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ORIGINAL ARTICLE

E2F1 drives chemotherapeutic drug resistance via ABCG2

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Multidrug resistance is a major barrier against successful chemotherapy, and this has been shown *in vitro* to be often caused by ATP-binding cassette (ABC) transporters. These transporters are frequently overexpressed in human cancers and confer an adverse prognosis in many common malignancies. The genetic factors, however, that initiate their expression in cancer are largely unknown. Here we report that the major multidrug transporter ABCG2 (BCRP/MXR) is directly and specifically activated by the transcription factor E2F1—a factor perturbed in the majority of human cancers. E2F1 regulates ABCG2 expression in multiple cell systems, and, importantly, we have identified a significant correlation between elevated E2F1 and ABCG2 expression in human lung cancers. We show that E2F1 causes chemotherapeutic drug efflux both *in vitro* and *in vivo* via ABCG2. Furthermore, the E2F1–ABCG2 axis suppresses chemotherapy-induced cell death that can be restored by the inhibition of ABCG2. These findings therefore identify a new axis in multidrug resistance and highlight a radical new function of E2F1 that is relevant to tumor therapy.

INTRODUCTION

During tumor development, a multitude of events occur, which influence disease progression and affect the efficacy of tumor therapy. An event that is considered extremely common in many cancers is perturbation of the transcription factor E2F1.[1,2] In normal cells, E2F1 is a major determinant of the commitment to cell cycle progression, and E2F1's activity is kept in check by the retinoblastoma protein, pRB.[3] In addition to its effects on cell cycle progression, E2F1 is best characterized for its role in apoptosis induction and has also been shown to regulate diverse processes such as differentiation and autophagy.[4,5] E2F1 mediates cell death largely by transcriptional activation of pro-apoptotic target genes or repression of anti-apoptotic genes and has been demonstrated to render cancer cells more susceptible to certain chemotherapeutic drugs.[6] It is currently not clear, however, as to what extent these and also as-yet unidentified activities of E2F1 may be relevant to E2F1's role in the pathogenesis and treatment of cancer.[7]

Multidrug resistance occurs in nearly all cancers and is a serious impediment to successful chemotherapy.[8] Major effectors of drug resistance are multidrug transporters that can be elevated and/or hyperactivated during the genesis of cancer. The ATP-binding cassette (ABC) multidrug transporters such as P-glycoprotein (MDR1/ABCB1), MRP1 (ABCC1) and ABCG2 (BCRP/MXR) are considered responsible for the majority of drug efflux in human cancer *in vitro*.[8,9] A high expression of ABC transporters has been repeatedly linked to an adverse prognosis in common forms of cancer.[10]

In order to understand drug resistance mediated by multidrug transporters, it is critical to know the factors that control their expression so that we can predict when the activity of any given transporter may be relevant. We report here the remarkable discovery that ABCG2 is under the control of E2F1 in multiple cell systems, and we show that the E2F1–ABCG2 axis regulates chemotherapeutic drug efflux and drug resistance. When coupled with the fact that E2F1 is frequently perturbed in human cancer, these findings represent a distinct new function for E2F1 that may be relevant for the development and treatment of human cancer.

RESULTS

To gain insights into the E2F1 function, we performed microarrays using tetracycline-inducible cells expressing either wild-type E2F1 or the DNA-binding defective mutant E2F1-E132 to identify genes induced by E2F1.[11] This intriguingly revealed that the highest E2F1-induced messenger RNA (mRNA) encodes the multidrug transporter ABCG2 (see Supplementary Information for microarray dataset). Direct mRNA and protein analyses in these cells showed that ABCG2 was strongly induced by wild-type E2F1 but not the mutant E2F1-E132 (Figures 1a and b and Supplementary Figures S1a and S1b). To test whether the induction of ABCG2 is a cell line-specific phenomenon, a panel of cell lines from different tissues were engineered to express E2F1 fused to the hormone-binding domain of the estrogen receptor. Tamoxifen-induced activation of E2F1-ER in these cells resulted in increased levels of ABCG2 mRNA and protein in all cases (Figures 1c and d). In contrast, no elevation of the other major ABC transporters was observed following the activation of E2F1 (Supplementary Figure S2).

To test whether ABCG2 is a direct target of E2F1, we used the E2F1-ER system. This revealed that E2F1 can induce ABCG2 mRNA levels in both the absence and presence of the protein synthesis inhibitor cycloheximide, indicating a direct relationship between E2F1 and ABCG2 (Supplementary Figure S3). In this regard, by infecting primary Tig3 lung fibroblasts with E1a, we also determined whether pRb function was modulating ABCG2 expression at different phases of the cell cycle, but this appeared not to be the case (Supplementary Figure S4). As a result, this caused us to consider whether ABCG2 is a direct transcriptional target of E2F1. Analysis of the ABCG2 promoter revealed a potential E2F1 binding site upstream of the transcription start site

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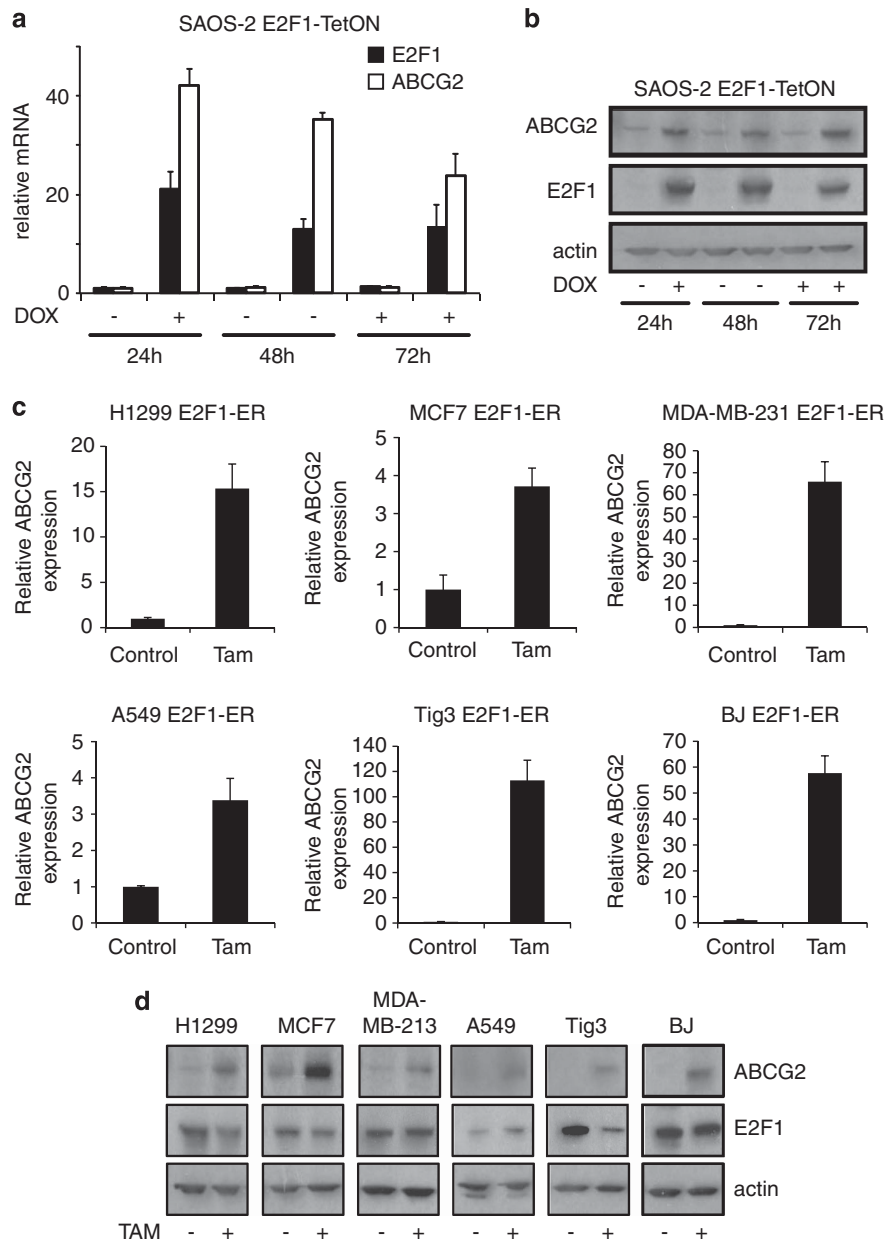E-mail: k.ryan@beatson.gla.ac.uk

Figure 1. E2F1 regulates ABCG2 expression. (**a, b**) Saos-2 TetOn E2F1 cells were induced with 1 μM doxycycline (DOX) for the indicated times. Levels of E2F1 and ABCG2 mRNA (**a**) and protein (**b**) were measured by western blotting. (**c, d**) A panel of human cell lines was infected with a retrovirus expressing E2F1-ER and were treated with 500 nM 4-hydroxytamoxifen (TAM) for 24 h to activate E2F1 or with EtOH as control. The effect on ABCG2 mRNA and protein levels was assessed by quantitative PCR (**c**) and western blotting (**d**).

(Figure 2a). To test whether this site was responsive to E2F1, cells were transfected with an expression vector for either wild-type E2F1 or E2F1-E132, together with luciferase reporter constructs containing increasing lengths from the ABCG2 promoter.[12] This revealed that the first 243 nucleotides upstream of the ABCG2 transcription start site are markedly responsive to E2F1 (Figure 2b). No activation was seen with the DNA-binding defective mutant E2F1-132E (Figure 2b). Moreover, deletion of the putative E2F1 binding site in the −243 construct rendered this construct unresponsive to E2F1 (Figure 2c). Finally, we performed chromatin immunoprecipitations (ChIPs) with E2F1, which confirmed that E2F1 indeed binds this region of the ABCG2 promoter (Figure 2d). The regulation of ABCG2 is a complex process, for example, alternative promotors and micro RNAs impact on ABCG2 expression in different cell types, and it cannot be ruled out that

E2F1 also interacts with these regulators.[10] However, our collective data show that ABCG2 is a direct target gene of E2F1.

The fact that E2F1 activates a multidrug transporter naturally caused us to consider whether E2F1 can cause efflux of chemotherapeutic drugs from cells via ABCG2. In this regard, we observed that ABCG2 accumulates at the cell membrane, following the activation of E2F1, making drug efflux highly likely (Supplementary Figure S5). Saos-2 TetOn E2F1 cells as well as MDA-MB-231 and BJ lung fibroblasts containing E2F1-ER were therefore tested for their ability to efflux the fluorescent chemotherapeutic mitoxantrone—a well characterized substrate of ABCG2.[9] In each case, activation of E2F1 with doxycycline (DOX) (TetOn E2F1) or 4-hydroxytamoxifen (TAM) (E2F1-ER) caused marked mitoxantrone efflux with a particularly marked effect being observed in BJ and MDA-MB-231 cells (compare blue line/
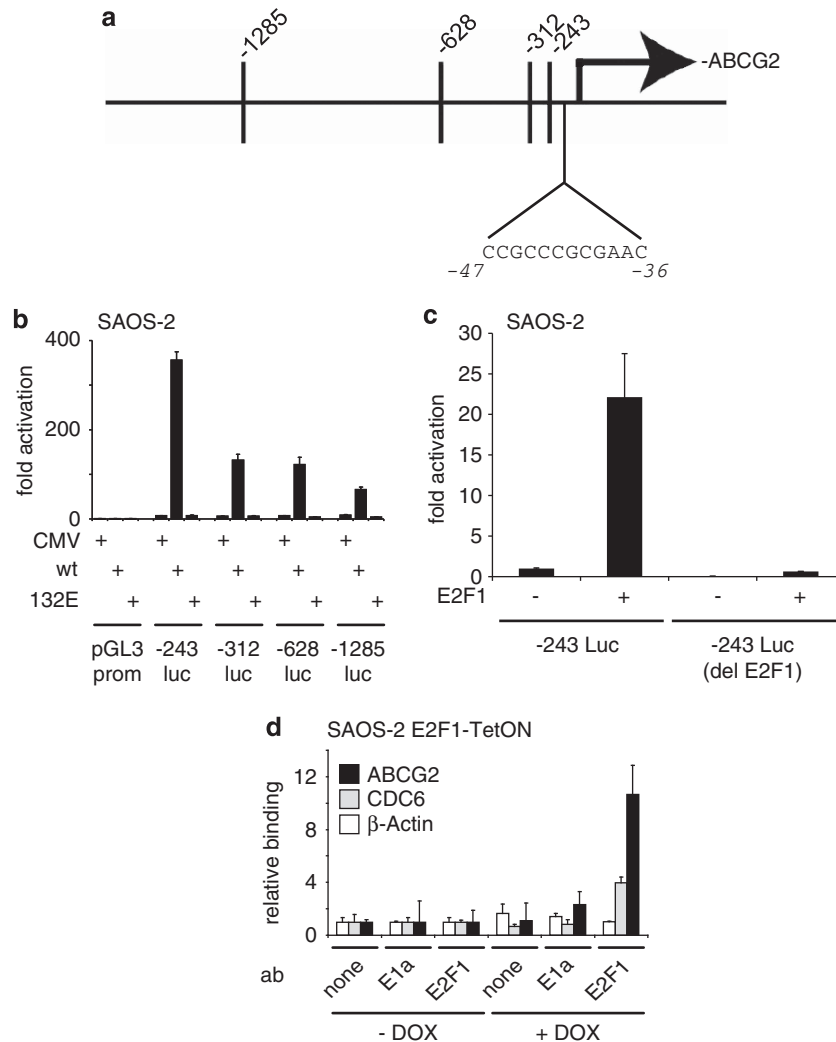
Figure 2. E2F1 regulates ABCG2 transcription via an element in the ABCG2 promoter. (**a**) Schematic of the ABCG2 promoter region. Reporter construct lengths are indicated. Sequence analysis also revealed a putative E2F1 binding site between − 36 and − 47 nucleotides upstream of the transcription start site. (**b**) Luciferase reporter constructs containing elements from the ABCG2 promoter as depicted in (**a**) were transfected into Saos-2 cells together with either wild-type E2F1 (wt), mutant E2F1 (E132) or CMV empty vector as control. Fold activation of each reporter is shown relative to CMV empty vector control. (**c**) − 243-Luc and a mutant of this construct with the putative E2F1 binding site deleted (del E2F1) were transfected into Saos-2 cells with either wild-type E2F1 or empty CMV vector as control. Fold activation of each reporter is shown relative to CMV-empty vector control. (**d**) ChIP assays were performed using the indicated antibodies (abs) on Saos-2 TetOn-E2F1 cells either in the absence or in the presence of 1 mM doxycycline (DOX) for 24 h. Promoter binding is shown relative to non-induced (− DOX) controls.

bar 'E2F1 ON' with green line/bar 'E2F1 OFF', $P < 0.01$) (Figure 3a). To investigate this effect further, efflux in MDA-MB-231 cells was compared with that in cells expressing E2F1-ER, E2F1-132E-ER, ABCG2 or an empty viral vector as control. As would be expected, maximal efflux was observed in cells expressing exogenous ABCG2, whereas no ABCG2 induction or efflux was observed in empty vector (pBabe) cells or cells expressing E2F1-132E-ER. This indicates that the E2F1-mediated induction of endogenous ABCG2 and drug efflux was specific for wild-type E2F1 (Figure 3b). Similar results were also obtained in BJ cells (Supplementary Figure 6).

To examine whether E2F1 can cause drug efflux *in vivo*, we took advantage of the fact that D-luciferin (the substrate for firefly luciferase) is a specific target of ABCG2, but not other drug transporters.[13] MDA-MB-231 cells were therefore engineered to express E2F1-ER and also the gene for luciferase. Cells were then injected into mice to form a xenograft. Groups of mice ($n = 5$ per group) were subsequently treated either with tamoxifen or ethanol as control. D-luciferin was then administered systemically to each group of mice, and luciferase activity was measured over time

in vivo as a readout of the efflux activity. Although the luciferase signal increased over time and plateaued after 40 min in control mice treated with ethanol, luciferase activity did not increase in mice that had been pre-treated with TAM to activate E2F1 (and therefore ABCG2) (Figure 3c). This indicates that the frequent elevation of E2F1 in tumors may well affect the availability, and therefore the efficacy, of a systemically administered drug.

To test whether E2F1-induced drug efflux was specifically mediated by ABCG2, we infected E2F1-ER cell lines with short hairpin RNAs (shRNAs) targeting ABCG2 or a scrambled hairpin (NTC) as control (Figure 4a).[14] shRNAs against ABCG2 effectively abolished ABCG2 induction, following the activation of E2F1 with TAM (Figure 4a). Strong efflux of mitoxantrone was observed following E2F1 activation in the cells containing the scrambled shRNA or the empty lentiviral vector (pLKO) (Figure 4b). In contrast, each of the lines containing a hairpin to knockdown ABCG2 underwent very little, if any, efflux following the activation of E2F1, confirming that efflux induced by E2F1 is mediated via ABCG2 (Figure 4b).
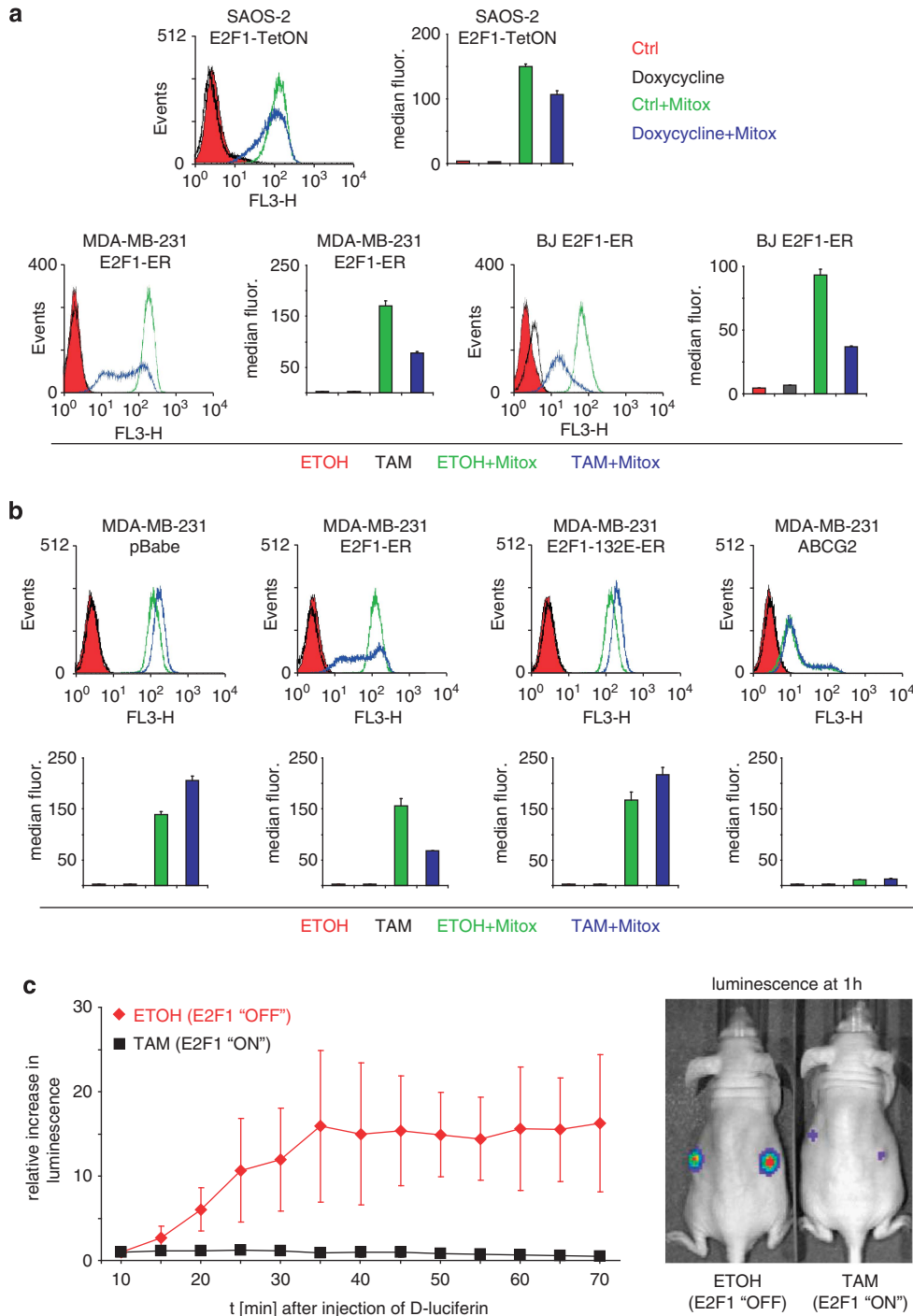
Figure 3. E2F1 causes drug efflux *in vitro* and *in vivo*. (**a**, **b**) Saos-2 TetOn-E2F1 cells as well as MDA-MB-231 and BJ cells that had been infected as indicated with retroviruses expressing E2F1, mutant E2F1 (132E), ABCG2 or pBabe as control were incubated with either doxycycline or 4-hydroxytamoxifen (TAM) as shown for 24 h. Cells were then incubated where indicated with 20 μM mitoxantrone for 1 h. Mitoxantrone was then removed and the extent of drug efflux (as determined by a decrease in fluorescence) was measured 3 h later by flow cytometry. Bar graphs (right of the corresponding histogram in (**a**) and below the corresponding histogram in (**b**)) represent the median fluorescence intensity of corresponding FACS profiles as a surrogate quantification of drug efflux. (**c**) Athymic mice ($n = 5$ in each group) were subcutaneously injected with luciferase- and E2F1-ER-expressing MDA-MB-231 cells to form xenografts. E2F1 was induced where indicated by intraperitoneal injection of TAM. Twenty-four hours later, mice were given an intraperitoneal injection of ᴅ-luciferin. After 10 min, mice were imaged every 10 min for 1 h and luminescence was recorded. Error bars indicate s.d. Representative images are of mice injected with either ethanol or TAM. The images are taken 1 h after injection of ᴅ-luciferin (**c**, right side).

A number of pharmacological inhibitors of multidrug resistance pumps have been described.[9] We decided to test, therefore, whether the ABCG2-specific efflux inhibitor Ko143[15] could revert efflux induced by E2F1. MDA-MB-231 E2F1-ER and BJ E2F1-ER cells

were treated with Ko143 and efflux of mitoxantrone measured as before. In both cell lines, the extensive efflux caused by E2F1 could be practically entirely reverted by treatment with Ko143, not only indicating that this effect of E2F1 can be successfully modulated
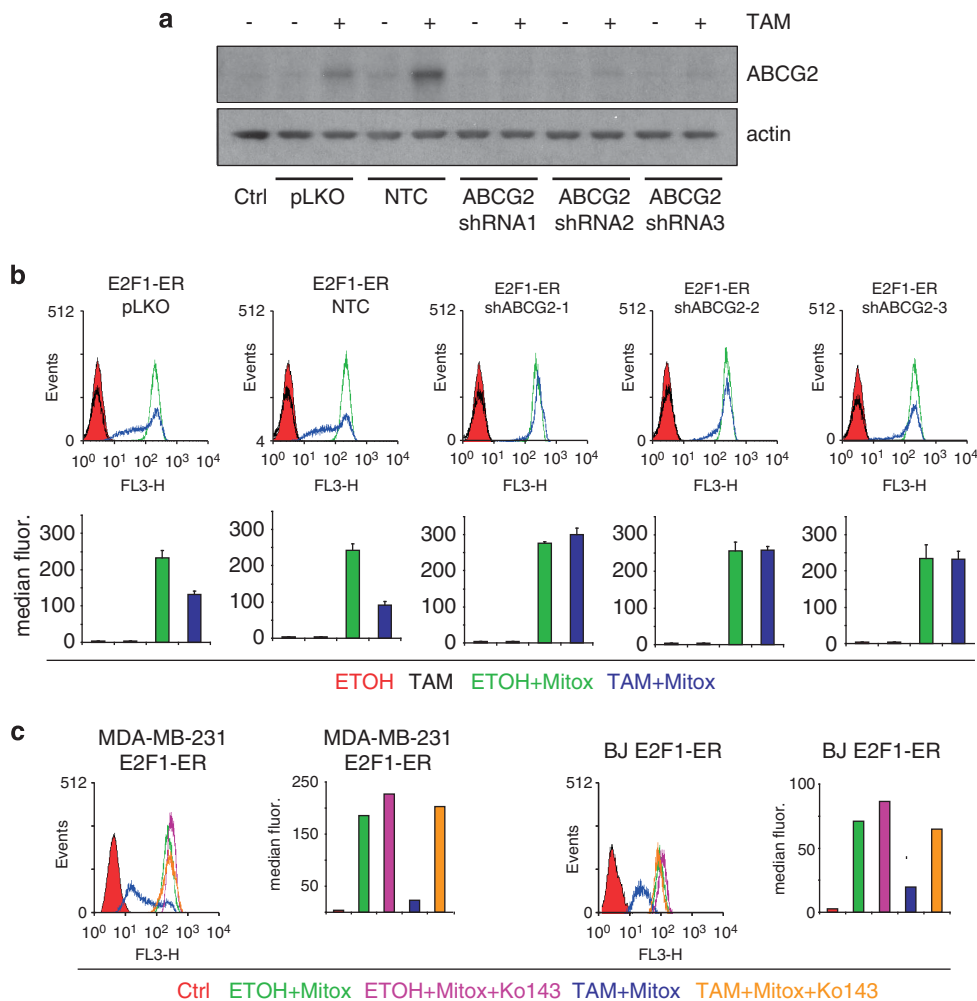
Figure 4. E2F1 causes drug efflux and resistance specifically via ABCG2. (**a**, **b**) MDA-MB-231 E2F1-ER cells were infected with shRNAs as indicated, and knockdown efficiencies were determined by western blotting (**a**). Cells were incubated with 4-hydroxytamoxifen (TAM) or EtOH as indicated for 24 h before incubation with 20 μM mitoxantrone (Sigma) for 1 h. Mitoxantrone was then removed and drug efflux measured 3 h later by flow cytometry (**b**). (**c**) MDA-MB-231 E2F1-ER and BJ E2F1-ER cells were incubated with TAM for 24 h. A concentration of 1 μM Ko143 was added 30 min before addition of mitoxantrone for 1 h. Mitoxantrone was then removed (Ko143 added back) and drug efflux measured 3 h later by flow cytometry. Bar graphs represent median fluorescence intensity of corresponding FACS profiles as a surrogate quantification of drug efflux. Quantification column graphs are beneath the corresponding histogram in (**b**) and to the right of the corresponding histogram in (**c**). pLKO, empty lentiviral vector. NTC, non-targeting control.

pharmacologically but also once again showing that E2F1-induced drug efflux is mediated significantly and primarily by ABCG2 (Figure 4c).

Our results implied that E2F1-induced drug efflux could actually reduce cell death caused by the administration of chemotherapeutic drugs. If so, the expression of E2F1 should cause resistance to a drug that is a known substrate of ABCG2, and this resistance should be reversed by inhibition of ABCG2. To test this BJ and MDA-MB-231 E2F1-ER cells were incubated with mitoxantrone either in the absence or presence of TAM, and cell death was determined by assessment of cell membrane permeability (Figures 5a and b). In both cases, activation of E2F1 could markedly reduce cell death from mitoxantrone ($P < 0.01$) and this cell death resistance could be reverted by the treatment with Ko143 ($P < 0.01$) (Figures 5a and b). Ko143 did not enhance cell death in the absence of mitoxantrone with or without the activation of E2F1. These data therefore show that the effectiveness of treating tumor cells with chemotherapeutic drugs can be markedly dampened due to the activation of ABCG2 by E2F1.

In light of our data showing that ABCG2 is regulated by E2F1 in many different cell types, we asked whether these observations
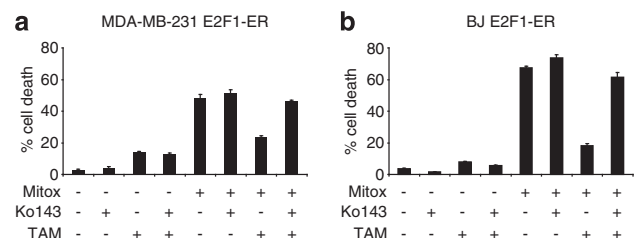

Figure 5. Activation of E2F1 impedes chemotherapy-induced death via ABCG2. (**a**, **b**) MDA-MB-231 E2F1-ER cells (**a**) and BJ E2F1-ER cells (**b**) were incubated with TAM or EtOH as indicated for 24 h. Mitoxantrone and Ko143 (or DMSO as control) were then added to cells for 14 h. Cells were collected and analyzed by flow cytometry for propidium iodide permeability (a sign of cell death).

were linked in human cancer. Lung cancer is the most common cancer worldwide, and lung tumors have been reported in separate studies to contain either high levels of E2F1 or ABCG2 with both markers individually indicating poor prognosis.[16,17] We therefore stained tissue microarrays containing non-small cell lung

cancer samples as well as normal lung for E2F1 and ABCG2. There was no significant staining for E2F1 and ABCG2 in normal lung tissue (Figure 6a). Similarly, tumor samples with normal levels of E2F1 rarely exhibited elevated staining for ABCG2 (Figure 6a). In contrast, tumors with areas of higher staining for E2F1 were frequently associated with high levels of ABCG2 in the same regions on sequential sections (Figure 6a). Across the entire tissue microarrays containing 54 tumors in total, 26 tumors had regions positive for high levels of E2F1 and 21 of these showed coincident elevated staining for ABCG2. Reciprocally, of 29 tumors with ABCG2 levels similar to normal lung, only 5 had regions with

elevated levels of E2F1 (Supplementary Tables S1 and S2). This E2F1 and ABCG2 association was found to be highly significant by Fisher's exact test ($P < 0.01$).

To test whether the correlation between E2F1 and ABCG2 levels in human lung cancer was causally related, we analyzed E2F1 and ABCG2 protein levels in a set of non-small cell lung cancer cell lines (Figure 6b). Knockdown of E2F1 by RNA interference in lines that express high levels of both E2F1 and ABCG2 caused a marked decrease of ABCG2, indicating that E2F1 was at least in part responsible for the elevated levels of ABCG2 in these cells (Figure 6c).
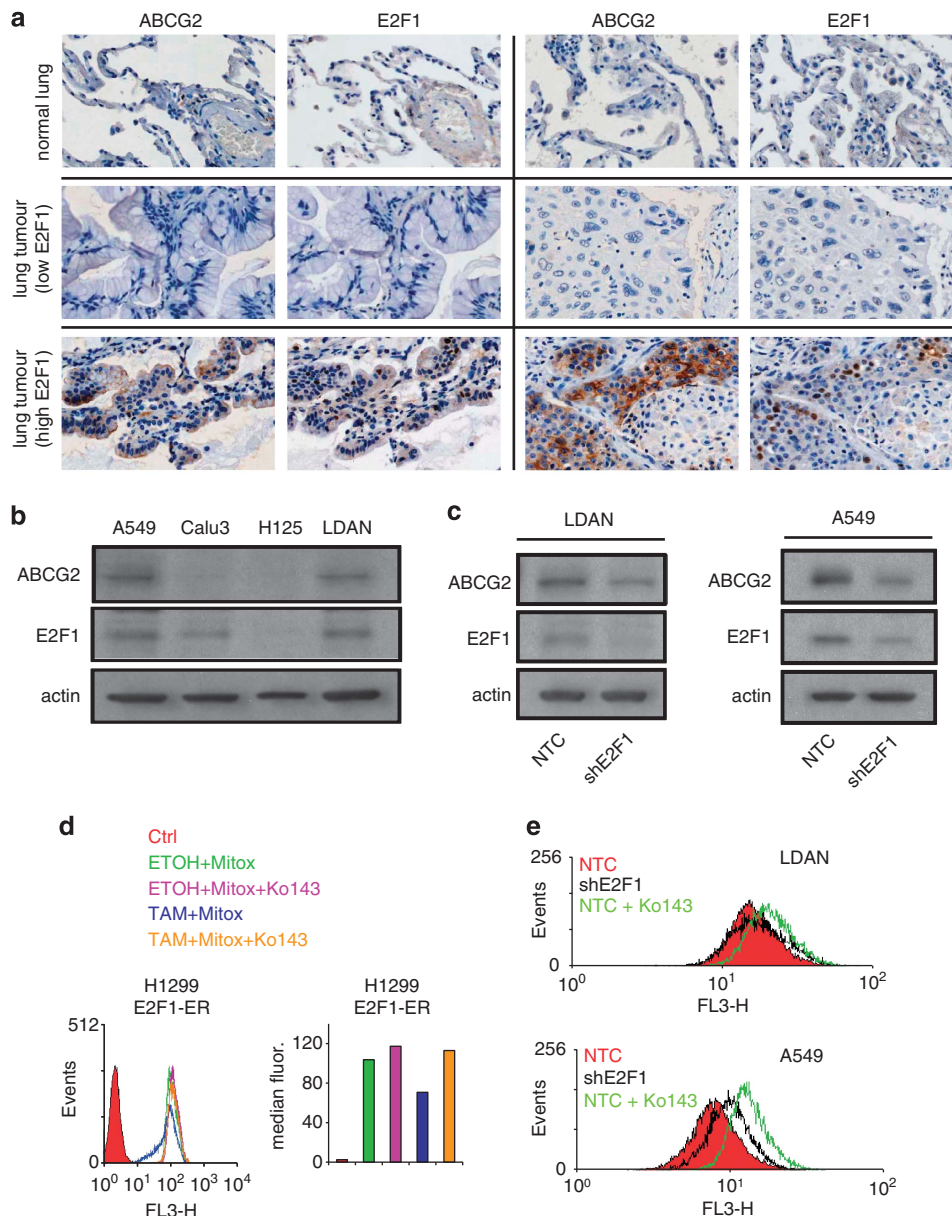

Figure 6. E2F1 is associated with ABCG2 expression in human lung cancer. (**a**) Tissue microarrays containing sequential sections from either normal lung ($n = 6$) or non-small cell lung cancer (NSCLC) tumors ($n = 54$) were stained with antibodies specific for E2F1 and ABCG2. (**b**) E2F1 and ABCG2 proteins levels were determined in a set of human lung cell lines by western blotting. (**c**) Lentiviral-mediated RNAi knockdown of E2F1 in A549 and LDAN cells results in a decrease in both E2F1 and ABCG2 protein levels as determined by western blotting. NTC, non-targeting control. (**d**) Non-small cell lung cancer H1299 cells were infected with E2F1-ER and were, where indicated, incubated with 4-hydroxytamoxifen (TAM) for 24 h. A concentration of 1 μM Ko143 was added 30 min before addition of mitoxantrone for 1 h, again where indicated. Mitoxantrone was then removed (Ko143 added back) and drug efflux measured 3 h later by flow cytometry. Bar graphs represent median fluorescence intensity of corresponding FACS profiles as a surrogate quantification of drug efflux. (**e**) LDAN and A549 cells containing E2F1 shRNA or control shRNA were incubated as indicated with Ko143 for 30 min before addition of mitoxantrone for 1 h, again where indicated. Mitoxantrone was then removed (Ko143 added back, where indicated) and drug efflux measured 3 h later by flow cytometry.

Finally, we tested whether E2F1 was involved in drug efflux in lung cancer cells. First, we infected H1299 non-small cell lung cancer cells with E2F1-ER, which revealed that the activation of E2F1 in these cells caused efflux of mitoxantrone and that this efflux could be completely reversed by the treatment with the ABCG2 inhibitor, Ko143 (Figure 6d). Similar results were also observed for the chemotherapeutic photosensitizer pheophorbide A (Supplementary Figure 7), which is a known ABCG2 substrate that has been shown to sensitize cells to apoptosis.[18,19] Second, we measured efflux in LDAN and A549 cells, as we had found that ABCG2 expression was at least partly dependent on E2F1 in these lines (Figure 6c). This showed that efflux was diminished as a result of E2F1 knockdown in both cell lines with E2F1 knockdown having an intermediate effect when compared with the treatment with Ko143, which causes maximal inhibition of ABCG2 (Figure 6e). When taken together and in line with the association between E2F1 and ABCG2 in human lung cancer, these data confirm that the E2F1/ABCG2 axis is active in human lung cancer cells.

DISCUSSION

In this study, we show that activation of ABCG2 by E2F1 causes drug efflux in multiple cell lines and in a xenograft model, and that this can lead to impedance of chemotherapy-induced cell death. Importantly, we also identify that E2F1 and ABCG2 protein levels are significantly colocalized in the most common form of lung cancer, and that endogenous ABCG2 levels are regulated by E2F1 expression in human lung cancer cell lines. Although both E2F1 and ABCG2 have previously been independently described to be poor prognostic indicators for lung cancer,[16,17,20] this is the first study to report that these two events are linked. Several other transcription factors have also been shown to regulate ABCG2,[10] but the fact that E2F1 is frequently elevated in human cancer means our findings add significantly to our understanding of how this multidrug transporter can be upregulated during tumor development. We therefore consider that the drug efflux via ABCG2 is a novel and may be a common phenotypic effect of activating E2F1.

A large number of reports have shown that E2F1 is a pro-death effector,[2] but more recently pro-survival roles for E2F1 have also been reported.[21] In particular, one report has shown that E2F1 can cause drug resistance via the activation of ΔN-p73, which leads to the repression of the microRNA, miR-205.[22] This microRNA controls the expression of Bcl-2 and the ABC transporters ABCA2 and ABCA5, such that when miR-205 expression is repressed, these factors are elevated and promote cell survival.[22] To the best of our knowledge, however, the study we present here is the first report to show that E2F1 directly activates one of the three major multidrug transporters implicated in cancer—ABCG2. As such, this study not only advances what we know about the regulation of ABCG2 but also highlights an extraordinary new function for E2F1 that may be relevant to both tumor development and cancer therapy. In this regard, this study may therefore help in the design of more targeted clinical trials that will ascertain the importance of the E2F1/ABCG2 axis in mediating impaired therapeutic responses and patient outcome following chemotherapy. In this regard, future studies should focus on ascertaining whether this efflux axis is active in a variety of tumor types and whether this affects the clinically relevant drugs for each disease.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Aldrich (Gillingham, UK) unless otherwise indicated. Ko143 was from Tocris Bioscience (R&D Systems Europe, Abingdon, UK) and D-Luciferin from Gold Biotechnology (St Louis, MO, USA). The ChIP Assay Kit was from Millipore (Watford, UK) and the human lung cancer tissue microarray was from Folio Biosciences (Columbus, OH, USA).

Plasmids

pTRE-E2F1 constructs were generated by cloning either wild-type E2F1 (pTRE-E2F1) or the DNA-binding defective E2F1 mutant 132E (TetOn-E2F1-132E) into the tetracycline-responsive plasmid pTRE (Clontech, Mountain View, CA, USA).

pBabe vectors (expressing E1a, wild-type E2F1 or E2F1-132E) fused to a tamoxifen-responsive element of the estrogen receptor (pBabe E1a-ER, pBabe E2F1-ER or pBabe E2F1-132E-ER) have been described previously.[23]

Luciferase reporter constructs were in pGL3 (pGL3 ABCG2 − 243, − 312, − 628 and − 1285) and have been described previously.[12] In order to generate a reporter construct lacking a potential E2F1 binding site, − 243-Luc was amplified with the following primers: E2F1 BS1 (− 36 to − 47) deletion primers: F, GTC GGG GCC ACG CCT CAC CCC CCG ACC TGG GGA AAC CCG G; R, CCG GGT TTC CCC AGG TCG GGG GGT GAG GCG TGG CCC CGA C

Antibodies

The following antibodies were used for western blotting (WB), immuno-precipitation in ChIP assays (IP), immunofluorescence (IF) and immunohis-tochemistry (IHC).

Target	Clone/cat no.	Manufacturer	Application
ABCG2	BXP-21	Millipore	WB, IF, IHC
ABCG2	5D3	Pharmingen (BD Biosciences, Oxford, UK)	IF
E1a	M58	Pharmingen	IP
E2F1	KH95 and KH20	Millipore	WB, IP, IHC
Estrogen receptor alpha (ER-α)	HC-20	Santa Cruz (Dallas, TX, USA)	IF
Actin	9212	Cell Signaling (Danvers, MA, USA)	WB

Cell culture and infections

Cells were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. Retroviral and lentiviral infections were performed as described.[23,24]

Cell line generation

Cell lines stably expressing inducible E2F1-ER or E2F1-132E-ER were produced by retroviral infection and antibiotic selection. Knockdown of ABCG2 or E2F1 was achieved by means of the lentiviral pLKO.1-system. TetOn E2F1-inducible cell lines were all generated in Saos-2 cells. Cells were first transfected with the TetOn regulator plasmid and cloned and tested for good inducibility of a Tet-responsive luciferase plasmid, Tet-Luc. A cell clone that showed good induction was subsequently transfected with either pTRE-E2F1 or pTRE-E2F1-132E together with pIRES-Hyg as a selectable marker. Clones were then again selected for good inducibility of E2F1 trans-genes.

Cell line authentication

All cell lines were authenticated using the Applied Biosystems AmpF/STR Identifiler Kit. Data were analyzed using Applied Biosystems Genemapper software and comparing the data with ATCC (LGC standards) and DSMZ databases.

Immunoblotting

Western analysis was done as previously described[25] using antibodies against: ABCG2 (Millipore, Watford, UK), E2F1 (Millipore) and actin (Cell Signaling) as outlined above.

Immunofluorescence

Cells grown on coverslips were fixed in 4% paraformaldehyde, permeabilized with PBS (1% BSA, 0.2% TritonX) and stained for ABCG2 (Chemicon) and estrogen receptor-α (Santa Cruz). Confocal microscopy was used to visualize ABCG2 expression and E2F1 cytoplasmic–nuclear translocation.

Quantitative real-time PCR

RNA isolation, complimentary DNA synthesis and quantitative real-time PCR were performed using the DynamoSYBRGreen 2-step quantitative

real-time PCR kit from Finnzymes (Thermo Fisher, Loughborough, UK) following manufacturer's instructions.

The following primers were used for quantitative real-time PCR of ABC-transporter transcripts and E2F1 mRNA, or for the amplification of genomic DNA from ChIP assays.

Target	Sequence 5′ to 3′ (forward, reverse)	Application
Human ABCG2	Qiagen, QuantiTect Primer Assay, Hs_ABCG2_1_SG*	qPCR of mRNA transcripts
Human E2F1	Qiagen, QuantiTect Primer Assay, Hs_E2F1_1_SG*	qPCR of mRNA transcripts
Human ABCB1/MDR1/PgP	Qiagen, QuantiTect Primer Assay, Hs_ABCB1_1_SG*	qPCR of mRNA transcripts
Human ABCC1/MRP1	QSiagen, QuantiTect Primer Assay, Hs_ABCC1_1_SG*	qPCR of mRNA transcripts
Human 18s	GTAACCCGTTGAACCCCATT, CCATCCAATCGGTAGTAGCG	qPCR of mRNA transcripts
Promoter region of human ABCG2	TCACCAGAAACCACCCATTT, TGGGCTGATCAGTACCTCGT	ChIP
Promoter region of human actin B	GGTGAACCCAGAAAAACTGG, GTGTGTGGAGATCCCTCCTG	ChIP
Promoter region of human CDC6	AAAGGCTCTGTGACTACAGCCA, GATCCTTCTCACGTCTCTCACA	ChIP

* Sequence not disclosed by the manufacturer.

Chromatin immunoprecipitation
Chromatin was isolated from Saos-2 TetOn-E2F1 cells treated with or without DOX for 24 h using the ChIP Assay Kit from Millipore following manufacturer's instructions. qPCR was used to detect ABCG2 promoter enrichment.

Drug efflux
Efflux assays were performed as described.[26] In brief, in vitro efflux was measured after incubation for 1 h (30 min for pheophorbide A) with fluorescent substrate mitoxantrone (pheophorbide A, 1 μM) followed by 3 h (1 h for pheophorbide A) efflux. In vivo efflux was quantified relative to the chemiluminescence 10 min after the injection of luciferin.[13] Statistical analyses were undertaken via a Student's t-test.

Cell death assay
After treatment for the indicated times, unfixed cells were analyzed by flow cytometry for propidium iodide permeability as an assessment of membrane integrity and cell viability. Statistical analyses were undertaken via a Student's t-test.

Cell cycle and ABCG2 expression analysis by flow cytometry
Following the designated treatments, cells were detached with PBS-EDTA and were stained with anti-ABCG2 antibody and subsequently with a FITC-conjugated secondary antibody before fixation in methanol. After a minimum of 2 h fixation at 4 °C, cells were re-suspended in PBS containing propidium iodide. Cells were then analyzed by flow cytomtery for ABCG2 levels and DNA content. ABCG2 expression for each phase of the cell cycle (identified by appropriate gating) was expressed relative to total ABCG2 expression after induction with tamoxifen.

Luciferase assay
Luciferase assays were performed using a panel of ABCG2 reporter constructs kindly provided by Doug Ross and were used as described.[12] SAOS-2 cells were transfected with 5 μg of ABCG2 Luciferase constructs and 0.1 μg of pcDNA3-E2F1 or empty vector. Twenty-four hours later, cells were lysed in luciferase lysis buffer according to manufacturer's instructions and analyzed for luciferase activity (Promega, Madison, WI, USA).

Immunohistochemistry
IHC was performed on formalin-fixed tissue microarrays using pH 6 heat-induced epitope retrieval. The DAKO Envision Kit was used to amplify and visualize antibody staining. Staining for ABCG2 was as described previously.[27]

RNA interference
The pLKO.1 lentiviral vector (expressing a blasticidine resistance) containing the following shRNA sequences was used to bring about knockdown of specific target genes.

shRNA (human)	Sequence (5′ to 3′)
shNTC (non-targeting control)	TAAGGCTATGAAGAGATAC
shABCG2-1	CCTTCTTCGTTATGATGTTTA
shABCG2-2	GCTGTGGCATTAAACAGAGAA
shABCG2-3	CCTGCCAATTTCAAATGTAAT
shE2F1	CATCCAGCTCATTGCCAAGAA

In vivo-efflux studies/xenograft experiments
Ethical approval to perform animal experiment was granted from the relevant institutions. Experiments were conducted on the basis of previously published data.[13] A number of 5×10^6 MDA-MB-231 E2F1-ER cells were injected into both flanks of 6- to 8-week-old athymic, nude mice. E2F1 was induced 24 h later by intraperitoneal injection of TAM (0.1 mg) or an equal volume of ETOH/sunflower oil solvent in control mice ($n = 5$ in each group). On the following day, D-luciferin (150 mg kg^{-1}) was injected intraperitoneally to anesthetized (2.5% isofluorane) mice and signal acquisition started immediately thereafter with readings every 10 min for 80 min. The relative increase in luminescence to the 10 min reading of each mouse was calculated and blotted against time as a readout of ABCG2—efflux function.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)