (73%) homology to ABCB1, has been detected in melanoma-derived cells, certain other cancers and in melanotic cells.[2,7−14] Heterologous expression of ABCB5 in the absence of confounding transporters would confirm the role of this protein as a functional transporter that could contribute to

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the inherent chemotherapy resistance of melanoma, and would also facilitate drug discovery for this important target.

ABCB5 is thought to be located in the plasma membrane,[11] and it has been proposed that its expression in melanoma cells[10,11,15] defines a sub-population of malignant melanoma-initiating cells.[15−18] ABCB5 is considered to contribute to the resistance of malignant melanomas to chemotherapeutics,[8,10] and specific silencing of ABCB5 by small interfering RNA increased sensitivity to several drugs in melanoma cells[19] and cells transfected with full-length ABCB5,[12] implicating ABCB5 as a chemoresistance factor. ABCB5 expression is also elevated in therapy-resistant tumor cells in colorectal cancer[20] and in several hematological malignancies.[21] However, ABCB5 is unusual in that the major transcripts expressed in melanomas and other cancers (designated ABCB5-α and ABCB5-β isoforms[9]) do not contain the complete domains required for either a full transporter or a typical half-transporter[22,23] and the 5′-untranslated region of the ABCB5-β mRNA does not contain an in-frame termination codon. Thus, it is difficult to assign transporter function to the unconventional structures of the ABCB5-α and ABCB5-β isoforms, although it has been suggested that the β form of ABCB5 may form a dimer to create a functional transporter.[23] A full-length transporter is, however, encoded by exons at the chromosomal locus of the β and α isoforms and the cDNA has been shown to confer resistance to multiple anticancer drugs when expressed in human cell lines.[24] Furthermore, a recent bioinformatic study of ABCB5 suggested that it has evolved as a functional transporter.[23]

To investigate a drug efflux function for ABCB5 we have cloned and expressed three ABCB5 ORFs heterologously in the model yeast *Saccharomyces cerevisiae* where any background contribution from other human proteins will be absent. We examined whether expression of ABCB5 conferred resistance to known substrates of the related human ABC transporter, ABCB1. Substrate compounds were selected which also inhibited yeast growth, so that resistance could be readily assessed. An important feature of the host *S. cerevisiae* strain[25,26] is that it is deleted in seven ABC transporters (*YOR1*, *SNQ2*, *PDR10*, *PDR11*, *YCF1*, *PDR5*, and *PDR15*). This results in a high sensitivity to a range (>18) of ABC transporter substrates.[27] Thus, cloned pump activity can be assayed in a background of depleted endogenous pumps. The gene encoding the transporter of interest is integrated at the *S. cerevisiae PDR5* genomic locus downstream of a promoter under the control of a mutant transcriptional regulator, Pdr1-3p, producing stable constitutive high-level expression of functional heterologous proteins in recombinant strains.[27] We have used this system to clone the ABCB5-β cDNA and a full-length cDNA.[24] In addition, because effective heterologous expression of human proteins often fails due to factors such as codon bias[28,29] we also cloned a synthetic DNA sequence that was codon-harmonized for expression in yeast.

EXPERIMENTAL SECTION

Strains and Media. *S. cerevisiae* strains used in this study are listed in Table 1 and were derived from AD1-8u$^-$.[25,26] Yeast strains were grown in 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose (YPD) medium (Difco Laboratories, Detroit, MI). Yeast transformants were selected on plates containing 0.077% (w/v) complete supplement mixture without uracil (CSM−URA) (Bio 101, Vista, CA), 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose. For assays of growth inhibition, yeast were grown in media containing complete supplement mixture (CSM) adjusted to pH 7.0 as described previously.[27] Where required for solid media, 2% (wt/vol) agar or 0.6% (wt/vol) agarose (Gibco: Invitrogen Corporation, Auckland, New Zealand) was included. Cultures of all strains reached the same maximum cell density (as determined by measuring OD_{600} of appropriate culture dilutions in a spectrophotometer) in the stationary phase of growth, and the parental and recombinant strains had equivalent growth rates.

Table 1. *S. cerevisiae* Strains Used in This Study

strain	genotype or description	source
ADΔ	MATα, pdr1-3, Δ*URA3*, yor1Δ::hisG, snq2Δ::hisG, pdr10Δ::hisG, pdr11Δ::hisG, ycf1Δ::hisG, pdr3Δ::hisG, pdr15Δ::hisG, pdr5Δ::hisG	ref 27
AD/ABCB1	AD1-8u$^-$, Δ*PDR5*::pABC3-HsABCB1	ref 27
AD/pABC3	ADΔ, Δ*PDR5*::pABC3	ref 27
AD/ABCB5-β	ADΔ, Δ*PDR5*::pABC3-HsABCB5β	this study
AD/ABCB5-f	ADΔ, Δ*PDR5*::pABC3-HsABCB5full	this study
AD/ABCB5-ch[a]	ADΔ, Δ*PDR5*::pABC3-HsABCB5ch	this study

[a]ch = codon-harmonized.

Materials and Compounds. Molecular biology reagents and restriction and modifying enzymes were from New England Biolabs (Beverly, MA) or from Roche Diagnostics NZ Ltd. (Auckland, New Zealand). High-performance liquid chromatography-purified DNA oligonucleotides were purchased from Hermann GbR Synthetische Biomolekule (Denzlingen, Germany). PCR and DNA fragments were purified using kits from Qiagen Pty. Ltd. (Clifton Hill, Victoria, Australia). Genomic DNA (gDNA) was isolated from yeast using the Y-DER yeast DNA extraction reagent kit from Pierce (Rockford, IL). PCRs used the high-fidelity KOD+ DNA polymerase (Toyobo, Osaka, Japan, or Novagen, San Diego, CA). Yeast were transformed using the alkali cation yeast transformation kit from Bio 101 (Vista, CA) modified as described previously.[27] Rhodamine-6G (R6G), rhodamine 123 (R123), tetramethylrhodamine (TMR), and daunorubicin (DAU) were purchased from Sigma-Aldrich Ltd. (Auckland, New Zealand).

Construction of Yeast Strains Overexpressing ABCB5 Proteins. ABCB5-β cDNA (GenBank BC104920) was purchased from Thermo Scientific Open Biosystems (Huntsville, AL) and was provided in vector pCR4-TOPO. Full-length ABCB5 DNA (GenBank AB353947) was generated from plasmid pcDNA3.1/ABCB5FL-V5-6His-TOPO.[24] Codon-harmonized ABCB5 synthetic DNA, giving the same translated sequence as AB353947, was purchased from DNA2.0 (Menlo Park, CA) and was provided in vector pJ246. This ABCB5 sequence has been submitted to GenBank (accession no. KC952952). All three ABCB5 ORFs were cloned using recombinant PCR into the *PDR5* locus of the host *S. cerevisiae* strain ADΔ upstream of a *URA3* selection marker gene, as illustrated in Figure 1. Overlapping DNA fragments were generated from the ORF-containing plasmids and from a cloning cassette based on the plasmid pABC3,[27] which enabled directional insertion into the *PDR5* locus of *S. cerevisiae* ADΔ. Primers used to amplify the DNA fragments are given in Table 2. Primers used to generate overlapping fragments of the β and full-length (non-codon-harmonized) ABCB5 sequences were designed such that the codon CGG encoding amino acid arginine, which has a very low usage frequency (1.7%) in yeast,

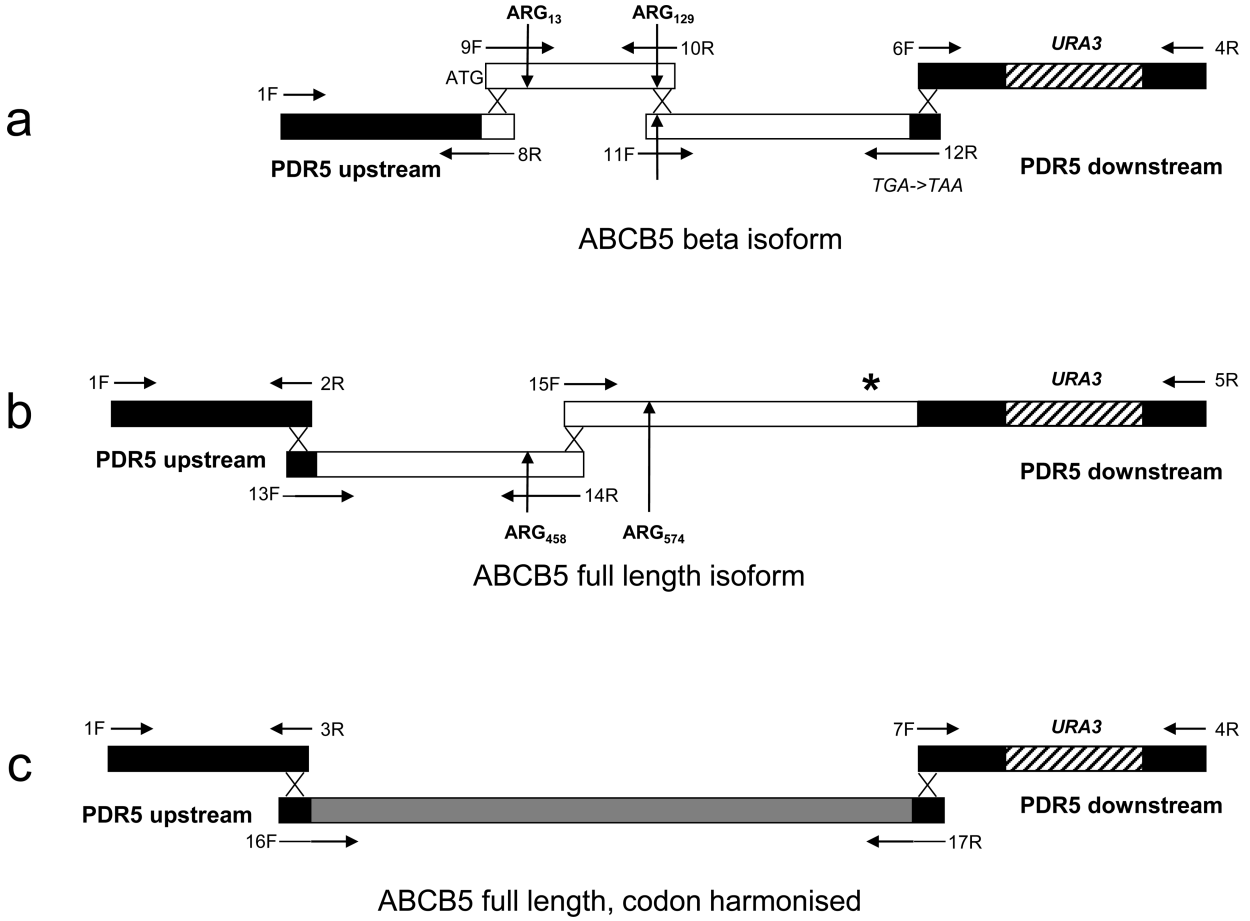

Figure 1. Generation of DNA fragments containing ABCB5 ORFs required for transformation of *S. cerevisiae*. PCR fragments were generated with primers (horizontal arrows) listed in Table 2 using pABC3 and plasmids containing ABCB5 as templates. Black bars include DNA homologous to a cassette containing *S. cerevisiae PDR5* and the terminator sequence from *PGK1* that directs integration at the *PDR5* locus.[27] Open bar is ABCB5-native cDNA; gray bar is codon-harmonized ABCB5 ORF. *URA3* is a marker for the selection of yeast transformants. PCR fragments overlapped by approximately 25 nucleotides, which allowed hybridization during recombinant PCR. (a) PCR fragments required to clone ABCB5-β isoform. Vertical arrows indicate positions of CGG arginine codons that were changed to CGT arginine codons. The TGA stop codon was changed to TAA. (b) PCR fragments required to clone ABCB5 full-length isoform. PCR fragment * was amplified from a template generated in (a). (c) PCR fragments required to clone full-length codon-harmonized isoform.

was replaced with the more frequently used CGT codon (6.4%). The CGG codons were at positions 13 and 129 in the ABCB5-β construct, and positions 458 and 574 in the full-length construct. The codons for arginine residues 458 and 574 were also changed in the codon-harmonized sequence provided by DNA2.0, but the replacement codon was AGA, which has a frequency in yeast of 21.3%. In addition, for all constructs, the stop codon TGA was replaced with TAA which is preferred in *S. cerevisiae*. Following generation of the overlapping fragments of the ORF and cassette upstream and downstream sequences, the complete cloning cassettes were assembled by a further round of PCR, before transformation of the host yeast strain, with selection of transformants on CSM-URA medium. The integrated cassettes in all recombinant yeast clones used were fully sequenced to confirm fidelity of PCR amplification and integration. Sequencing primers are given in Table 2.

Yeast Cell Extract Preparation and Cell Membrane Isolation. Total cell protein extracts (TE) were prepared by alkaline lysis and trichloroacetic acid precipitation as described previously.[30] For SDS-PAGE and immunoblot analysis, the precipitate was resuspended in a 6× sample buffer which contained 35 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 10% (w/v) SDS, 0.4 g/L bromophenol blue, and 2% DTT.

Two preparations of yeast membranes, comprising either a crude membrane fraction (CM), which included mitochondrial membranes, or a partially purified membrane fraction enriched in plasma membranes (PM), were separated from other cellular material as described previously.[31] Briefly, cells were grown in YPD liquid medium at 30 °C to a cell density of $OD_{600} \approx 6.0$ before harvesting by centrifugation. Cells were homogenized in 20% (w/v) glycerol, 0.5 mM EDTA, and 50 mM Tris pH 7.5 containing 1 mM PMSF using a Bead-Beater (BioSpec Products, Inc., Bartlesville, OK). Crude membranes were pelleted by differential centrifugation and an enriched plasma membrane preparation obtained after an acid precipitation step.[32] All extracts (TE, CM, and PM) were analyzed by SDS-PAGE separation, Western blotting, and immunodetection as described below.

SDS-PAGE, Western Blotting, and Immunodetection. SDS-PAGE was performed according to the Laemmli method[33] using 8% (wt/vol) acrylamide separating gels. Following determination of protein content by a micro-Bradford assay (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as the standard, samples (15 μg) were mixed with 6× SDS-PAGE sample buffer. Separated polypeptides were visualized by using Coomassie blue R250 or electroblotted (100 V, 1.5 h, 4 °C)

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Table 2. Primers Used in This Study

primer	sequence (5′−3′)
	Primers for Amplification of DNA Fragments for Recombinant PCR
	pABC Cassette Primers
1F	GAACATGAACGTTCCTCAGCGCG
2R	CATTTTTGTCTAAAGTCTTTCGAACGAGCGGAT
3R	CATTTTTTAATTAAGTCTTTCGAAC
4R	ACTAGACTTGGCGCGCCTACCGTTC
5R	CCGTAAGGCACAGTTAAGAAATAATG
6F	TAAATTCGGCCGCTTCTTTGGAATTATTG
7F	TTCTTTGGAATTATTGGAAGG
	β Form Construct (AD/ABCB5-β) Primers
8R	GATGTCATTCTCATCCACCATTTTTGTCTAAAGTCTTTCGAACG
9F	ATGGTGGATGAGAATGACATCAGAGCTTTAAATGTGCGTCAT
10R	CCACGATTGTAGTACGACCTTTGCTCGC
11F	GCGAGCAAAGGTCGTACTACAATCGTGG
12R	CCAAAGAAGCGGCCGAATTTACTGCACTGACTGTGCATTCACTAAC
	Full-Length Construct (AD/ABCB5-f) Primers
13F	CGTTCGAAAGACTTTAGACAAAAATGGAAAATTCAGAAAGAGCTGAAG
14R	GCTCTTGACTAACCACTCCAATATGGTCTCGATAATGACGCACATTTAAAGCTCTGATGTC
15F	ATTGGAGTGGTTAGTCAAGAGC
	Full-Length Codon-Harmonized Construct (AD/ABCB5-ch) Primers
16F	CTCGTTCGAAAGACTTAATTAAAAAATG
17R	CAATTCCTTACCTTCCAATAATTCCAAAGAAGC
	Sequencing Primers
	Common to All Constructs
18F	GAGCATAAAACAGAGAGGCGATATAGG
19F	TTGGCAACTAGGAACTTTCG
20R	TCGGATAAGAAAGCAACACCTGG
21R	TATGAGAAGACGGTTCGCCATTCGGACAG
	For β or Full-Length Clones
22R	TCCCTTGCTGCTCTCTCCATCTC
23F	ATTGGAGTGGTTAGTCAAGAGC
24F	GCGAGCAAAGGTCGTACTACAATCGTGG
25F	TATGGATGGGAGATGACATTC
26R	TGCTTAAGTTCTTGCTTATCTTTG
	For Codon-Harmonized Clones
27F	CTAACATCAAAGGAGCTGTCTGCC
28F	TACTGATGAGGAGATGGAACGTGC
29F	TTCGTCATTCTAGGAGTGATCTGT
30F	TGCTCACTTATTCGCACTATTAGAG

onto nitrocellulose membranes (Hybond-ECL; GE Healthcare, Auckland, NZ). Immunodetection of recombinant proteins was performed as described previously.[30] Anti-human ABCB5 antibodies, anti-human ABCB1 antibodies, and anti-*S. cerevisiae* Adh1p antibodies, were obtained from Abcam (Cambridge, UK). An anti-ABCB5 antibody (Abcam catalog no. ab80108) was a polyclonal antibody, produced in rabbits inoculated with a KLH-conjugated synthetic peptide selected from the N terminal region of human ABCB5-β. Although it reacted with the recombinant β protein, it did not react with the full-length ABCB5 protein (Figure S1). However, another Abcam antibody, a goat polyclonal to ABCB5 (Abcam catalog no. ab77549) reacted with both the β protein and the full-length ABCB5. The immunogen was a synthetic peptide: C-QTQHRNTSKKAQ, corresponding to amino acids 460−471 of human ABCB5-β (NP_848654.3). This antibody did not cross-react with ABCB1. The anti-ABCB1 antibody was a mouse monoclonal [C219] (catalog no. ab3364) that recognizes peptides VQEALD and VQAALD in ABCB1. This antibody was cross-reactive with both the β and full-length ABCB5 (Figure S1), presumably recognizing the similar sequences (VQHALD and VQAALE) present in equivalent positions in the ABCB5 sequence. Immunoreactivity in all blots was detected by enhanced chemiluminescence[34] and images were developed on ECL Hyperfilm (GE Healthcare). In some cases, in order to probe with another antibody, developed immunoblots were treated with "stripping" buffer containing 62.5 mM Tris (pH 6.7), 2% SDS, and 100 mM 2-

mercaptothanol at 50 °C for 30 min. After being rinsed in phosphate-buffered saline, blots were subjected to another round of immunodetection as described above.

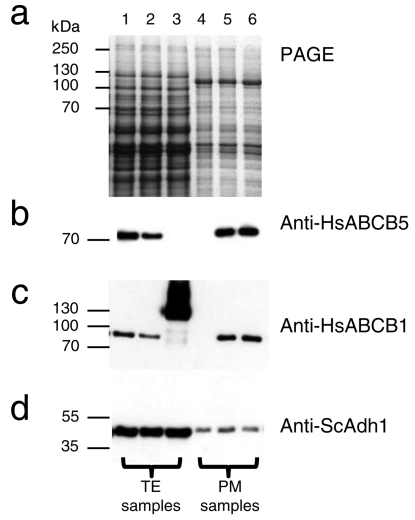
Drug Susceptibility Assays. *Liquid Microtiter Plate Dilution Determination of Minimum Growth Inhibitory Concentrations (MICs).* The MICs of compounds for the inhibition of yeast growth were determined in accordance with the CLSI microdilution reference method (CLSI guidelines document M27-A3 except that the method was modified by using a CSM-based medium[26] because *S. cerevisiae* AD1-8u$^-$, and its derivative strains, do not grow in the RPMI medium used in the CLSI method). Briefly, 200 μL of CSM pH 7.0 in microtiter plate wells was inoculated with 4×10^3 yeast cells per well and incubated at 30 °C for 48 h with shaking (150 rpm) in the presence of a series of two-fold dilutions of a compound. Cell growth was measured at 600 nm using a Synergy 2 microplate reader (Biotek, Winooski, VT).

Agar Plate Drug Resistance Assays. Serial two-fold dilutions of exponential phase yeast cells (initial cell concentration 1.0×10^6 cells/mL) were prepared in YPD. A 5 μL portion of each dilution was spotted onto YPD agar medium containing the indicated concentration of a test compound or a control plate with no added compound. Growth of yeast colonies within the spot area was monitored after incubation at 30 °C for 48 h.

Agarose Diffusion Drug Resistance Assays. Test compounds were examined by agar disk diffusion for their ability to inhibit growth of yeast strains. Sterile plastic lidded rectangular plates containing CSM (pH 7.0) solidified with 0.6% agarose (20 mL) were seeded with 1×10^6 mid-log phase cells suspended in 5 mL of melted (50 °C) top-agarose medium (CSM plus 0.6% (w/v) agarose). After the plates were seeded, filter disks containing the test compounds were placed onto the solidified top-agarose and the plates were incubated for 24 h at 30 °C or until clear growth inhibition zones were visible. Values presented are means of at least three independent measurements ± standard error (SE). Differences between values with a $p \leq 0.05$, as assessed by standard two-tailed Student's t test, were considered significant.

RESULTS

ABCB5 Protein Encoded by the β cDNA Isoform Was Expressed in Yeast Cells and Detected in Cell Extracts by Immunodetection but Did Not Confer Resistance to Pump Substrates. Expression of ABCB5 was detected in both total cell protein extracts (TE) and partially purified PM preparations of two AD/ABCB5-β clones by immunodetection using a specific anti-ABCB5-β antibody (Figure 2b, lanes 1, 2, 5, and 6). The ABCB5 protein was approximately the size predicted for the β isoform (89 kDa). When the same blot was stripped and re-probed with an antibody to ABCB1, as well as detecting ABCB1 (Figure 2c, lane 3) there was some cross-reactivity of this antibody with proteins from the ABCB5-expressing strains (Figure 2c, lanes 1, 2, 5, and 6). In contrast, the anti-ABCB5 antibody did not cross-react with ABCB1 (Figure 2b, lane 3). Neither antibody reacted with preparations from the control empty cassette strain AD/pABC3 (Figure 2b,c, lane 4) or with a strain (AD/CaCDR1) expressing the *Candida albicans* ABC transporter Cdr1p (not shown) and the blots showed no evidence of degradation of ABCB1 or ABCB5. The blot was stripped again and re-probed with an antibody to the control yeast cytoplasmic "housekeeping" protein Adh1p (alcohol dehydrogenase; Figure 2d). As expected, this protein



e Susceptibility to drugs (MIC; μg/ml)

Strain	R6G	ITC	R123	DAU
AD/pABC3	0.25	0.03	10.0	1.0
AD/ABCB5-beta	0.25	0.03	10.0	1.0
AD/ABCB1	1.0	0.25	30.0	8.0

Figure 2. Expression of ABCB5-β in *S. cerevisiae*. (a) SDS-PAGE analysis of total cell extract samples (TE; lanes 1−3) and plasma membrane-enriched fractions (PM; lanes 4−6) from lanes 1 and 5, AD/pABCB5-β clone 1; lanes 2 and 6, AD/pABCB5-β clone 2; lane 3, AD/ABCB1; lane 4, AD/pABC3. (b−d) Western blots of gel shown in (a) incubated with anti-HsABCB5 antibodies (ab80108) (b), anti-HsABCB1 antibodies (ab3364) (c), and anti-ScAdh1p antibodies (ab34680) (d). (e) Susceptibility of yeast strains to potential pump substrates: R6G, rhodamine-6G; ITC, itraconazole; R123, rhodamine 123; DAU, daunorubicin.

was detected in all samples (lanes 1−6) and the intensity of the signal was equivalent for lanes 1−3 and for lanes 4−6, indicating equal loading of proteins from the different strains, including the control strains. Adh1p was detected in greater amounts in the TE preparations (lanes 1−3) than in PM-enriched preparations (lanes 4−6), as expected for a cytoplasmic protein, whereas ABCB5-β was present in similar amounts in both TE and PM preparations (Figure 2b,c).

Despite the detection of the ABCB5-β protein in total cell extracts and partially purified PM preparations from yeast strains, the expression of ABCB5-β in *S. cerevisiae* did not confer resistance to putative pump substrates itraconazole, R6G, R123, or DAU (Figure 2e). In contrast, the strain expressing human ABCB1 showed a 3−8-fold increase in resistance to these compounds compared to a control strain (AD/pABC3) containing an empty cloning cassette.

Expression of Full-Length ABCB5 cDNA (Non-Codon-Harmonized) in *S. cerevisiae* Conferred a Limited Resistance to R123 but Not to Other Potential Pump Substrates. Expression of the full-length ABCB5 cDNA was detected by immunodetection of extracts of total cell protein (TE) using the cross reactive anti-ABCB1 antibody (Figure 3a, lane 1), although the reactivity was less than that for the extract from the β recombinant (Figure 3a, lane 2). Both extracts showed considerably less reactivity than the ABCB1 recombinant extract (Figure 3a, lane 3). The ABCB5 protein was

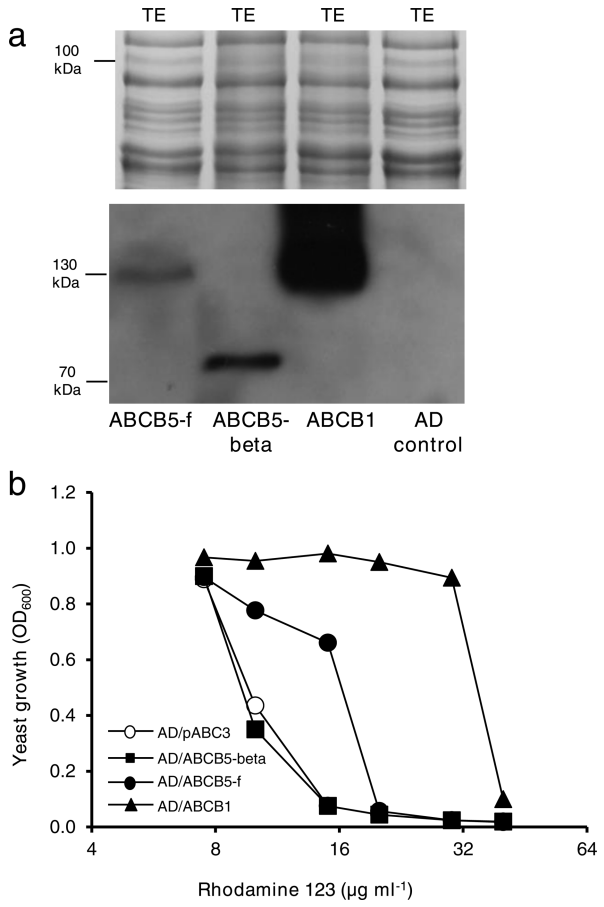


Figure 3. Expression of full-length ABCB5 (ABCB5-f) in *S. cerevisiae*. (a) PAGE and immunodetection with anti-HsABCB1 antibody (ab3364) of ABCB5-β (lane 2), ABCB5-f (lane 1), or ABCB1 (lane 3) in alkaline extracts of total cell protein (TE) from strains AD/ABCB5-β, AD/ABCB5-f or AD/ABCB1, respectively. Lane 4 shows that the antibody did not react with extracts containing equivalent total protein from the host strain AD/pABC3. (b) Susceptibility of the growth of *S. cerevisiae* strains AD/pABC3, AD/ABCB5-β, AD/ABCB5-f, and AD/ABCB1 in liquid culture to increasing concentrations of rhodamine 123. Values shown are the means of triplicate determinations and SEs did not exceed 10%.

approximately the size predicted for the full-length protein (130 kDa).

The AD/ABCB5-f strain showed a slight, but reproducible, increase in resistance to R123 (Figure 3b) but not to DAU (not shown) compared to the host strain whereas the AD/ABCB5-β strain possessed the same MICs as the control AD/pABC3 strain to these substrates (Figures 2e and 3b).

Expression of Codon-Harmonized Full-Length ABCB5 in *S. cerevisiae* Conferred Resistance to Potential Substrates R123, DAU, TMR, FK506, and Clorgyline. A codon-harmonized ABCB5 sequence was transformed into the ADΔ host strain to generate the strain AD/ABCB5-ch. CM and PM preparations from AD/ABCB5-ch reacted more strongly with the anti-ABCB5 antibody than those from the AD/ABCB5-f strain (Figure 4a). The immunoblots in Figure 4a were all from a blot of the same PAGE gel on which all lanes had equivalent loadings of protein. The increased ABCB5 protein expression level in AD/ABCB5-ch was reflected in an increased resistance of the strain, relative to the AD/ABCB5-f strain, to R123, DAU, TMR, FK506 or clorgyline (Figures 4b,c and 5a). Resistance to these compounds was measured by growth inhibition in liquid medium (Figure 4b) or on solid medium (Figures 4c and 5a). The resistance phenotypes were not strong, as growth differences between the control strain and strains expressing ABCB5 were only detected at drug concentrations close to the MIC for the control strain, and did not reach the levels observed for the AD/ABCB1 strain (Figures 2e and 3b). We were not able to detect efflux into the culture medium mediated by ABCB5 expression nor to detect specific ATPase activity (results not shown) as demonstrated previously for fungal ABC transporters.[27] However, when grown in parallel on the same agarose solid medium plate containing a sub-MIC concentration of R123 (5 μg/mL) colonies of the strains expressing either ABCB1 or ABCB5 had lower fluorescence per cell than colonies of the control AD/pABC3 strain, indicating a lower accumulation of R123 (Figure S2). Furthermore, the resistance to R123 conferred by ABCB5 expression was reversed in a dose-dependent manner (Figure 5b) by the known ABC-transporter inhibitor beauvericin[35] at concentrations that were 2- or 4-fold lower than the MIC (10 μM) of beauvericin for either the control host strain AD/pABC3 or the ABCB5-expressing strain in the same assay medium (Figure S3). In addition, beauvericin at 2.5 μM and 5 μM chemosensitized the AD/ABCB5-ch strain to R123 (Figure 5b); 5 μM beauvericin caused a 2-fold decrease in the R123 MIC (20 μg/mL reduced to 10 μg/mL).

The observation that the MIC of beauvericin (10 μM) is the same for the host strain as for the ABCB1- and ABCB5-expressing strains (Figure S3) also suggests that that it is not a substrate of either ABC protein. The results of disk assays (Figure 5a) also confirmed that expression of ABCB5 did not confer resistance to beauvericin whereas, in contrast, the compounds clorgyline and FK506 showed differential activities against the host AD strain and AD/ABCB5-ch, indicative of being substrates of ABCB5. Beauvericin has been shown to inhibit ABCB1-mediated efflux of R123 from human cell lines[36] and is an inhibitor, but not a substrate, of fungal ABC transporters.[35]

DISCUSSION

Human ABC proteins represent important drug targets. They are responsible for the transport of anti-cancer drugs out of tumor cells and thus prevent the attainment of therapeutic concentrations of the drugs within cells. Thus, there is a need to discover pump inhibitors that would allow combination therapy with anti-cancer drugs to prevent ABC-transporter-mediated resistance. If ABCB5 could be confirmed as conferring drug resistance, then heterologous expression of this protein would facilitate targeted drug discovery screens for such inhibitors to allow future combination therapy of melanoma. Heterologous expression in yeast represents a convenient platform for inhibitor screening. It is a host without other human proteins that in transfected human cell lines may interact with ABCB5, affecting interpretation of functional assays. The *S. cerevisiae* AD host has the added advantage over other eukaryotic expression systems, such as insect cell lines,[12] that seven endogenous ABC transporters have been deleted.[27] Another important feature is that several compounds that are known substrates of human ABC transporters show toxicity in yeast, and therefore the resistance conferred by expression of an efflux pump can be assessed by simple yeast growth inhibition experiments. The compounds selected have different targets in eukaryotic cells; R6G, R123, and TMR accumulate in mitochondria[37] and exert toxicity in yeast by affecting mitochondrial function,[38] whereas

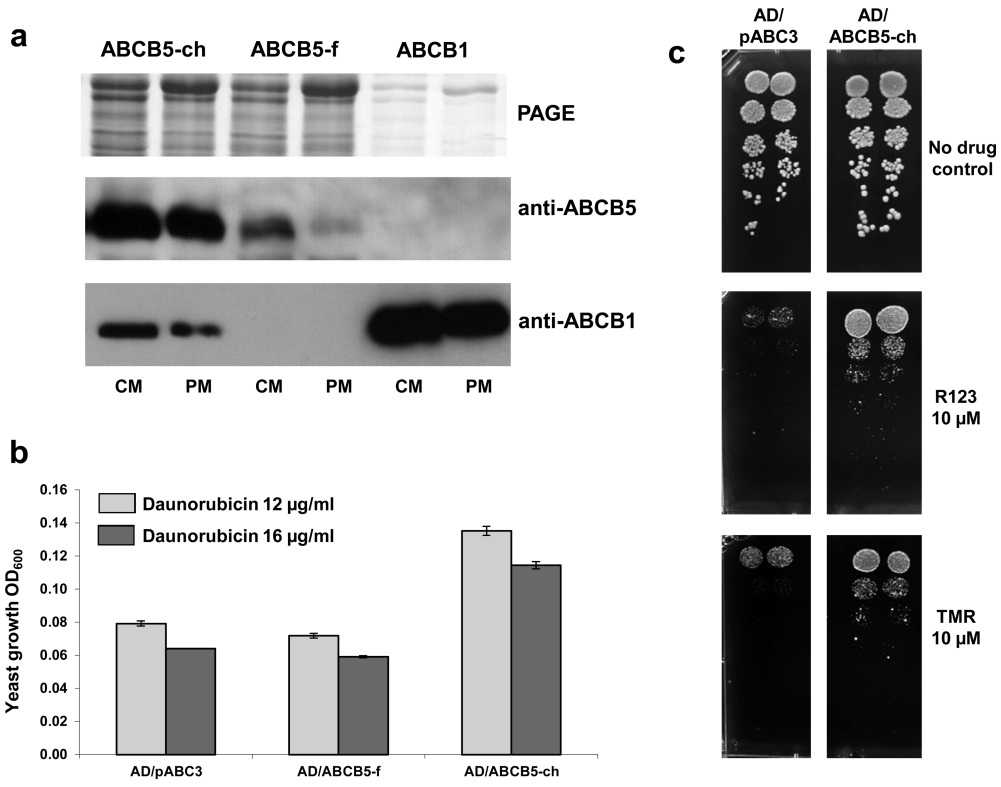


Figure 4. Expression of codon-harmonized full-length ABCB5 (ABCB5-ch) in *S. cerevisiae*. (a) PAGE and immunodetection of replicate blots with either anti-HsABCB5 antibody (ab77549) or anti-HsABCB1 antibody (ab3364). Lanes were loaded with crude membrane fractions (CM) or plasma membrane-enriched fractions (PM) extracted from strains AD/ABCB5-f (lanes 3 and 4), AD/ABCB5-ch (lanes 1 and 2), and AD/ABCB1 (lanes 5 and 6). (b) Susceptibility of the growth of *S. cerevisiae* strains in liquid culture to two sub-MIC concentrations of daunorubicin. (c) Susceptibility of the growth of *S. cerevisiae* strains on agar containing no drug, rhodamine 123 (10 μM), or tetramethylrhodamine (10 μM).

the DNA-intercalator daunorubicin exerts toxicity in *S. cerevisiae* by competing for the DNA binding sequences of important transcription factors.[39] FK506 is an immunosuppressive drug that is a substrate of ABCB1 and acts via inhibition of calcineurin.[40] Clorgyline, a monoamine oxidase inhibitor, has not previously been reported as a substrate of human ABC transporters, but was recently shown to be an inhibitor of fungal ABC transporters.[41]

The ability of human ABC transporters such as ABCB1 and ABCB5 to confer drug efflux properties is evidenced by expression studies using human and insect cell lines.[10,12,24,42] However, such an approach has the inherent problem that the background of other endogenous transporters may confound analysis of putative substrates or inhibitors of the cloned transporter. Heterologous expression in the *S. cerevisiae* AD strain avoids this issue, and allows ABC protein function to be investigated in a more readily controlled environment. Heterologous protein expression in yeast has other well-established advantages[43] for investigation of the biology of transporters such as substrate specificity and binding[44] as well as for protein purification and structure determination[45] and in assay development for screening purposes.[46] Heterologous expression of membrane proteins has, however, proved difficult.[47,48] The commonly used bacterial systems are often unable to express eukaryotic membrane proteins.[47] Existing eukaryotic systems such as insect cell lines give variable expression levels and are difficult and expensive to culture. Expression in the yeast *Pichia pastoris* can result in pump protein hyperglycosylation[49] and requires induction for long periods with methanol which may damage proteins. The model yeast *S. cerevisiae* is often preferred[43] because of ease of genetic manipulation, the availability of many strains including entire deletion libraries, and its robust growth properties in small-scale and large-scale culture.

We have demonstrated previously that the human ABC transporter ABCB1 can be expressed using the *S. cerevisiae* AD/pABC3 yeast platform, conferring ≥8-fold increases in resistance to multiple known substrates.[27] In the host AD strain, seven ABC transporters that might contribute to the efflux of pump substrates have been deleted, however, the *STE6* transporter, a highly specific transporter for the **a**-factor mating pheromone, was not deleted in the AD strain. Although *STE6* has the same topology as the cloned human ABCB1 and ABCB5 genes, it could not contribute to any observed resistance, as the AD strain is MATα, and *STE6* is not expressed in this mating type.[50,51] Also, the upstream region of *STE6* does not contain the Pdr1p/Pdr3p response element (PDRE) and therefore this transporter cannot be constitutively expressed under the control of the *PDR1-3* mutation present in the AD host strain. The increase in resistance to pump substrates in the recombinant strains was significantly better than the 2-fold increase for the same substrates, relative to a vector control strain, for plasmid-based ABCB1 expression in *S. cerevisiae*.[44] Thus, given our success with heterologous expression of ABCB1 using chromosomally integrated rather than plasmid-based expression, we investigated the expression of ABCB5-β and full-length ABCB5 in yeast. Expression of ABCB1, ABCB5-β, or full-length ABCB5 in cell extracts and membranes purified from recombinant strains was confirmed by immunoblot analysis using an ABCB5-specific antibody

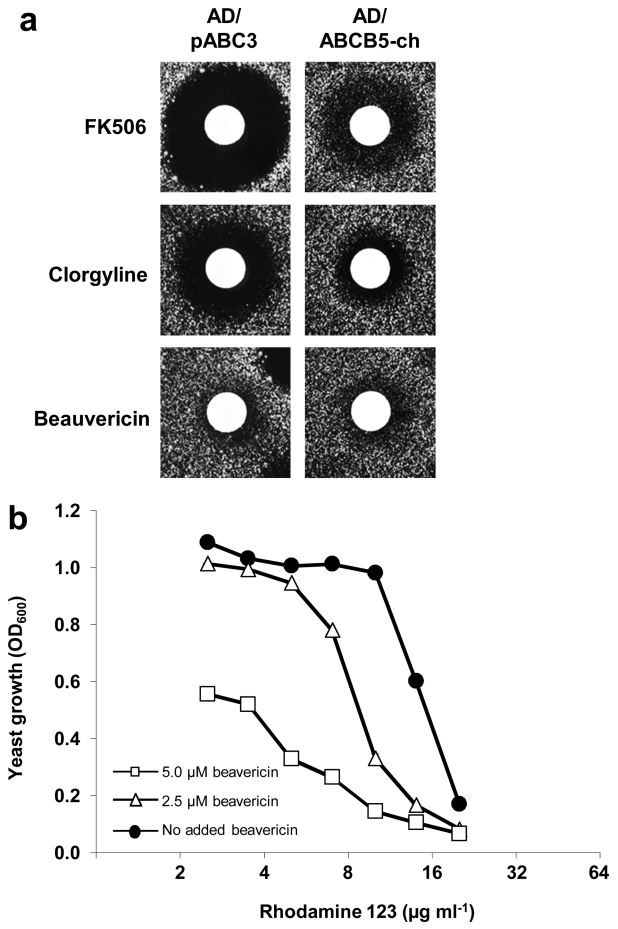


Figure 5. Phenotype conferred by expression of codon-harmonized full-length ABCB5 (ABCB5-ch) in *S. cerevisiae*. (a) Comparison of the inhibitory effects of compounds on the growth of the control *S. cerevisiae* strain AD/pABC3 or the recombinant strain AD/ABCB5-ch as demonstrated by agarose disk diffusion assays. Paper disks containing FK506 (0.5 nmol/disk), clorgyline (74 nmol/disk), or beauvericin (19 nmol/disk) were placed on the surface of agarose plates seeded with yeast. Plates were incubated at 30 °C for 24 h. (b) Reversal of resistance to R123 conferred by codon-harmonized full-length ABCB5 in the presence of beauvericin. Values shown are the means of triplicate determinations for growth in liquid culture after 24 h in the presence of R123 and beauvericin at the concentrations indicated. SEs did not exceed 10%.

(Abcam ab77549). Equivalent expression of ABCB5 was observed either in total cell extracts (TE) or in plasma membrane-enriched preparations of the AD/ABCB5-f strain (Figure 2b), whereas there was much lower expression of the cytoplasmic protein Adh1p in the plasma membrane-enriched preparations relative to total extracts. This could indicate at least partial enrichment of ABCB5 in plasma membranes. The full-length ABCB5 protein has a 76% similarity to the ABCB1 protein sequence, and cross-reactivity with an antibody raised to an ABCB1 peptide was observed (Figures 2 and 3). The reactivity of the anti-ABCB1 antibody with the homologous ABCB1 protein in the AD/ABCB1 strain was greater than the cross-reactivity with the ABCB5 protein (Figure 2c). This difference in antibody reactivity could reflect either an effect of the slightly different sequences of antibody recognition sites or lower total expression of the ABCB5 protein than ABCB1 when cloned in the host yeast strain. The anti-ABCB1 antibody (Abcam ab3364) recognizes two sequences in ABCB1 (VQEALD and VQAALD); the ABCB5 protein contained two similar sequences at equivalent positions in the full-length protein (VQHALD and VQAALE). Interestingly, we found that one commercially produced ABCB5 antibody (Abcam ab80108) reacted with extracts from the strain expressing the ABCB5-β construct, but not with extracts from the full-length ABCB5-expressing strain (Figure S1). Few papers describing expression of ABCB5 protein confirm protein size with immunoblot analysis, but it is feasible that a lack of detection of the full-length protein in some studies may reflect a defect in the antibody used.

Although the full-length and β constructs were both expressed in yeast, with the β protein apparently expressed in greater amounts (Figure 3a) only the full-length construct conferred a phenotype of resistance to known ABCB5 substrates (Figure 3b), whereas expression of the β protein did not alter the drug resistance of the host strain (Figure 2e). It has been suggested that the β form of ABCB5 may form a dimer to create a functional transporter, as the sequence contains coiled-coil motifs that may be potential dimerization sites[23] although no experimental evidence for functional dimerization of ABCB5-β has been reported. Indeed, there was no evidence for the functional dimerization of the β protein in our recombinant yeast strain, although heterologous expression of true human half transporters in yeast can result in the formation of functional homodimers. For example, heterologous expression of the human peroxisomal ABC half transporter ALDP (normally a homodimer) has been shown to rescue the mutant phenotype of a *S. cerevisiae* strain in which the transporter proteins Pxa1p and Pxa2p (which form a heterodimer involved in peroxisomal fatty acid transport) were deleted.[52] Given that a phenotype was only observed for the strain expressing the full-length transporter, and the sequence of the ABCB5-β isoform is unconventional for a true half-transporter, only the full-length sequence was subjected to codon harmonization.

Codon harmonization of the full-length ABCB5 DNA sequence resulted in increased ABCB5 expression in *S. cerevisiae* (Figure 4a) and conferred greater resistance to ABCB5 substrates than non-harmonized ABCB5 (Figure 4b,c). Interestingly, despite being closely related to ABCB1, ABCB5 protein expression was significantly lower under the experimental conditions used (Figures 3a and 4a). ABCB1 can be detected in Coomassie blue-stained plasma membrane-enriched preparations from AD/ABCB1[27] but ABCB5 expression was only detected by immunoblot (this study). In addition, there appeared to be a smaller proportion of ABCB5 correctly trafficked to the plasma membrane than occurred for ABCB1 (Figure 4a). Thus, the lower levels of resistance observed for strains expressing ABCB5 compared to those expressing ABCB1 may reflect a lower level of protein present in the plasma membrane rather than any inherent defect in ABCB5 as a drug efflux pump. We were not able to detect efflux into the culture medium mediated by ABCB5 expression, nor specific ATPase activity, as we have previously demonstrated for fungal efflux pumps expressed in the same *S. cerevisiae* expression system.[27] We speculate that ABCB5 is expressed in internal membranes and that the resistance conferred by expression of ABCB5 reflected sequestration of the substrate within the cell, such that the substrate concentration at the site of toxic activity was reduced. This is in accord with previously published results showing that melanosomes contribute to the refractory properties of melanoma cells by sequestering cytotoxic

drugs.[53] However, we observed that colonies of the AD/ABCB5-ch strain, or the AD/ABCB1 strain, when grown on sub-inhibitory concentrations of R123, retained less fluorescence than colonies of the control AD/pABC3 strain (Figure S2). This could indicate an efflux function, although the results may also reflect the different spectroscopy readings of cells in which R123 is sequestered by the action of ABCB5 or ABCB1 in the respective recombinant strains compared to the control cells. However, this observation is further confirmation of an efflux-related function conferred on yeast cells by the expression of ABCB5.

The increased resistance observed for the ABCB5-expressing strain compared with the otherwise isogenic control strain indicated that this resistance was ABCB5-specific. Furthermore, codon harmonization, which resulted in increased ABCB5 expression in *S. cerevisiae* (Figure 4a), also conferred greater resistance to ABCB5 substrates than did non-harmonized ABCB5 (Figure 4b). In addition, the drug resistance phenotype of the ABCB5-ch expressing strain could be reversed by the inhibitor beauvericin (Figure 5b).

In summary, although expression of full-length, codon-harmonized ABCB5 in yeast was lower than that observed for the related ABCB1 transporter without codon harmonization, we were able to demonstrate a resistance phenotype for the ABCB5-expressing strain. We showed for the first time that the β construct does not confer a drug resistance function, at least when expressed in yeast. However, we cannot exclude the possibility that differential post-translational modification of the β and full-length isoforms in yeast cells is responsible for the lack of function for the β construct. Our results confirm that expression of full-length ABCB5 in *S. cerevisiae* can confer resistance to substrates of human ABC transporters and therefore this protein may be involved in the insensitivity of melanomas to many chemotherapy drugs. Further optimization of our *S. cerevisiae* expression system may result in sufficient ABCB5 expression that the strain can be used in fluorescence-based high-throughput screening to identify ABCB5 inhibitors.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1–S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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