A stress-responsive enhancer induces dynamic drug resistance in acute myeloid leukemia

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Abstract

The drug efflux pump ABCB1 is a key driver of chemoresistance, and high expression predicts for treatment failure in acute myeloid leukemia (AML). In this study, we identified and functionally validated the network of enhancers that controls expression of ABCB1. We show that exposure of leukemia cells to daunorubicin activated an integrated stress response-like transcriptional program to induce ABCB1 through remodeling and activation of an ATF4-bound, stress-responsive enhancer. Protracted stress primed enhancers for rapid increases in activity following re-exposure of cells to daunorubicin, providing an epigenetic memory of prior drug treatment. In primary human AML, exposure of fresh blast cells to daunorubicin activated the stress-responsive enhancer and led to dose-dependent induction of ABCB1. Dynamic induction of ABCB1 by diverse stressors, including chemotherapy, facilitated escape of leukemia cells from targeted third-generation ABCB1 inhibition, providing an explanation for the failure of ABCB1 inhibitors in clinical trials. Stress-induced up regulation of ABCB1 was mitigated by combined use of pharmacologic inhibitors U0126 and ISRIB, which inhibit stress signalling and have potential for use as adjuvants to enhance the activity of ABCB1 inhibitors.

Keywords

Acute myeloid leukemia; ABCB1; ATF4; integrated stress response; drug resistance.
Introduction

Resistance of leukemia cells, including leukemia stem cells (LSC) with disease reconstituting activity, to the chemotherapy drugs used in standard induction and consolidation regimens is the most common cause of treatment failure in acute myeloid leukemia (AML). Primary drug resistance is certainly linked to the genetic lesions driving AML; for example, leukemias with an NPM1\textsuperscript{mut} FLT3-ITD genotype are substantially more difficult to cure with chemotherapy alone than NPM1\textsuperscript{mut} AMLs that lack FLT3-ITD, although the reasons for such differential sensitivity remain obscure. Levels of expression of drug detoxifying enzymes, topoisomerase II, microRNAs, and the propensity of cells to undergo autophagy have all been suggested to contribute to intrinsic drug resistance (1).

Most significantly, high expression of the ABCB1 drug efflux pump (also known as MDR1 or P-glycoprotein) which actively exports anthracyclines, predicts for treatment failure in AML (2, 3). More generally, ABCB1 is highly expressed in many poor risk malignancies as well as in normal gut, liver, kidney and the blood-brain barrier (4). Inhibitors of ABCB1 have been tested in clinical trials in AML but with limited success. Nevertheless, in view of its significant role in the disease, the rationale for targeting ABCB1 remains strong (3). Furthermore, given the abundance of preclinical evidence supporting a role for ABCB1 in drug resistance, the failure in clinical trials of inhibitors of ABCB1 has not been adequately explained.

A greater understanding of the cancer-specific regulation of ABCB1 and its role in drug resistance is required to facilitate the design of new therapeutic strategies. Specifically, it is unclear how ABCB1 expression is established and maintained in human AML. Whether expression is constitutive or dynamic is of critical relevance to the clinical application of ABCB1 inhibitors where previous trials have assumed constant expression (5). Advances in enhancer biology have established that these distal regulatory elements govern cell-type specific gene expression and frequently respond to environmental conditions and homeostatic perturbations (6, 7). Critically, the enhancer landscape of ABCB1 has yet to be defined.
Results

Resistance to daunorubicin due to stereotypical induction of \( ABCB1 \)

We initially set out to evaluate mechanistic heterogeneity in the acquisition of resistance to daunorubicin, which is the mainstay drug of AML induction chemotherapy regimens. To do this we generated multiple daunorubicin-resistant K562 leukemia cell lines in parallel. K562 cells are derived from the pleural effusion of a patient with chronic myeloid leukemia in terminal myeloid blast crisis (8) and, unmanipulated, they undergo apoptosis in response to daunorubicin with an IC\(_{50}\) of \( \sim 40 \text{nM} \). We selected this line in view of its extensive use as a model system by the ENCODE consortium.

Three separate vials of early passage K562 cells were thawed and cultured separately for two weeks. The three drug-sensitive lines were designated K562_S1-3 and aliquots were cryopreserved for later use. Each line was then exposed to escalating doses of daunorubicin in continuing culture until they were able to expand in 500nM (Figure 1A). Resistant lines were designated K562_R1-3 and the time taken to acquire this level of resistance was 106 days (K562_R1 and R3) or 117 days (K562_R2). The daunorubicin IC\(_{50}\) values were 2.3\( \mu \text{M} \), 4.7\( \mu \text{M} \) and 9.9\( \mu \text{M} \) respectively with increases versus drug sensitive lines K562_S1-3 of 55-fold, 101-fold and 249-fold respectively (Figures 1B and 1C).

To evaluate changes in gene expression, we performed RNA sequencing. To avoid detecting transient changes in gene expression associated with recent daunorubicin exposure or contamination with apoptotic cells, each line was propagated for a further 10 days without daunorubicin prior to RNA extraction. RNA sequencing was performed using a single replicate for each sensitive line (K562_S1-3) and two replicates for each resistant line (K562_R1-3). When each drug-resistant line was compared with the sensitive lines, the most highly up regulated protein coding gene in each case was \( ABCB1 \) (mean 4,700-fold) even though the lines had been cultured separately from one another for at least four months (Figures 1D, 1E and Table S1). Increased \( ABCB1 \) expression was confirmed by quantitative PCR and this correlated well with increased cell surface \( ABCB1 \) protein (Figures 1F and 1G). To confirm the up regulated protein expression was functional we performed fluorescent dye efflux experiments. Drug-sensitive K562_S lines did not efflux calcein AM whereas drug-
resistant K562-R lines exhibited robust drug efflux (Figures 1H and 1I). Efflux was completely reversed by either verapamil (a non-specific ABC transporter substrate) or tariquidar (a highly specific inhibitor of ABCB1) (5). This confirmed that all drug efflux was due to ABCB1 (Figure 1J). No other ABC transporter gene was up regulated by more than 2.5-fold in resistant cells (Table S1). Thus even when chemoresistance is induced in separate lines, the mechanism of acquisition (i.e. ABCB1 up regulation) is stereotypical.

**Daunorubicin-resistant leukemia cells express a common ISR-like gene signature**

Unsupervised hierarchical clustering analysis, using cosine distance and average linkage, of 5,953 expressed protein coding genes revealed that transcriptomes of sensitive and resistant lines differed substantially from one another (Figure 2A). Interestingly, principal component (PC) analysis revealed differences in the transcriptome of K562_S3 compared to both K562_S1 and K562_S2 (PC2) which were preserved as cells developed resistance (Figure 2B). PC1 accounted for 50% of the variance and defined the transition from sensitive to resistant in each case. Differential gene expression analysis identified 223 and 154 genes as significantly upregulated or downregulated respectively (t-test, \( P<0.01 \), fold change >2 or <0.5) (Figure 2C, Table S2). Among the upregulated gene set there was significant enrichment for Gene Ontology Biological Process terms reflecting cellular stress including “response to endoplasmic reticulum stress” and “endoplasmic reticulum unfolded protein response”; among the downregulated gene set there was enrichment for “rRNA processing” and “mRNA splicing, via spliceosome” (9). Gene set enrichment analysis (GSEA) revealed that, of the 2,414 curated gene sets from the Molecular Signatures Database tested (v6.2) (10), those reflecting the response of HL-60 promyelocytic leukemia cells to the aminopeptidase inhibitor tosedostat (11), and arterial endothelial cells to hypoxia (12) were the most significantly enriched among both up and down regulated genes in daunorubicin-resistant versus sensitive K562 cells (Figures 2D and S1A). Tosedostat is an aminopeptidase inhibitor that induces intracellular amino acid deprivation and consequent activation of the Integrated Stress Response (ISR). Likewise, hypoxia activates the ISR by impairing disulfide bond formation causing protein misfolding and endoplasmic reticulum stress (13).
To identify candidate regulators of high level \textit{ABCB1} transcription, and more generally the associated ISR-like transcriptional programme, we identified transcription factor genes upregulated in resistant versus sensitive cells (Tables 1, S1 and S2). The most highly expressed was \textit{ATF4}. Others included \textit{ATF4}-bound transcriptional targets such as \textit{ATF3}, \textit{XBP1} & \textit{CEBPB} (Table S2) or \textit{ATF4} binding partners including JUN, JUNB, CEBPB, CEBPG, DDIT3 & \textit{ATF3} (14, 15). Consistent with \textit{ATF4} being a core driver of the upregulated ISR-like transcriptional programme, GSEA demonstrated highly significant enrichment for \textit{ATF4} target genes among genes up regulated in daunorubicin-resistant versus sensitive K562 cells (Figure 2E). In this analysis, \textit{ATF4} target genes were those identified as genes closest to the strongest 500 \textit{ATF4} binding peaks identified by ChIPseq in K562 cells (Table S2) (14, 16). Similar analyses using sets of genes located closest to the 500 strongest CEBPB, CEBPG, \textit{ATF3}, JUN or JUNB binding peaks in K562 cells also revealed significant enrichment in daunorubicin-resistant K562 cells (Table S2) (14, 16). Notably, however, enrichment scores were lower than for the analysis using \textit{ATF4} target genes (Figure S1B). All together these data demonstrate that the acquisition of an \textit{ABCB1}-dependent daunorubicin-resistant cellular state in myeloid leukemia cells is associated with sustained upregulation of an ISR-like transcriptional programme, with the transcription factor \textit{ATF4} at its core.

**Expression of \textit{ABCB1} is regulated by a stress-responsive enhancer**

Despite its clinical significance as a critical regulator of chemoresistance, knowledge of the transcriptional control of \textit{ABCB1} is incomplete. Constitutive expression of its promoter requires motifs within 250bps of the transcription start site that facilitate binding of Nuclear Factor-Y and SP1, and promoter binding sites for EGR1, WT1, HIF1A, CEBPB, FOXO factors and TCF7 have been reported (17). TP53 may repress or activate the \textit{ABCB1} promoter depending on whether it is wild-type or mutant; promoter DNA methylation represses \textit{ABCB1} expression; and genetic translocations may activate \textit{ABCB1} expression through juxtaposition of the native promoter to that of more active but unrelated genes (4). \textit{ABCB1} expression may also be induced by stressful stimuli and roles for the AP-1 transcription factor family and Nuclear Factor-κB have been suggested, but supporting data are indirect (4, 17). There is no
knowledge as to whether \textit{ABCB1} is regulated by enhancer elements, and if so which factors control these.

To identify candidate \textit{ABCB1} enhancer elements, we performed ChIPseq for H3K27 acetylation, a histone modification that marks active enhancers (18), using sensitive (K562\_S1-3) and resistant (K562\_R1-3) lines. We searched a 2MB region centred on \textit{ABCB1} for differentially acetylated regions in resistant versus sensitive lines; the great majority of cis-regulatory elements lie within 1MB of target genes (19). Consistent with the dramatic increase in transcription there was strong promoter acetylation in drug resistant lines which was not observed in sensitive lines. In addition, we identified four acetylation peaks, designated E1-4, in intronic sequences of \textit{ABCB1} (E1-3) or upstream of the promoter (E4) in resistant but not sensitive cell lines (Figure 3A).

Using H3K27Ac ChIPseq data from ENCODE (14) we also searched for candidate enhancer elements in normal liver and adrenal gland, the tissues with the highest constitutive levels of \textit{ABCB1} expression (Figure 3A). The pattern was tissue-specific, although putative \textit{ABCB1} enhancers from K562\_R1-3 lines were acetylated in liver (E1 & E3) or adrenal gland (E1, E2 & E3). Normal human CD34\textsuperscript{+} hematopoietic stem and progenitor cells (HSPCs) express intermediate levels of \textit{ABCB1} and H3K27 acetylation of E3 was observed (Figure 3A). Interestingly, E3 and four additional sites were marked by H3K4 monomethylation in CD34\textsuperscript{+} HSPCs, a histone modification which marks poised as well as active enhancers.

To determine the nature of candidate regulatory elements contacts, we next performed 4C-sequencing in drug-resistant cells with a viewpoint centered on the \textit{ABCB1} promoter. There were particularly strong interactions between E3 and E4 and the promoter, and lower level interactions between E1 and E2 and the promoter (Figures 3A, S2A). Strong contact was also observed between three additional regions and the promoter, termed C1, C2 and C3. C1 is H3K4 monomethylated and weakly H3K27 acetylated in CD34\textsuperscript{+} HSPCs, and strongly acetylated in liver; C2 is H3K4 monomethylated in CD34\textsuperscript{+} HSPCs; and C3 is acetylated in adrenal gland. These observations suggest C1-C3 may exhibit tissue specific enhancer activity, although the presence of constitutive contact with the promoter in K562\_R cells may be explained by C1 being bound by CTCF and cohesin (Figure S2B). The reason for contacts between C2, C3 and the promoter were not apparent. Thus, the \textit{ABCB1} promoter
exhibits a network of physical contacts with nearby enhancers in drug resistant K562 leukemia cells.

To confirm that putative enhancers were functional, we next performed targeted silencing using a CRISPR-dCas9-KRAB system. We designed multiple sgRNAs for each region (Figures S3A-D) and screened them in K562_R3 cells, using loss of cell surface ABCB1 expression or increased calcein AM retention as a measure of activity (Figures S3E-F). The most active guides were then selected for use in all resistant cell lines. K562_R1-3 cells were dual infected with pHR-SFFV-dCas9-BFP-KRAB and pLKO5.sgRNA.EFS.tRFP657, the latter expressing an sgRNA targeting an enhancer or the promoter, or a non-targeting control (Figure 3B). We used ChIPseq for H3K9 trimethylation to confirm that silencing was discrete and accurate: induced regions of heterochromatin ranged in size from 3-8kb, were centred on the target sequence for each guide, and did not target the promoter, even where 4Cseq had shown the enhancer region to be in close physical proximity (Figure 3C). Quantitative PCR and flow cytometry assessment of the effect of ABCB1 promoter silencing revealed substantial repression of transcription (Figures 4A, 4B). The enhancer silencing experiments revealed either modest or no significant contribution to ABCB1 expression from E1, E2 and E4. The most extensive reductions in expression of ABCB1 transcripts and protein were observed following silencing of E3, demonstrating that this was the most active enhancer, consistent with its high level of H3K27 acetylation and close contact with the promoter.

Within E3 is a DNAse1 hypersensitive site (Figure 4C) (14). Motif analysis of the 30 base pair sequence revealed consensus binding sites for several of the transcription factors up regulated in drug-resistant cells, including ATF4, JUN and CEBPB (Table 1 and 2, Figure 4C). We used ENCODE ChIPseq data from unmanipulated K562 cells to characterise binding of those factors to each enhancer (Figure 4C and S4A). Datasets were available for six of the 12 factors upregulated in resistant cells (Table S3), all of which were bound to the E3 enhancer suggesting it to be stress responsive (Figure 4C). There was some modest ATF3 and ATF4 binding at E1 and adjacent to E4. Critically, binding of AP-1 transcription factors to the promoter was absent (Figure S4A). Interestingly, E2 exhibited TAL1 and GATA2 binding, key hematopoietic stem cell (HSC) transcription factors that are active in AML and associated
with poor clinical outcome (20). To confirm the ENCODE data and to determine whether there was increased binding of stress responsive transcription factors at E3 in drug resistant cells, we performed ChIP PCR. We observed significant increases in the binding of ATF4, ATF3, CEBPB, JUND and JUN to E3 in K562_R1 compared to K562_S1 cells (Figure 4D). These data together demonstrate that acquisition of daunorubicin-resistance is associated with activation of a stress responsive, AP-1 bound enhancer element in intron 4 of ABCB1.

**Dynamic induction of ABCB1 by diverse cellular stressors**

To explore further the relationship between cell stress and expression of ABCB1, but over a shorter timescale, we induced intracellular amino acid depletion using the aminopeptidase inhibitor tosedostat (11). Tosedostat is able to induce cellular stress in both sensitive and resistant cells because it is not an ABCB1 substrate subject to cellular extrusion in ABCB1 high cells (Figure S5A). There was significant up regulation of ABCB1 expression in all K562 lines after 48 hours, although the absolute level of increase was far greater in drug-resistant lines (Figure 5A). Activation of the ISR upregulates ATF4 through a translational mechanism (15) so it was unsurprising that changes in ATF4 transcript levels were modest (Figure S5B). Instead, as a surrogate measure of ATF4 activity, we quantified expression of three genes that are known direct targets of ATF4: DDIT3, DDIT4 and CEBPB (21). Expression of all three was robustly induced by tosedostat, again with the absolute level of increase being greater in drug-resistant lines (Figure 5A). Tosedostat also induced expression of the AP-1 transcription factor JUN in all lines (Figure S5C). Similar observations were made following treatment of unmanipulated early passage K562 cells with alternate stressors: thapsigargin, which activates the ISR through blockade of the endoplasmic reticulum Ca\(^{2+}\) ATPase (Figure S5D) (22), and high density culture (cell density of >10\(^6\)/ml for 48 hours; Figure S5E). Thus, diverse cellular stressors induce dynamic up regulation of ABCB1 and other direct targets of ATF4.

Exposure of sensitive and resistant K562 lines to 100nM and 500nM daunorubicin respectively for 72 hours also induced ABCB1 (Figure 5B). As for tosedostat, the greatest absolute levels of increase were observed in drug-resistant lines, and they correlated with significant increases in DDIT3, DDIT4 and CEBPB (Figure 5B). By contrast with tosedostat, the fold-change increases in DDIT3, DDIT4 and CEBPB induced by 500nM daunorubicin
were lower and increased expression of \textit{ATF4} was not observed, suggesting that daunorubicin may be a somewhat less efficient activator of the ISR pathway (Figure S5F). Daunorubicin also induced expression of \textit{JUN} in resistant lines (Figure S5G). The differences in response to both tosedostat and daunorubicin of drug-sensitive versus drug-resistant K562 lines are in keeping with the observed enhancer remodelling at the \textit{ABCB1} locus induced by prolonged (>100 days) daunorubicin exposure.

These data demonstrate that brief daunorubicin exposure also induces \textit{ATF4} target gene expression, including \textit{ABCB1}. Importantly, \textit{ABCB1} expression in daunorubicin-resistant K562 lines was dynamic and diminished over time if cells were not continuously exposed to drug (Figure 5C). Loss of \textit{ABCB1} expression was more pronounced when cells were propagated at low density (<200k/ml) emphasising the need for rigorous control of cell culture conditions when performing stress experiments. Even modest elevations of cell density (>200k/ml) were sufficient to cause significant increases in \textit{ABCB1} compared to low density controls (Figures 5C & D). Re-exposure of K562\_R1-3 cells to daunorubicin (100 or 500nM for 7 days) following a 24 day daunorubicin-free period of culture led to a dose-dependent reestablishment of \textit{ABCB1} expression, an effect that was dependent on the activity of the \textit{ATF4}-bound E3 enhancer because it was attenuated when E3 was silenced with dCas9-KRAB (Figure 5E). All together these data demonstrate that expression of the daunorubicin drug export pump \textit{ABCB1} is dynamically regulated in leukemia cells though the \textit{ATF4}-bound E3 enhancer.

\textbf{Daunorubicin activates a stress-responsive \textit{ABCB1} enhancer in primary AML cells}

We next examined \textit{ABCB1} enhancer accessibility and usage in primary AML. We identified cases of relapsed or refractory AML from Manchester Cancer Research Centre’s Tissue Biobank with high \textit{ABCB1} expression by quantitative PCR (Figure 6A and Table S4) and performed ChIP sequencing for H3K27Ac in high expressing cases where sufficient cryopreserved bulk blast cells were available (red bars in Figure 6A). We also made use of a recently published DNAse-seq primary AML dataset (23). Quantitative PCR analysis revealed that \textit{ATF4} expression correlated significantly with \textit{ABCB1} (Figure 6B, \(r=0.53, p=0.005\)). Considering the genomic region encompassing the coding sequence of \textit{ABCB1} and
sequences 20kB upstream and 10kB downstream, we identified five DNase1 hypersensitive sites (DHS) (in addition to the DHS observed at the promoter) in multiple cases of AML (Figures 6C and 6D). These included E1 and E3 (accessible in 12/36 and 13/36 primary AML cases respectively) which became strongly acetylated in drug-resistant K562 cells, and the CTCF binding site C1 (accessible in 32/36 primary AML cases) (Figures 3A, 6C & 6D). Regions E2 and E4 (Figure 6D and data not shown) were not accessible. Two additional sites (A & B; accessible in 13/36 and 14/36 cases respectively) which were not acetylated in drug-resistant K562 cells were also DNase1 hypersensitive. DHS site B was adjacent to other confirmed ABCB1 enhancers (E1 and E2; Figure 6D) and acetylated in ABCB1-expressing adrenal tissue. Importantly this site also contains binding motifs for ATF4, JUN and CEBPB suggesting that it too may serve as a stress-responsive enhancer (Figure 6E). Across the totality of primary AML samples profiled by Assi et al. (23) 6/36 exhibited DHS at both B and E3 sites, 8/36 at B only, 7/36 at E3 only and 15/36 at neither B nor E3. Together these data show that stress-responsive regulatory elements are accessible in bulk primary AML cells. Our own H3K27Ac ChIP-sequencing analyses further demonstrated that ABCB1-expressing samples exhibited peaks of acetylation surrounding these sites: of the 10 samples analyzed, four had discernible H3K27Ac peaks at B only, 2 at E3 only, 1 at both and 3 at neither. In one case there was a peak of acetylation at A. Thus, in a substantial proportion of primary AML cases, stress-responsive ABCB1 regulatory elements are accessible and active.

To determine whether primary AML cells respond to stress in a similar manner to drug-resistant K562 cells we exposed fresh bulk primary AML blasts from bone marrow or blood (Table S4) to daunorubicin (10nM, 100nM and 1000nM) for 18 hours. We observed dose dependent induction of ATF4 target genes ABCB1, DDIT3, DDIT4, CEBPB and JUN, although as before changes in ATF4 transcripts were modest or absent (Figures 6F, S6A). It was of note that this response was not observed where similar analyses were performed using cryopreserved AML samples following a freeze-thaw cycle (Figure S6B). Vehicle treated freeze-thawed samples exhibited substantially higher levels of ATF4 and DDIT3 compared to vehicle treated fresh samples (Figure S7A), suggesting that the freeze-thaw process activates cellular stress pathways consequently obscuring the response to daunorubicin exposure. Two additional fresh primary AML samples were treated with 1000nM
daunorubicin or vehicle for 18 hours (Figure S7B) and subjected to ChIP-PCR for H3K27ac surrounding E3 (Figure 6G). Significant increases in acetylation were observed, confirming acute stress-induced regulation of E3. By contrast, daunorubicin had no effect on the acetylation of the CTCF binding site C1 (Figure 6G).

We also assessed the effect of daunorubicin exposure on two other ABC transporter genes previously associated with chemoresistance in AML (3). ABCG2 expression increased significantly in 4/5 fresh samples following daunorubicin exposure, but absolute levels of expression were very low as judged by cycle threshold (Figure S7C). Induction was not observed in 3/4 freeze-thawed samples (Figure S7D). ABCC1 was more highly expressed and its expression increased significantly in all fresh samples (Figure S7E) with responses again smaller or absent in freeze-thawed samples (Figure S7F). The change in expression of these efflux pumps in response to daunorubicin mirrors that of ABCB1, suggesting regulation by similar mechanisms. Interestingly, ENCODE data in unmodified K562 cells shows intronic binding of CEBPB, CEBPG, JUND, JUN, ATF4 and ATF3 within ABCC1, suggesting that this efflux pump may also be stress responsive (Figure S8) (14).

ABCB1 is also expressed in normal HSCs and down regulated during differentiation. Indeed, extrusion of rhodamine 123 or Hoechst 33342 by ABCB1 has been used to identify long-term repopulating HSCs (24). HSCs also make use of the ISR and ATF4 to protect against homeostatic cellular stress and to preserve the integrity of the stem cell pool (25). Given this account of an adaptive, pro-survival ISR signature in HSCs we examined the expression of K562 resistance-associated transcription factor genes across normal hematopoiesis (26). As previously described, ABCB1 expression diminished as cells differentiated, with the highest expression seen in early HSCs (Figure S9A). ATF4 expression followed a similar pattern and was highly correlated with ABCB1 (Figures S9A-C, r = 0.91, p<0.001). Given the predominantly translational regulation of ATF4 we also studied its transcriptional target DDIT3: changes in expression correlated even more closely with that of ABCB1 (Figures S9A-C, r = 0.95, p<0.001). Indeed, all of the TFs that were upregulated in daunorubicin-resistant K562 cells were significantly correlated with ABCB1 expression across normal hematopoiesis (Figure S9B). Reflecting this observation, GSEA revealed highly significant enrichment of expression of the 223 genes upregulated in drug-resistant versus
sensitive K562 cells in normal HSC/MPPs versus downstream myeloid progenitor populations (Table S2, Figure S9D), suggesting a common gene expression program driven by adaptive pro-survival stress signalling. Analysis of H3K27Ac, H3K4me1 ChIPseq and DNAse-seq data (ENCODEnormal CD34+ HSPCs confirmed that regulatory elements A, E1, E3 and, to a lesser extent, B were accessible in CD34+ HSPCs and marked by H3K4 monomethylation and, in the case of E3, by H3K27 acetylation (Figure S9E).

These data demonstrate the close link between expression of a stress responsive genetic programme and resistance to daunorubicin through up regulation of ABCB1; they further demonstrate that chemotherapy treatment with daunorubicin activates a stress-responsive enhancer and induces up regulation of a drug-resistance mechanism in AML blast cells that may contribute to therapeutic failure and disease relapse.

**Activation of an ISR-like response facilitates escape from ABCB1 inhibition**

Pharmacologic inhibitors of ABCB1 have been tested in clinical trials as adjuncts to AML therapy but without significant success (3). Trials of the third generation inhibitor tariquidar used doses of 2mg/kg (resulting in plasma concentrations of ~4nM), based on maximal inhibition of rhodamine 123 efflux in CD56+ NK cells which exhibit relatively high, stable levels of ABCB1 expression (5). Our observation of dynamic, stress responsive ABCB1 expression raised a question as to whether the dose of ABCB1 inhibitors used to inhibit steady-state cells might be ineffective under conditions of cellular stress.

We re-exposed drug-resistant K562 cells (lines R1-3) which had been cultured without daunorubicin for 24 days to 500nM daunorubicin or vehicle for 72 hours and assessed the ability of 5nM tariquidar to inhibit efflux of calcein AM. As expected, re-exposure of cells to daunorubicin further induced ABCB1 expression (Figure 7A). Concomitant treatment of cells with 5nM tariquidar abolished calcein AM efflux in vehicle-treated cells but in daunorubicin re-exposed cells, where ABCB1 had been further induced, in each case a population of cells was observed that failed to retain calcein AM. This demonstrates continued activity of ABCB1 drug efflux in a sub-population of cells despite exposure of the cell population to levels of tariquidar approximating those achieved in clinical trials (Figures 7B and 7C). This effect became yet more apparent where daunorubicin exposure was extended to seven days but
could be overcome by increasing the concentration of tariquidar (Figures 7A-C), indicating that the effect was due to differential expression of ABCB1. We confirmed this by flow sorting tariquidar-treated K562_R1-3 cells into calcein AM<sup>neg</sup> and calcein AM<sup>pos</sup> populations and evaluating ABCB1 expression (Figures 7D-F). Similar observations were made when K562_R1-3 cells were exposed to tosedostat demonstrating that this effect was not specific to daunorubicin and likely consequent upon activation of an ISR-like programme (Figures S10A-C). To identify an approach to overcome the phenomenon of daunorubicin and stress-induced escape from ABCB1 inhibition, we evaluated stress pathway inhibitors. U0126 antagonizes AP-1 target gene transcription via inhibition of MEK1/2, and ISRIB (integrated stress response inhibitor) antagonizes the consequences of eIF2a phosphorylation through a mechanism involving binding of eIF2B to restore normal translation of factors including ATF4 (27, 28). Treatment with 10µM of U0126 was suggestive of reduced ABCB1 induction in K562_R1 exposed to 500nM daunorubicin for 72 hours compared to vehicle (Figure 7G). Whilst ISRIB alone did not have an effect, combined treatment with U0126 led to significant dose-dependent suppression of ABCB1 induction (Figure 7G).

Thus daunorubicin and stress-induced acute induction of ABCB1 can overcome pharmacologic inhibition of ABCB1 leading to leukaemia cell survival and this can, at least in part, be mitigated by concomitant treatment of cells with inhibitors of stress signalling.
Discussion

Efflux of chemotherapeutic agents by ABCB1 is an important cause of treatment failure in human cancer. High expression levels may be an intrinsic feature of the cell type, or due to promoter translocations between ABCB1 and genes with strong constitutive expression, such as those found in patients with breast or ovarian cancer who relapse following prior therapy (29, 30). We found that primary AML cells displayed dynamic expression of ABCB1, suggesting physiological regulation rather than control by constitutively active captured promoters. Our analyses reveal the network of enhancers that controls intrinsic expression of ABCB1 in human leukaemia cells; the gene is a direct target of the transcription factor ATF4 which is activated through a chemotherapy-induced cellular stress response.

Drug resistance in cancer can arise through multiple mechanisms, including genetic events and stochastic transcriptional changes in rare cells (31). We observed significant transcriptional differences in one of our cell lines (K562_3) that persisted as the line acquired resistance. These differences are likely due to the genetic and transcriptional divergence that frequently accompanies the propagation of cancer cell lines (32). Indeed, this was our primary motivation for creating three independent resistant cell lines and it is significant that in spite of these differences all lines developed daunorubicin resistance through induction of ABCB1.

For cancer cells to survive they must adapt to stressful stimuli. The ISR is activated by endoplasmic reticulum stress, hypoxia, amino acid deprivation and oxidative stress, common consequences of uncontrolled proliferation and outgrowth of the vascular supply. The transcription factor ATF4 is a critical effector of the ISR and is highly expressed in many cancers as a result of extrinsic stress or direct activation by constitutive oncogene expression (33). During stress it is efficiently translated as a result of eIF2α phosphorylation, permitting heterodimer formation with transcription factors such as JUN, FOS and CEBPB, and binding of transcriptional targets (15, 34). We found that adaptation of leukemic cells to prolonged daunorubicin exposure (> 100 days) involved expression of an ATF4-centred, ISR-like transcriptional programme which led to sustained upregulation of ABCB1. ATF4 and its interaction partners bind a stress-responsive enhancer in intron 4 suggesting that this element responds specifically to stress signaling with an adaptive, pro-survival output. The dramatic differences in histone acetylation surrounding, in particular, enhancer E3 are
indicative of enhancer remodelling, although the molecular mechanisms underlying this process remain unclear. It is also unclear how the various active enhancers cooperate to regulate transcriptional activity at the promoter. Uncovering these mechanisms would be of great interest, not least because the adaptive molecular changes surrounding E3 appear to serve as the basis for the epigenetic memory of prior cellular stress, at least as far as expression of \(ABCB1\) is concerned.

Previous reports of \(ABCB1\) responses to cellular stress have been contradictory, demonstrating induction or repression, even after exposure to the same stressor (35). These conflicting results might be consistent with the pleiotropic function of ATF4, which is able to orchestrate adaptation and survival or apoptosis depending on cellular context and the severity of the insult. Indeed, down regulation of \(ABCB1\) appears to precede cell death, suggesting that the gene is negatively regulated by ISR signaling where apoptosis is the result (36).

Even in the era of targeted therapies, tumour bulk continues to predict treatment failure for many cancers (37) and the total white cell count in blood at presentation is strongly predictive of outcome in AML (38). Our observation that prolonged daunorubicin exposure elicited a transcriptional response that was shared by cells exposed to amino acid deprivation or hypoxia suggests that extrinsic stress applied experimentally has similar consequences to environmental stresses experienced by cancer cells in vivo. We speculate that protracted cellular stress primes stress-responsive \(ABCB1\) enhancers for both strong constitutive activity and augmented responses following exposure to additional stressors, such as chemotherapy, leading to increased drug efflux: chemotherapy may induce its own chemoresistance mechanism. Whilst steady-state expression of ABC transporters is seen only in a subset of resistant AML cases we found that dynamic up regulation of \(ABCB1\) following daunorubicin exposure occurred in all fresh primary samples tested (39). Rapid adaptation to therapy may therefore represent a more common mechanism of resistance than previously appreciated, especially considering that the biopsies that provide primary material for research are seldom taken during treatment.

The importance of \(ABCB1\) in hematopoiesis is well established. Its expression is a hallmark of hematopoietic stem cells (HSCs) and accounts for their reduced staining with
Hoechst 33342. HSCs display high levels of pro-survival ISR activity which we found to correlate with the expression of ABCB1 and the transcription factor combination expressed in our resistant cells (25). Leukemic stem cells (LSCs) can also be identified by their capacity for ABCB1-mediated dye efflux (40, 41); LSCs occupy hypoxic bone marrow niches which may contribute to ABCB1 expression and chemoresistance (42). Given the abundance of evidence supporting a role for ABCB1 in drug resistance, the lack of success of clinical trials of ABCB1 inhibitors is puzzling. A potential explanation is suggested by our observation that exposure of leukemia cells with primed ABCB1 enhancers to daunorubicin leads to rapid and substantial upregulation of ABCB1, with escape of a leukemia cell sub-population from the effects of drug efflux pump inhibition.

The emerging role of the ISR as driver of adaptation and survival in cancer has led to interest in pharmacological manipulation of this pathway. We found that stress-induced upregulation of ABCB1 could be mitigated by use of the Mek inhibitor U0126 alone or in combination with ISRIB, suggesting a possible therapeutic strategy for testing in early phase trials. Given that the output of the ISR is dependent on the precise state of each cell there is a risk that a therapy designed to promote apoptosis may inadvertently drive adaptation and survival in a subset of cells. The precise function of ABCB1 as an effector of adaptive stress signalling also needs to be defined. We also found that ABCC1 expression was induced by daunorubicin exposure in fresh primary AML cells and that intronic regions likely bind the same transcription factors that drive ABCB1 expression. The evolution of the ABC superfamily has involved gene duplication and members presumably share previously unrecognised regulatory features (43). ABC transporters are also highly evolutionarily conserved, contributing to both nutrient import and multidrug resistance in bacteria (44). These pumps efflux a wide range of endogenous compounds and have been shown to influence paracrine signaling, membrane lipid composition and cellular redox state (45). It is therefore likely that expression of ABCB1 has physiological effects that mitigate certain forms of stress. In fact, the removal of chemotherapy from leukemia cells may simply be an unfortunate by-product of its primary function.

In summary, we show that cellular stress can drive chemoresistance through ABCB1 enhancers, providing an explanation for the failure of clinical trials of ABCB1 inhibitors and
suggesting an approach to overcome drug resistance. This study has implications for the study of resistance mechanisms more generally as these data demonstrate that the behaviour of cancer cells is highly dependent on cell context and environmental factors. Studies of cells in steady state alone may be potentially misleading.
Methods

Cell culture
K562 cells were from DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 2mM L-Glutamine (Life Technologies, Carlsbad, CA) and 10% fetal bovine serum (Sigma Aldrich). Whilst under drug selection cells were counted and replated every third day. Cell lines were confirmed mycoplasma-free and authenticated by short tandem repeat DNA profiling.

Primary AML samples
Primary human AML samples were from the Manchester Cancer Research Centre Tissue Biobank (approved by the South Manchester Research Ethics Committee). Their use was authorized by the Tissue Biobank’s scientific sub-committee, with the informed consent of donors. For ChIP, selected samples were thawed or collected fresh and immediately crosslinked. For treatment with daunorubicin, fresh leukemic blast cells were obtained by density gradient centrifugation of bone marrow or peripheral blood. Cells were treated in α-MEM medium supplemented with 12.5% heat-inactivated FBS, 12.5% heat-inactivated horse serum, 2mM L-glutamine, 57.2µM β-mercaptoethanol, 1µM hydrocortisone (Sigma Aldrich) and IL-3, G-CSF and TPO (all at 20ng/ml; Peprotech, Rocky Hill, NJ).

Reagents
Daunorubicin, verapamil and ISRIB were from Sigma Aldrich; tosedostat and tariquidar were from Generon (Slough, UK); and thapsigargin and U0126 were from Merck (Kenilworth, NJ). Compounds were resuspended in DMSO (tosedostat, tariquidar, thapsigargin, ISRIB, U0126) or ddH2O (verapamil and daunorubicin), aliquoted and stored at -20°C. Final DMSO concentration was <0.5% in all experiments.

Cell viability assays
5x10^3 cells were plated in each well of a 96-well plate with media containing a serial dilution of daunorubicin. Plates were incubated for 72hr at 37°C. 20µl of 140µg/mL resazurin (Sigma
Aldrich) was added to each well. Plates were then incubated for a further four hours and read using a POLARstar Omega plate reader (BMG Labtech, Aylesbury, UK).

**RNA sequencing and data analysis**

Total RNA was extracted from 5x10^5 cells using QIAshredder spin columns and an RNeasy® Plus Micro kit (Qiagen, Manchester, UK). Prior to sequencing RNA integrity was checked using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). PolyA libraries were prepared using a SureSelect PolyA kit (Agilent Technologies), samples were then barcoded and pooled. Sequencing was performed using a NextSeq desktop sequencing system (Illumina, San Diego, CA). A single run (400M reads) of 75bp paired-end sequencing produced a mean of 45.7M reads per sample. Reads were aligned to the human genome (hg38) using STAR v2.4.2a (46). DEseq2 was used to perform differential gene expression analysis and calculate FPKM (fragments per kilobase of transcript per million mapped reads) values for each transcript (47). Hierarchical clustering, similarity matrix and heatmap visualisations were created using clustergrammer (48). Principal component analysis (PCA) was performed using ggplot2 (49). Gene set enrichment analyses (10) were performed with GSEA v2.0 software (http://www.broad.mit.edu/gsea) using signal-to-noise for gene ranking and 1000 data permutations. To identify ATF4, CEBPB, CEBPG, ATF3, JUN or JUNB target genes, K562 ChIPseq datasets were downloaded from the ENCODE consortium (14). The strongest peaks by pileup value were identified by Model-based Analysis of ChIPseq v2 (MACS2) using default parameters (Table S2) (50). Gene expression and ABCB1 correlations in sorted cord blood populations were analysed using data from Laurenti et al. (26). Raw data files for RNA sequencing are available at the Gene Expression Omnibus with the accession number GSE131825.

**Quantitative PCR**

cDNA was generated using a High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA). qPCR reactions were performed in MicroAmp® optical 384-well reaction plates and analysed using a QuantStudio® 5 PCR system (Applied Biosystems). Reactions were performed in triplicate or quadruplicate and included primers for β-Actin (ACTB) as a
housekeeping gene. Primers were designed using the Universal Probe Library (UPL) Assay Design Center (Roche, Basel, Switzerland) and purchased from Integrated DNA Technologies (Coralville, IA). Raw fluorescence data was converted to Ct values using the Thermo Fisher Cloud facility (Waltham, MA) and normalised to ACTB. For primer sequences and associated probes see Table S5.

**Fluorescent-Activated Cell Sorting (FACS), flow cytometry & assessment of calcein-AM retention**

Flow cytometry was performed using an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ). A FACS Aria II (BD Biosciences) was used for cell sorting experiments. FlowJo v10.1 (BD Biosciences) was used to analyze data. To assess calcein AM retention 5x10^5 cells were resuspended in PBS containing 10nM freshly prepared calcein AM (Biolegend, San Diego, CA) with 40µM verapamil, 5 or 50nM tariquidar or vehicle. Samples were incubated for 20 minutes at 37°C, then resuspended in pre-warmed culture medium and incubated for a further 10 minutes to ensure optimal retention. Calcein AM accumulation was assessed by flow cytometry. ABCB1 expression was assessed using CD243-PE or CD243-APC (clone UIC2, eBioscience, Waltham, MA), the latter being used when cells were treated with daunorubicin which has similar excitation and emission spectra to PE.

**Chromatin immunoprecipitation (ChIP) and next generation sequencing**

ChIP was performed using anti-H3K27Ac (ab4729, Abcam, Cambridge, UK) and anti-H3K9me3 (ab8898, Abcam). 10^8 cells were used for each precipitation using the method described by Lee et al. (51). Briefly, cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature before the reaction was quenched with 0.125M glycine. Cell pellets were washed twice with PBS and nuclear lysates sonicated for 6 cycles using a Bioruptor® Pico (Diagenode, Liege, Belgium). 10µg of antibody bound to 100µl of magnetic beads (Dynabeads Protein G, Invitrogen, Carlsbad, CA) was added to each sample and immunoprecipitation performed overnight on a rotator at 4°C and 20rpm. After five washes with RIPA buffer (50mM HEPES pH 7.6, 1mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5M LiCl), chromatin IP-bound fractions were extracted by incubating for 15 min at 65°C with elution buffer (50mM TrisHCl pH8, 10mM EDTA, 1% SDS). Crosslinking was then reversed
by incubation at 65°C for 6 hours. RNaseA (1mg/ml) and proteinase K (20mg/ml) were added to eliminate RNA and protein from the samples. DNA was extracted using phenol:chloroform:isoamyl alcohol and precipitated by adding 2 volumes of ice-cold 100% ethanol, glycogen (20µg/µl), 200mM NaCl and freezing at -80°C for at least 1hr. Pellets were washed with 70% ethanol and eluted in 50µl 10mM TrisHCl pH8.0.

Libraries were prepared for sequencing using a Microplex Library Preparation Kit (Diagenode). 200-800bp fragments were selected using AMPure beads (Beckman Coulter, Brea, CA) and quantified by qPCR with a KAPA Library Quantification Kit (Kapa Biosystems, Basel, Switzerland). Sequencing was performed using a NextSeq desktop sequencing system (Illumina) with 75bp, paired-end high output generating 40-65M reads per sample. Reads were aligned to the human genome (hg38) using BWA-MEM v0.7.15 (52). Read duplicates were removed using Picard v2.1.0. Reads were further filtered using Bedtools v2.25.0 to keep only paired reads that mapped to standard chromosomes and to remove reads with a mapping quality of less than 10. Reads mapped to blacklisted regions defined by ENCODE were then removed using Bedtools (http://mitra.stanford.edu/kundaje). To define H3K9 trimethylation caused by dCas9-KRAB we subtracted non-targeting control reads from each sgRNA track using the BAMcompare function from deepTools2 (53). Results were correlated with ChIPseq from ENCODE (Table S3) and publically available DNase I hypersensitivity site (DHS) data (14, 23). Motif analysis was performed using JASPAR (http://jaspar.genereg.net). Raw data files for ChIP sequencing are available at the Gene Expression Omnibus with the accession number GSE131825.

Chromatin immunoprecipitation (ChIP) PCR
ChIP was performed using anti-H3K27Ac (ab4729, Abcam), anti-ATF4 (ab23760, Abcam), anti-ATF3 (D2Y5W, Cell Signaling Technology, London, UK), anti-c-JUN (60A8, Cell Signaling Technology), anti-JUND (D17G2, Cell Signaling Technology) and anti-CEBPB (ab322588, Abcam). Cells were cross-linked using ChIP Cross-link Gold (C01019027; Diagenode) for 30 mins in PBS with 1mM MgCl₂ and then with 1% formaldehyde for 10 min. The reaction was then quenched with 0.125M glycine. Cell pellets were washed twice with cold PBS containing protease inhibitors (Complete EDTA-free tablets, Roche). 10 million cells
were used per ChIP, as described in the protocol reported by Lee et al. (51). Nuclear lysates were sonicated using a Bioruptor Pico (Diagenode) for either 10 (K562) or 8 (BB953 and BB946) cycles. Immunoprecipitation was performed overnight at 20 rpm and 4°C, with 10ul magnetic beads (Dynabeads (Protein G), Invitrogen) per 1µg antibody. Washing and DNA extraction was performed as for ChIP sequencing. For ChIP quantitative PCR assays were performed in 384-well MicroAmp optical reaction plates using Taqman Fast Universal PCR Mastermix (Life Technologies) and Universal Probe Library System designed primers and probes (Roche). Signal was detected using an ABI PRISM 7900HT Sequence Detection System (Life Technologies). For primer sequences and associated probes see Table S6.

4C sequencing

4C primer sequences and enzyme combinations were selected using the University of Chicago online tool (http://mnlab.uchicago.edu/4Cpd) with co-ordinates from the ABCB1 promoter active in K562_R1-3 cells (hg38, chr7:87,598,302-87,601,399). 4C sequencing was performed according to the protocol developed by Splinter et al. (54). Briefly, 10^7 cells were cross-linked with 2% formaldehyde for 10 minutes at room temperature before the reaction was quenched with 0.125M glycine. Cells were lysed with buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1% TX-100 and 1x complete protease inhibitors (Roche, #11245200). The cross-linked nuclear preparation was then incubated with DpnII. Digestion was confirmed by reversing crosslinking for an aliquot and running on a 0.6% agarose gel. Samples were then ligated overnight at 16°C using T4 DNA ligase (Roche, #799009). Ligation efficiency was again confirmed with 0.6% agarose gel. Crosslinking was reversed and DNA extracted using phenol-chloroform, samples were then subjected to a second digestion using Csp6I. Ligation was again performed overnight at 16°C using T4 DNA ligase, DNA was then extracted using phenol-chloroform and purified with a QIAquick PCR purification kit (Qiagen, #28104). PCR primers were designed to incorporate 4C primers with a barcode and Illumina adapter sequences:

Reading primer: 5’ P5-Barcode-Primer 3’
Non-reading primer: 5’ P7-Primer 3’
CRISPR-dCas9-KRAB enhancer silencing

CRISPR guides were designed using Off-Spotter (https://cm.jefferson.edu/Off-Spotter/), using putative enhancer sequences from K562_R1-3 H3K27ac ChIP sequencing data. Several guides were selected for each enhancer and the promoter to allow preliminary screening of sgRNA activity. Guides were chosen to provide relatively even coverage across each enhancer and targeting of both DNA strands (Figures S3A-D). Primers incorporating the sgRNA sequence were designed as follows (primer sequences are shown on Table S7):

\[
\begin{align*}
5' & \text{CACC} & \text{XXXXXXXXXXXXXXXXXXXXXXX} & 3' \\
3' & \text{XXXXXXXXXXXXXXXXXXXXXXXXX} & \text{AAAA} & 5'
\end{align*}
\]
Primers were annealed by heating reagents A (Table S8) to 98°C for 5 minutes then allowing slow cooling by removing the heat block from the heating element until equilibrated to room temperature. Annealed primers were then ligated into pLKO5.sgRNA.EFS.tRFP657 (Addgene (Watertown, MA) #57824) using combined digestion-ligation with BsmBI and T4 DNA Ligase (Promega (Madison, WI) #M180A). Reagents B (Table S8) were heated to 55°C for 2 hours, reagents C (Table S8) were then added and the temperature reduced to 37°C for 1 hour. Lentivirus was produced using 293FT packaging cells (Life Technologies) cultured in DMEM (Sigma Aldrich) with 10% fetal bovine serum. 4µg of vector was added to 1ml DMEM with 21µl polyethylenimine (Polysciences, Warrington, PA), 2µg pCMVd8.91 and 1µg pMD2.G. The mixture was incubated for 30 minutes at room temperature then added drop wise to a 10cm dish containing 75% confluent 293FT cells, medium was replaced after 24 hours. Conditioned medium containing lentivirus was collected at 48 and 72 hours after transfection, packaging cells were removed using a 0.45µm filter. K562_R1-3 were reselected for 7 days with 500nM daunorubicin to ensure high-level ABCB1 expression prior to lentiviral transduction with pHHR-SFFV-dCas9-BFP-KRAB (Addgene, #46911). Transduction was performed by resuspending 2x10⁶ K562 cells in fresh viral supernatant containing 8µg/ml polybrene. After 24 hours the medium was exchanged to remove the virus. Seven days later a second transduction was performed using lentivirus containing ligated pLKO5.sgRNA.EFS.tRFP657. After five days expression of mTagBFP, tRFP657, ABCB1 and calcein AM retention were assessed by flow cytometry. All sgRNAs were screened for activity using K562_R3 (Figures S3E-F). The most active guide for each enhancer was then used to transduce dCas9-KRAB+ K562_R1-3. Flow cytometry, RNA extraction and ChIP were then performed on days 5, 7 and 10 following transduction respectively. A further assessment of mTagBFP, tRFP657 and ABCB1 expression were made on day 13 to confirm stable expression (Figure 3B).

Assessment of ABCB1 ATPase activity

The Pgp-Glo Assay System™ (Promega, #V3601) was used to assess the ability of tosedostat to induce ABCB1 ATPase activity. The assay was performed as described in the product literature. Briefly, Na₃VO₄ (0.1mM), verapamil (0.2mM) or tosedostat (0.2mM) were
incubated for 40 minutes at 37°C with 5mM ATP and membranes containing recombinant ABCB1. Residual ATP was then assessed by adding Ultra-Glo™ luciferase and incubating at room temperature for 20 minutes. Luminescence was quantified using a GloMax-Multi detection system (Promega). Na3VO4 inhibits ABCB1 ATPase activity providing a negative control. Verapamil is a known ABCB1 substrate, inducing ATPase activity and providing a positive control.

**Statistics**

For flow cytometry, quantitative PCR, ChIP-PCR and luciferase assays, statistical significance was determined using the unpaired, two-tailed Student’s t-test when comparing two experimental groups, or with one-way ANOVA with Tukey’s correction when comparing 3 or more groups. All tests were performed in Prism 8 (GraphPad). p-values of <0.05 were considered statistically significant. The statistical methods used to analyze next generation sequencing data are detailed in the relevant sections of the methods.

**Study approval**

Primary human AML samples were from the Manchester Cancer Research Centre Tissue Biobank (approved by the South Manchester Research Ethics Committee). Their use was authorized by the Tissue Biobank’s scientific sub-committee, with the informed consent of donors.
Author contributions

MW & TS designed the study and MW & FS performed experiments. MW, FA and TS performed bioinformatics analyses. MW and TS wrote the manuscript. All authors read and approved the final version of the manuscript.

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Declaration of interests

The authors have declared that no conflict of interest exists.
References


Figure 1

A

K562_S1
K562_S2
K562_S3
NO DRUG
ESCALATING DAUNORUBICIN
NO DRUG
14 days
106-117 days
10 days

Twice weekly cell count

Cell numbers increased → Increase [dauno] by 25-50%
Cell numbers unchanged → No change in [dauno]
Cell numbers reduced → Reduce [dauno] by 25-50%

B

% Survival

[Daunorubicin] µM

0.001
0.01
0.1
1
10
100
1000
10000
100000
1000000

0
20
40
60
80
100
120

C

Daunorubicin IC₅₀ (µM)

Sensitive
Resistant

D

-log₁₀ p-value

ABC B1

E

Fold change

F

log₁₀ relative ABCB1 expression

K562_1
K562_2
K562_3

Sensitive
Resistant

H

Count

Cell numbers increased
Increase [dauno] by 25-50%
Cell numbers unchanged
No change in [dauno]
Cell numbers reduced
Reduce [dauno] by 25-50%

I

Calcein

Sensitive
Resistant
Resistant + verapamil
Resistant + tariquidar

J

Log₁₀ relative ABCB1 MFI

Relative ABCB1 MFI

K562_1
K562_2
K562_3

Sensitive
Resistant
Resistant + verapamil
Resistant + tariquidar

***
***
***
***
***
***
***
***
***
***

G

Log₁₀ calcein MFI

K562_1
K562_2
K562_3

Sensi/ve
Resistant
Resistant + verapamil

H

log₁₀ fold change

K562_S1
K562_S2
K562_S3

***
***
***
***
***
***
***
***
***
***

I

Count

Calcein

Log₁₀ calcein MFI

K562_1
K562_2
K562_3

Sensi/ve
Resistant
Resistant + verapamil
Resistant + tariquidar

***
***
***
***
Figure 1. Resistance to daunorubicin due to stereotypical induction of ABCB1

(A) Outline of experiment. (B) Dose response curves for sensitive and resistant lines following 72hr treatment with the indicated dose of daunorubicin. (C) Bar chart shows mean±SEM IC_{50} values for daunorubicin for all lines (n=4). ***p<0.001 by unpaired t-test. (D) Volcano plot shows differential gene expression between sensitive (K562_S1-3) and resistant (K562_R1-3) cell lines. (E) ABCB1 is the most highly up regulated gene in each resistant line compared with their sensitive parental line. (F) Mean±SEM fold increase in ABCB1 expression, as determined by quantitative PCR (n=4). ***p<0.001 by unpaired t-test. (G) Mean±SEM fold increase in ABCB1 median fluorescence intensity (MFI), as determined by flow cytometry (n=3). ***p<0.001 by unpaired t-test. (H-I) Representative flow histograms show calcein AM retention in the indicated lines in the presence or absence of (H) verapamil 40µM or (I) tariquidar 50nM. (J) Summary of calcein AM retention data for all three line pairs for verapamil and tariquidar (n=3).
Figure 2

A

B

C

D

E

<Figure 2>

Sample Clustering

##[,1] [,2] [,3]
## [1,] 255 0 0
## [2,] 255 51 51
## [3,] 255 102 102
## [4,] 255 153 153
## [5,] 255 204 204
## [6,] 255 255 255
## [7,] 204 204 255
## [8,] 153 153 255
## [9,] 102 102 255
## [10,] 51 51 255
## [11,] 0 0 255
## [1] "#FF0000" "#FF7F7F" "#FFFFFF" "#7F7FFF" "#0000FF"

K562
KAS1
MOLM13

resistance
sensitive
resistant

Higher expression in resistant

Higher expression in sensitive

Higher expression in resistant

ATF4 TARGET GENES

NES 3.2 FDR 0.0%

Higher expression in resistant
Figure 2. Daunorubicin-resistant leukemia cells express a common ISR-like gene signature

(A) Similarity matrix and hierarchical clustering of samples by differential gene expression. (B) Principal component (PC) analysis of gene expression from all sensitive and resistant cell lines. (C) Heatmap shows differentially expressed genes (223 upregulated and 154 genes as downregulated; t-test, p<0.01, fold change >2 or <0.5). (D-E) Gene set enrichment analysis plots.
Figure 3

A

50 kb | hg38

ABCB1

E1 E2 C1 E3 C2 P C3 E4

H3K27ac

H3K4me3

4Cseq

K562_S1
K562_S2
K562_S3
K562_R1
K562_R2
K562_R3
Liver
Adrenal
CD34+ HSPCs
CD34+ HSPCs

B

D-15 D-7 D0 D5 D7 D10 D13

K562_R1-3

DAUNO

pHR-SFFV-dCas9-BFP-KRAB

pLKO5.sgRNA.EFS.tRFP657

C

50 kb | hg38

ABCB1

E1 E2 E3 P E4

H3K27ac

H3K4me3

98.5%

median 20th centile 80th centile 10.1 0.01 0.001
Figure 3. Regulatory element landscape of ABCB1

(A) ChIPseq tracks for H3K27Ac and H3K4Me1 surrounding ABCB1 (chr7:87,495,508-87,626,404; hg38) in the indicated human cells and tissues, including CD34+ hematopoietic stem and progenitor cells. Putative enhancers (E1-4) are highlighted in blue. Lower track shows a local contact profile generated from 4C sequencing of K562_R1 using a viewpoint centred on the ABCB1 promoter. Regions of contact that do not contain an active enhancer in K562_R1-3 are highlighted in red (C1-3). (B) Experimental outline (left panel); and representative flow cytometry plot (right panel) showing double positive population (blue - K562_R1 BFP+ RFP+) and negative control population (red). (C) H3K9me3 ChIPseq tracks for each sgRNA; signal from empty vector was subtracted to show only histone methylation resulting from presence of the guide. Red arrows indicate the position of the target sequence. H3K27Ac ChIPseq tracks from (A) are included for reference.
**Figure 4**

(A) Relative ABCB1 expression for different treatments. Bars indicate the mean ± SD. 

(B) Relative ABCB1 MFI for different treatments. Bars indicate the mean ± SD. 

(C) Regulation of transcription factors CEBPB, ATF4, and JUN. 

(D) Relative ChIP signal for different treatments and transcription factors. Bars indicate the mean ± SD.
Figure 4. Expression of ABCB1 is regulated by a stress-responsive enhancer

(A) Mean±SEM ABCB1 expression by quantitative PCR in dCas9-KRAB+ resistant cell lines (K562_R1-3) expressing sgRNAs targeting the indicated putative enhancer elements (E1-4) or the promoter (P), relative to control cells expressing a non-targeting guide (Ctl). **p<0.01 by one way ANOVA with Tukey post hoc test (n=4). (B) As for (A), but with mean±SEM ABCB1 median fluorescence intensity (MFI) by flow cytometry. **p<0.01 by one way ANOVA with Tukey post hoc test (n=3). (C) ChIPseq tracks for H3K27Ac, H3K9Me3 (our data) and the indicated transcription factors in K562 cells (ENCODE); and DNAse-seq (ENCODE) at the E3 enhancer. Sites of AP-1 binding motifs are indicated. (D) Mean±SEM relative ChIP PCR signal for the indicated transcription factors for K562_R1 and K562_S1 using primers for the E3 enhancer. **p<0.01, ***p<0.001 by unpaired t-test (n=3).
**Figure 5. Dynamic induction of ABCB1 by diverse cellular stressors**

(A-B) Mean±SEM expression of the indicated genes by quantitative PCR relative to a fresh aliquot of unmanipulated drug-sensitive K562 cells (n=3) following exposure to (A) tosedostat 50µM for 48hr or (B) daunorubicin (100nM for sensitive or 500nM for resistant lines, for 72hr). *p<0.05, **p<0.01, ***p<0.001 by unpaired t-test. (C) ABCB1 mean fluorescence intensity (MFI) over time in K562_R1 cells maintained in high or low density culture. Numbers indicate cell density (k/ml). (D) ABCB1 MFI in K562_R1-3 following 14 days of high or low density culture (n=3). **p<0.01, ***p<0.001 by unpaired t-test. (E) Mean±SEM ABCB1 MFI in dCas9-KRAB⁺ resistant cells (K562_R1) expressing either an E3 targeting sgRNA or a non-targeting sgRNA (EV) following 7 days of exposure to the indicated dose of daunorubicin (n=3), ***p<0.001 by unpaired t-test.
Figure 6

A

Log$_{10}$ relative ABCB1 expression

10 nM

100 nM

1000 nM

B

Relative ATF4 expression

100

10

1

0.1

Log$_{10}$ relative ABCB1 expression

r = 0.53

p = 0.005

C

10 kb

hg38

ABCB1

K562_R1

Liver

Adrenal

t(8;21)-1

inv(16)-3

ITD/NPM1-5

ITD-1

- TGA TGG TAA TCC

C1

E3

DHS

H3K27ac

D

10 kb

hg38

ABCB1

K562_R1

Liver

Adrenal

RUNX1-T-7

CEBPA(x2)-1

t(8;21)-1R

ITD-1

CEBPA(x2)-3

H3K27ac

CHIS

H3K27ac

E

CEBPB

ATF4

JUN

F

Vehicle

100 nM

10 nM

1000 nM

BB889 1B

G

Relative ChIP signal

Vehicle

Daunorubicin

C1

E3_1

E3_2

BB946 2B

BB953 2B
Figure 6. Daunorubicin activates a stress-responsive ABCB1 enhancer in primary AML cells

(A) ABCB1 expression by quantitative PCR in primary AML samples (n=3). H3K27Ac ChIP sequencing was performed on the samples highlighted in red. (B) Correlation of ATF4 and ABCB1 expression. $r =$ Pearson product-moment correlation coefficient. (C-D) ChIPseq (our data) and DNase-seq tracks (23) surrounding (C) C1 and E3 (chr7:87,561,371-87,579,610; hg38) and (D) A, B, E1 and E2 (chr7:87,494,187-87,522,854; hg38) from the indicated cell line, human tissue (ENCODE) and primary AML samples. (E) TF binding motifs identified at the centre of site B. (F) Mean±SEM relative expression of the indicated genes following exposure of fresh primary AML blast cells to the indicated doses of daunorubicin for 18hr (n=3). BB numbers indicate Biobank identifier. (G) Mean±SEM relative ChIP PCR signal for H3K27ac using fresh primary AML blast cells exposed to 1000nM daunorubicin or vehicle for 18hr (n=3). Data from two patients (BB946 & BB953) are shown. PCR was performed using two primer sets for the E3 enhancer (E3_1 & E3_2) and one for the CTCF binding site C1. *p<0.05, **p<0.01, by unpaired t-test. BB numbers indicate Biobank identifier.
Figure 7. Activation of an ISR-like response facilitates escape from ABCB1 inhibition

(A) Mean±SEM relative ABCB1 MFI in K562_R1-3 following exposure to 500nM daunorubicin or vehicle for 72hrs or 7 days (n=3). (B) Proportion of cells that are calcein AM negative following exposure of K562_R1-3 to the indicated conditions as determined by flow cytometry (n=3). (C) As for (B), but showing individual flow histograms for each of the indicated conditions. (D) Experimental outline depicting FACS of calcein AMneg and calcein AMpos populations. (E) Mean±SEM ABCB1 expression by quantitative PCR of calcein AMneg and calcein AMpos populations (n=3). **p<0.01, ***p<0.001 by unpaired t-test. (F) As for (E), but with relative ABCB1 median fluorescence intensity (MFI) by flow cytometry (n=3). (G) Mean±SEM ABCB1 expression by quantitative PCR in K562_R1 following exposure to 500nM daunorubicin or vehicle for 72hrs with the indicated inhibitors (n=3-6). *p<0.05 by one way ANOVA with Tukey post hoc test (n=3).
Table 1. The most significantly upregulated transcription factors in resistant versus sensitive lines. Genes are ranked by mean expression in resistant lines (fragments per kilobase of transcript per million mapped reads; FPKM).

<table>
<thead>
<tr>
<th>Gene</th>
<th>FPKM</th>
<th>Fold change</th>
<th>P-value</th>
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<tr>
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Table 2. Transcription factor binding motifs identified in a 30bp sequence taken from the H3K27 acetylation nadir at the centre of enhancer E3.

<table>
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<th>MOTIF</th>
<th>Score</th>
<th>Strand</th>
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<tbody>
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<tr>
<td>JUND(var.2)</td>
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<tr>
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<tr>
<td>ATF4</td>
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<td>HLF</td>
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<tr>
<td>GATA2</td>
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<tr>
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<tr>
<td>FOXC1</td>
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<tr>
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<tr>
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