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Loss of O^6-methylguanine-DNA methyltransferase confers collateral sensitivity to carmustine in topoisomerase II-mediated doxorubicin resistant triple negative breast cancer cells

Selina Raguz[a], Caroline Adams[b], Nahal Masrour[b], Sabeena Rasul[b], Panagiotis Papoutsoglou[b], Yunhui Hu[c,b], Giulia Cazzanelli[b], Yuan Zhou[1b], Naina Patel[b], Charles Coombes[b], Ernesto Yagüe[b*]

[a] *Clinical Sciences Centre, Medical Research Council, London, UK*

[b] *Division of Cancer, Imperial College London, UK*

[c] *Institute of Hematology and Blood Diseases Hospital, Tianjin, China*

* Corresponding author: Dr Ernesto Yagüe, Division of Cancer, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, Great Britain. Tel: +442075942802. Email: ernesto.yague@imperial.ac.uk

[1]Present address: State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Science and Peking Union Medical College,288 Nanjing Road, Tianjin 300020, P. R. China

Email addresses: s.raguz@csc.mrc.ac.uk (S.Raguz); adamsce@umich.edu (C.Adams); n.masrour@imperial.ac.uk (N.Masrour); s.rasul@imperial.ac.uk (S.Rasul); p.papoutsoglou11@imperial.ac.uk (P. Papoutsoglou); y.hu@imperial.ac.uk (Y.Hu); giulia.cazzanelli11@imperial.ac.uk (G.Cazzanelli); cams_yuan@hotmail.com (Y.Zhou); naina.patel@imperial.ac.uk (N.Patel); c.coombes@imperial.ac.uk (C.Coombes).

Abstract

Triple-negative breast cancer is characterized by aggressive tumors whose cells lack estrogen and progesterone receptors and do not over-express HER2 . It accounts for approximately 10-15% of breast cancer cases. We sought to generate a cellular model of chemotherapy drug resistance for this type of disease to provide the tools for the development of new therapies. Doxorubicin is a component of some chemotherapy regimes used to treat this form of cancer but resistance preventing disease eradication frequently occurs, mainly due to over-expression of drug transporters such as P-glycoprotein. CALDOX cells were generated by exposure of CAL51 to doxorubicin. Resistance to doxorubicin did not involve drug transporters, as the both parental and resistant cells accumulated doxorubicin to comparable levels. CALDOX cells had slower proliferation rate and an extended G1 cell cycle stage than the parental line, mainly due to an intrinsic activation of *CDNK1* (p21), but this cell cycle block was not involved in the mechanism of resistance. CALDOX cells had reduced levels of *TOP2A* (topoisomerase IIα) and were cross resistant to the topoisomerase II inhibitors~~poison~~s etoposide and mitoxantrone. CALDOX cells showed collateral sensitivity to carmustine due to the lack of O^6 -methylguanine-DNA-methyltransferase (MGMT) expression, related to the hypermethylation of its promoter. The collateral sensitivity of CALDOX cells to carmustine provides the rationale to evaluate *MGMT* promoter methylation status to design better therapeutic strategies for triple negative breast cancer.

Keywords

Triple negative breast cancer, collateral sensitivity, TOP2A, MGMT

1. Introduction

Cancer chemotherapeutic treatment was introduced into clinical practice at the end of the Second World War when Gilman and colleagues used nitrogen mustard to treat a patient with advanced lymphoma. Since then, chemotherapy has been one of the main therapeutic strategies in cancer treatment. However, in a significant proportion of cases, and after an initial response, relapse occurs. This is accompanied by phenotypic changes giving the chemotherapy-surviving cancer cells the capacity to invade other organs and become resistant to further treatment, normally with fatal consequences. Standard chemotherapeutic strategies have used compounds such as alkylating agents, antimetabolites, antibiotics, as well as synthetic and hormonal agents that interfere with the basic machinery of the cell. However, the unravelling at the molecular level of many of the alterations present in the cancer cell [1] has enabled the development of targeted therapies for some forms of cancer such as tamoxifen [2] or imatinib [3].

Phenotypic changes associated with drug resistance *in vitro* often involve the development of collateral sensitivity to other drugs and have been reported since the first cellular models of drug resistance were generated [4],[5]. Drug resistance due to P-glycoprotein up-regulation has been unequivocally demonstrated to predict the chemotherapy response in leukaemia patients and good evidence of its involvement in breast cancer exists [6],[7], [8]. P-glycoprotein is a member of a large group of membrane proteins, ABC transporters, which use the energy provided by ATP hydrolysis to pump out of the cell a variety of cytotoxic compounds [9], although other ABC transporters have also been associated with drug resistance. However compounds, generally inhibitors, targeting P-glycoprotein have not passed multiple clinical trials [10]. Importantly, resistance appears not only to traditional chemotherapy (through P-glycoprotein-dependent or –independent mechanisms), but also to targeted therapy such as tamoxifen, that targets the estrogen receptor

(ER) in breast cancer [11], or imatinib, that targets the kinase activity from the translocated *BCR-ABL* in chronic myelogenous leukemia [12]. For these, aromatase inhibitors or second generation inhibitors targeting imatinib-insensitive ABL kinase mutations, such as dasatinib, are in use. However, due to the genetic flexibility of the cancer cell [1], the capacity to develop resistance is intrinsic to cancer development, as the environmental pressure of drug treatment selects for cells no longer constrained by molecular and cellular checkpoints [13]. Collateral sensitivity might offer another therapeutic strategy for cancer treatment, as tumours which have developed resistance to one or several drugs could be effectively treated with the drug to which cancer cells have acquired sensitivity. Although such a therapeutic strategy has not been applied as such, the combined use of several drugs to treat many cancers may have fortuitously, and in an empirical fashion, made use of the collateral sensitivity phenotype [14]. With the advent of personalized medicine it is envisaged that cancer phenotyping and patient tailored treatment will be mainstream in the not-so-distant future [15]. Thus, an understanding of the mechanisms involved in collateral senstivity will be important in the development of future cancer therapeutics.

Treatment of breast cancer patients is currently stratified according to the presence of several markers. ER-positive patients receive, in the first instance tamoxifen and later aromatase inhibitors, whereas those HER2-positive are treated with Herceptin. Triple negative (ER, progesterone receptor and HER2) patients, who represent between 10-15% of cases, receive standard systemic chemotherapy, as well as all patients who develop progressive disease [16],[17]. Most cellular models of breast cancer have traditionally used the ER-positive cell line MCF7. Although this has been important for many preclinical studies, other cellular models, especially of ER-negative breast cancer are necessary to study mechanism of drug resistance and collateral sensitivity before initiating clinical studies. Here, we describe the generation of a new model of drug resistance using the triple negative cell line CAL51,

which has a normal karyotype [18]. Resistance to doxorubicin, etoposide and mitoxantrone was associated with down-regulation of topoisomerase II. We found that collateral sensitivity to carmustine, an alkylating agent, was due to lack of O^6-methylguanine-DNA methyl transferase (MGMT) expression by methylation of its promoter.

2. Material and Methods

2.1 Cell culture, transduction and transfection

The breast cancer CAL51 cell line (German Resource Centre for Biological Material, DSMZ, Braunschweig, Germany) was grown in low glucose (1 g/L) DMEM-glutamax (Invitrogen, Carlsbad, California, USA) supplemented with 10% foetal calf serum [18]. CAL51 authentication by short tandem repeat profiling was performed by LGC Standards (Teddington, UK). For the generation of CALDOX cells, 0.4 µM doxorubicin (Tocris Bioscience, Bristol, UK) ~~(0.4 µM)~~ was added to CAL51 cultures until a stable population of cells emerged that could proliferate in the presence of doxorubicin. Thereafter, CALDOX cells were routinely maintained in low glucose DMEM-glutamax supplemented with 10% foetal calf serum and 0.4 µM doxorubicin. The multidrug resistant cell line NCI/ADR-Res [19] was cultured in the presence of 1 µM doxorubicin. GP2-293 pantropic retroviral packaging cells (Clontech Laboratories, Mountain View, California, USA) and HEK293T cells (American Type Culture Collection, LGC Standards, Teddington, UK) were maintained in DMEM supplemented with 4.5 g/L glucose, 10% foetal calf serum and 4 mM L-glutamine (Invitrogen). All cells were routinely tested for the presence of mycoplasma using the Lonza MycoAlert kit (Basel, Switzerland).

2.2 DNA procedures

Retroviral plasmids pRetroSuper-puromycin (pRSp) expressing short hairpins targeting *TP53* (5′-CTACATGTGTAACAGTTCC-3’) and non-targeting scrambled (5'-TAGCGACTAAACACATCAA -3’) have been described [20]. Lentiviral vector pGIPZ

targeting *TOP2A* (5'-CAGTAGAGAATACAAGAAA-3') was obtained from Thermo Fisher Scientific (Whaltham, Massachussets). A commercial plasmid carrying the *MGMT* coding sequence (Origene, Rockville, Maryland)) was digested with *Eco*RI and *Not*I and the approximately 900-bp fragment was ligated into a modified MSCVpuro (Clontech) retroviral vector digested with the same restriction enzymes, transformed in *E. coli* and the recombinant sequence verified. This plasmid was used to transfect CALDOX cells to generate CALDOX-MGMT and CALDOX-EV (carrying only empty vector).

2.3 Viral transduction

Viral transductions were essentially as described [20]. Briefly, retroviral transfections were performed using 20 µg retroviral plasmid, 2 µg pVSV-G (Clontech) and 55 µg polyethylenimine 25000 (Polysciences, Warrington, Philadelphia, USA) prior to cotransfecting into GP2-293 pantropic retroviral packaging cells. Lentiviral transfections were performed using 20 µg lentiviral plasmid, 2 µg pVSV-G, 8 µg pPAX2 (Addgene, Cambridge, Massachusetts, USA) and 75 µg polyethylenimine (Sigma-Aldrich, St. Louis, Missouri, USA) 25000 prior to cotransfecting into HEK293T cells. Viral supernatants were collected 24-48 hours after transfections, filtered through 0.45 µm cellulose acetetate filters and supplemented with 8 µg/mL polybrene Polybrene (Sigma-Aldrich8 µg/mL) prior to adding to recipient cells. A maximum of 3 rounds of infections were carried out. Puromycin (1 µg/ml; Sigma-Aldrich) was added 96 hours after first infection to recipient cells for selection of stable transgene expression.

2.4 Antibodies

Antibodies for immunodetection following standard immunoblotting procedures were DO-I for p53 (Santa Cruz Biotechnology, Santa Cruz, CA), EA10 for p21 (EMD Biosciences, San Diego, CA) and AC-15 for β-actin (Sigma). Appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma) were detected by enhanced chemiluminescence (GE Healthcare Life Sciences, Pittsburgh, Philadelphia, USA~~A~~). Immunoblots were quantified using Image J [21].

2.5 Gene expression analysis

Total RNA (isolated using RNAzol B, Biogenesys, Poole, UK) was reverse transcribed with avian myeloblastis virus reverse transcriptase (Roche, Burgess Hill, UK) and real-time quantitative PCR was performed using SYBR-Green (Bioline Reagents, London, UK) and gene specific primers (Table 1) on an ABI Prism 7700 detection system (PerkinElmer Life Sciences, Waltham, Massachusetts, USA). A comparative threshold cycle was used to determine the relative gene expression as previously described [22]. PCR reactions followed the MIQE guidelines [23] and included three normalizers (*RPS6*, *RPS14* and *RPLP0*). The two showing the least variability were used normalization.~~At least two normalizers were used in all calculations, as recommended in the MIQE guidelines [22].~~

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2.6 IC_{50}

A sulphorhodamine B (Sigma-Aldrich) assay [24] was used to screen for drug cytotoxicity essentially as previously described [25]. IC_{50} was considered as the drug concentration that killed 50% of cells in a time equivalent to three cell doublings.

2.7 Flow cytometry

Cell surface P-glycoprotein was determined by flow cytometry using the phycoerythrin-conjugated UIC2 antibody (Immunotech, Marseille, France) or the corresponding isotype control (Sigma-Aldrich) essentially as described [22]. Functional drug efflux was performed using 0.1 µM BODIPY-taxol (Invitrogen) in the presence or absence of 1 µM cyclosporin A (Sigma-Aldrich) essentially as described [13]. For the analysis of intracellular doxorubicin accumulation, cells were incubated with 0.4 µM doxorubicin (Tocris) for 15 min and detected in the flow cytometer red channel. For cell cycle analysis, cells were stained with propidium iodide (Sigma-Aldrich) after fixation with ice-cold 70% methanol and the DNA content estimated determined by flow cytometry essentially as described [25].

2.8 Pyrosequencing

The methylation status of specific cytosine residues in the *TOP2A* and *MGMT* gene promoters was determined following bisulphite modification of DNA essentially as described [26]. Briefly, genomic DNA was extracted from CAL51 and CALDOX cells with Genomic DNA extraction kit (Sigma-Aldrich), bisulphite treated using EpiTect Bisulfite kit (Qiagen, Crawley, UK) and PCR amplified with FastStart Taq DNA Polymerase (Roche) using annealing temperature of 59°C for *TOP2A*, 53°C for *LINE1* and 57°C for *MGMT* (Table 2). The biotinylated strand of the amplicons was purified with streptavidin-coated Sepharose beads (GE Healthcare, Chalfont St Giles, UK) and Vacuum Prep Tool (Qiagen) and subsequently annealed to the sequencing primer. Pyrosequencing was performed using PyroGold Reagent kit (Qiagen) with Pyromark Q96 MD instrument. The percentage methylation at individual CG sites for each gene was analysed using Pyro Q-CpG software

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(Qiagen). Each assay was performed in triplicate for each sample. All runs contained a standard curve, prepared by diluting hypermethylated DNA (CpGenome Universal Methylated DNA, Millipore, Whatford, UK) in normal human DNA (Promega, Madison, Wisconsin, USA). Whole genome amplified DNA (GE Healthcare) was used as unmethylated standard. *LINE1* methylation analysis was performed to assess the global methylation levels of the sample [27].

3. Results

3.1 Doxorubicin resistance in CALDOX cells is not mediated by membrane transporters

Cell sensitivity assays indicated that CALDOX cells were approximately 30 fold more resistant to doxorubicin than CAL51 cells with IC_{50} values of 0.6 µM and 0.02 µM, respectively (Fig. 1A). The drug resistance phenotype was stable, as CALDOX cells grown in the absence of doxorubicin for up to 20 passages where able to grow again in the presence of 0.4 µM doxorubicin (data not shown). Since ABC transporters are frequently up-regulated in drug resistant cells [6], we asked whether that would also be the case in CALDOX cells. P-glycoprotein, which is responsible for doxorubicin, among many other drugs, efflux was not up-regulated in CALDOX cells (Fig.1B). In addition, we used an efflux assay with a fluorescent substrate (BODIPY-taxol) which is pumped by P-glycoprotein and some other ABC transporters, again with negative results (Fig. 1B). Since doxorubicin is fluorescent we also tested whether CALDOX cells could pump out doxorubicin and thus were not fluorescent. Results showed that both CAL51 and CALDOX cells accumulated doxorubicin to the same degree, whereas NCI-ADR/RES cells, which express high levels of ABC transporters, did not (Fig. 1B). Thus, doxorubicin resistance in CALDOX cells in independent of membrane transporters.

3.2 p53 and the doxorubicin response in CALDOX cells

While drug naïve CAL51 cells showed a doubling time of 18 h, CALDOX cells showed a slower proliferation rate, with a doubling time of approximately 32 h (Fig. 2A). This slow proliferation rate was accompained by an increase in the percentage of cells at the G1 stage of the cell cycle (approximately 50% in CAL51cells and 75% in CALDOX cells)

and a concomital decrease in the percentage of cells in the S and G2/M phases (Fig. 2B). However, this extended G1 period in CALDOX cells did not affect cell viability (absence of sub-G1 population, Fig 2B).

Next, we asked whether p53 might have a role in blocking cells at this stage of the cell cycle by up-regulating p21. For this, we treated first both cell lines with increasing doses of doxorubicin. Drug sensitive CAL51 cells, as expected, responded with a G1 arrest and apoptosis, which was clear even at 0.1 µM doxorubicin and increased with increasing drug concentrations (Fig. 3A). However, there was no noticeable alteration in the cell cycle in response to up to 1 µM doxorubicin, and only a small percentage (5-10%) of cells underwent apoptosis at 10 µM doxorubin in CALDOX cells. This resistance of CALDOX cells to doxorubicin was confirmed when the p53 and p21 levels were monitored. As p53 transcriptionally activates *CDKN1A* (coding for p21), we used p21 levels as a surrogate of p53 activation due to genotoxic stress. As expected, p21, both at mRNA and protein levels, increased in response to doxorubicin in CAL51 cells (Fig. 3B,C). However, CALDOX cells showed an intrinsic activation of p21 in the absence of doxorubicin which only increased very slightly with higher doxorubicin doses (Fig. 3B,C). Thus, higher concentrations of doxorubicin are necessary to trigger a p53-driven DNA damage response in drug resistant CALDOX cells than in sensitivy CAL51 cells.

We hypothesized that a p53-p21 controlled cell cycle regulation at the G1 stage could be responsible for maintaining the cells for longer at G1 and this would allow cells to repair DNA before entering into the S phase of the cell cycle, contributing thus to the drug resistant phenotype. To test this hypothesis, we knocked-down *TP53* (coding for p53) expression *via* stable expression of short hairpins in CALDOX cells. As before, we used changes in *CDKN1A* mRNA levels as a surrogate for p53 activity. A high efficient knock-down of *TP53* mRNA was obtained (approximately 95%) and, as expected, this led to down-regulation of

CDKN1A mRNA levels by approximately 80% (Fig. 4A). This decrease in the p21 levels restored the CALDOX cell cycle profile to that of the parental, drug sensitive CAL51 cells (Fig. 4B) without altering their sensitivity to doxorubicin (Fig. 4C) or their doubling time (data not shown). Thus, although the cell cycle profile of CALDOX cells is controlled by p53 *via* p21, the p53-DNA damage response pathway is not responsible for the drug resistance phenotype.

3.3 CALDOX cells are resistant to topoisomerase II inhibitor~~poison~~s and show collateral sensitivity to carmustine

Mechanistic insight into the effectors of drug resistance can be gained by performing drug sensitivity screens, as different drugs have different target molecules and modes of action. Doxorubicin cytotoxicity is multifactorial, and formation of free radicals, membrane damage as well as DNA intercalation has been described as some of its mechanisms of action [28]. However, doxorubicin also binds topoisomerase II leading to an increase in the levels of topoisomerase II:DNA complexes, formation of DNA strand breaks and triggering of the apoptotic cascade [29]. Thus, to gain insight into the effectors of drug resistance in CALDOX cells, we screened a small number of drugs with different modes of action to doxorubicin, as well as another two topoisomerase IIα ~~poison~~inhibitors, etoposide and mitoxanthrone. Doxorubicin-resistant CALDOX cells were cross-resistant to the other two topoisomerase II ~~poison~~inhibitors used, showing greater resistance index to mitoxantrone (166 fold) and etoposide (greater than 150 fold) than to doxorubicin (45 fold). However, CALDOX cells were not resistant to camptothecin, a topoisomerase I ~~poison~~ inhibitor (Table 1). CALDOX cells did not display cross resistance to the DNA cross linking agent cisplatin and were marginally cross resistant to the microtubule depolymerizing agent, taxol (2.6 fold). Interestingly, CALDOX cells showed collateral sensitivity to carmustine, a drug forming O^6-

guanine alkyl adducts (with a resistance index of less than 0.16, that is CAL51 cells were more than 6 fold resistant to carmustine than CALDOX cells). Thus, CALDOX cells are cross resistant to topoisomerase IIα inhibitor~~poison~~s with collateral sensitivity to the alkylating agent carmustine.

3.4 Down-regulation of TOP2A leads to drug resistance

Down-regulation of topoisomerase II causes resistance to drugs such as doxorubicin and etoposide in a variety of cell systems [30],[31],[29]. In CALDOX cells we also found that expression of *TOP2A* was down-regulated by approximately four fold (Fig. 5A). Initial attempts to over-express *TOP2A* in CALDOX cells in order to rescue the drug resistance phenotype were unsuccessful (data not shown), as it is well established that experimental up-regulation of *TOP2A* has deleterious effects [32]. Therefore, we decided to down-regulate *TOP2A* levels in CAL51 by RNA interference using a lentiviral vector. CAL51-shTOP2A cells expressed approximately 50% less *TOP2A* mRNA than the corresponding control cells transfected with the empty vector (Fig. 5A). Down-regulation of *TOP2A* led to a notable increase in the number of doxorubicin resistant colonies (Fig. 5B). Thus, down-regulation of *TOP2A* leads to drug resistance in CALDOX cells.

3.5 Methylation of MGMT promoter in doxorubicin-resistant cells leads to collateral sensitivity to carmustine

Carmustine, and similar alkylating agents, cause cytotoxicity through the formation of O^6-methylguanine, genotoxicity and apoptosis. These adducts are removed from the O^6-guanine by O^6-methylguanine-DNA methyl transferase (MGMT; E.C. 2.1.1.63) in a reaction that transfers the alkyl group from the DNA to an internal cysteine residue in the MGMT, thus restoring the integrity of DNA [33]. Since it is well established that cells with low levels

of MGMT are sensitive to alkylating agents [34],[35], we asked whether the collateral sensitivity of CALDOX cells to carmustine could be due to MGMT down-regulation. Indeed, *MGMT* mRNA was undetectable in CALDOX cells, while expressed at abundant levels in CAL51 cells (Fig. 6A). Epigenetic modifications in *MGMT* have been described in colorectal [36] and more recently in triple negative breast cancer [37]. We, thus, asked whether *MGMT* promoter was methylated in CALDOX cells. We analyzed methylation at 8 CpG sites in the CpG island on the *MGMT* promoter in both CAL51 and CALDOX cells. Hypermethylation of these 8 sites was found in CALDOX cells (approximately 75%) whereas CAL51 cells only showed background levels (around 5%) as these cells express *MGMT* (Fig. 6B). In contrast, *TOP2A* promoter remained unmethylated in both cell lines. Methylation values of *LINE1*, which have been reported as a surrogate for global methylation [27], did not change either between cell lines and when compared to normal human genomic DNA. This indicates that during the generation of CALDOX cells there has been no major change in global methylation and that the changes found are specific for *MGMT*. Thus, the collateral sensitivity to carmustine in doxorubicin-resistant cells is due epigenetic changes involving hypermethylation at the *MGMT* locus.

In order to confirm the role of MGMT in the collateral sensitivity of CALDOX cells to carmustine, we generated a stably transfected CALDOX derivative, CALDOX-MGMT, overexpressing MGMT by retroviral transduction. These cells expressed approximately 70% *MGMT* mRNA levels than those found in the parental CAL51 cells (Fig. 7A). Reintroduction of MGMT resulted in the reversal of carmustine sensitivity (Fig. 7D), without altering resistances to etoposide (Fig. 7C) or doxorubicin (Fig. 7B). Thus, down-regulation of *MGMT* is responsible for the collateral sensitivity to carmustine in doxorubicin-resistant CALDOX cells.

4. Discussion

The use of drug resistant derivatives from cell lines is of paramount importance for the characterization of the molecular mechanisms involved in cancer drug resistance. Many of the traditional drug resistant lines were generated by exposition to increasing concentration of the chemotherapeutic agent in a step-wise fashion, generating drug resistant derivatives with several-hundred-fold greater resistance to the drug relative to the parental cell line [38],[39], and is a method that is still in use these days [40]. These drug resistant lines were very important before the techniques to ectopically express foreign DNA were of routine used, since they over-expressed one or several of the molecular effectors conferring drug resistance, such as P-glycoprotein, and biochemical assays could be easily performed with them [41],[42],[43]. However, such drug resistant cell lines are far from ideal when studying regulatory mechanisms controlling drug resistance since the concentration of drug used vastly exceeds that found by cancer cells. Current protocols for the generation of resistant sublines involve the continuous use of lower doses of drug than those used in the above studies [22],[13] or the treatment with single, or repeated, doses of drug for a very short period (hours), mimicking the burst of drug in the serum of chemotherapy-treated patients [44]. It has been argued that the protocol used in the generation of resistant cells influences the mechanisms of resistance selected in such cells [45], being this, in the majority of cases, up-regulation of P-glycoprotein. We have used a moderate concentration of doxorubicin (0.4 μM) to generate doxorubicin resistant derivatives from CAL51, a breast cancer cell line with a normal diploid karyotype and luminal phenotype negative for ER, PgR and ERBB2 [18], although subtle genomic alterations, as expected in a cancer cell, have been described [46],[47]. Very few cellular models, if any, for drug resistance in triple negative breast cancer are available. As this form of cancer is particularly aggressive and currently lacking any

targeted therapy, the development of cellular models is of paramount importance to unravel molecular mechanisms of drug resistance and to test novel drugs. Surprisingly CALDOX cells have not up-regulated P-glycoprotein expression, or other drug transporter, being thus an excellent model for P-glycoprotein-independent triple negative breast cancer.

Drug sensitivity assays confirmed the lack of involvement of ABC transporters, as CAL51 and CALDOX cells were equally sensitive to the P-glycoprotein substrate camptothecin [48]. However, cross-resistance to topoisomerase II, but no topoisomerase I, ~~poison~~inhibitors was observed. Topoisomerase II performs essential DNA topologic changes in proliferating eukaryotic cells decatenating the newly replicated sister DNA molecules. Doxorubicin and mitoxantrone intercalate in DNA and trap topoisomerase II as a covalent complex on DNA, whereas etoposide interaction is weaker and other protein-drug interactions have been suggested to play key roles in the formation of topoisomerase II trapped complexes [49],[29]. As this process ultimately leads to DNA damage, activation of apoptosis and cell death, it is clear that the overall level of topoisomerase II is a determinant of drug sensitivity. Decreased levels of the enzyme have been found to cause doxorubicin resistance in cell systems and mouse tumour models [50] due to differential expression during the cell cycle [51] and promoter methylation [52]. In addition, mutations in the coding region in both cell lines and clinical samples resulting in drecreased activity lead to drug resistance [30]. Conversely, high levels of topoisomerase II, due to gene amplification, positively correlates with response to anthracyclin neo-adjuvant chemotherapy in locally advance primary breast cancer [53]. In the triple negative breast cancer model presented here, down-regulation of *TOP2A* mRNA is a determinant for the generation of doxorubicin resistant cells. The mechanism by which *TOP2A* expression is reduced in this drug resistance model is not due to either mutations in the coding region (data not shown) or altered CpG methylation in the -200

to -166 promoter region, although methylation at other sites or mutations affecting gene expression could have arisen in an as yet unidentified region of the locus.

Collateral sensitivity in drug resistant cells has been considered a case of "synthetic lethality" in which the genetic alterations that confer resistance to a drug sensitizes it to other drugs [5]. Doxorubicin-resistant cells expressing P-glycoprotein have been described to show collateral sensitivity to cisplatin, cytarabine or gemcitabine [54],[55]. In addition, other drug resistant cells by down-regulation of topoisomerase II have been described to show collateral sensitivity to gemcitabin, but not to alkylating agents [54]. No systematic studies screening drug resistant cells for collateral sensitivity have yet been reported. There are reports in the literature describing colateral sensitivity in a variety of drug resistant models, but none include sensitivity to carmustine [4].

Although alkylating agents, such as cyclophosphamide, form part of routine breast cancer chemotherapy, those producing O^6-methylguanine or O^4-methylthymine (which are inactivated by MGMT) are used in the treatment of glioblastoma and to a lesser extent in treating malignant melanoma, gastointestinal and pancreatic cancer, Hodgkin's and non-Hodgkin's lymphoma [33]. However, some trials using temozolomide have been reported [56], and carmustine has been used in stage II-III and metastatic breast cancer [57],[58]. Loss of MGMT has been found in breast tumours recurring locally after operation, indicating that MGMT is a positive predictive factor for relapse-free survival [59], and *MGMT* promoter methylation has been recently reported in breast cancer [37]. Our data highlights the link between doxorubicin resistance and colateral sensitivity to carmustine and offers the potential new avenue of evaluating of *MGMT* promoter methylation status to design better therapeutic strategies for triple negative breast cancer.

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Figure captions

Figure 1. Doxorubicin resistance in CALDOX cells is not mediated by membrane transporters**.** (A) CALDOX cells (IC_{50} 0.6 μM) are 30 fold more resistant to doxorubicin than CAL51 cells (IC_{50} 0.02 μM). Sulphorhodamine B staining after 3 cell doublings. (B) Absence of membrane pumps in CALDOX cells. *Left panels*, Flow cytometric analysis of P-glycoprotein expression using the UIC2-phycoerythrin-conjugated antibody (*filled peaks*) and the corresponding IgG isotype control (*clear peak*). For CAL51 and CALDOX cells, the IgG and UIC2 peaks overlap, indicating that active P-glycoprotein is not present at the cell surface. NCI/ADR-Res cells are used as a positive control. *Middle panels*, Flow cytometric analysis of BODIPY-taxol efflux. The incubation of cells in the presence of a membrane pump inhibitor (cyclosporin A, *filled peaks*) produces an increase in the fluorescence of the cells since they cannot extrude the fluorescent dye out of the cell. *AF*, autofluorescence. For CAL51 and CALDOX cells, the fluorescence peaks in the presence and absence of cyclosporin do overlap, indicating absence of cyclosporin-inhibitable membrane pumps. NCI/ADR-Res cells are used as a positive control. *Right panels*, Flow cytometric analysis of intracellular doxorubicin accumulation. Cells were incubated with 0.4 μM doxorubicin for 15 min and detected in the flow cytometer red channel (*filled peaks*); *AF*, autofluorescence. In both CAL51 and CALDOX cells doxorubicin accumulates intracellularly and the cells fluoresce. Cells with active membrane pumps (NCI/ADR-Res) do not accumulate doxorubicin intracellularly. Experiments were repeated at least twice, and one representative experiment is shown.

Figure 2. CALDOX cells have a slow proliferation rate and abnormal cell cycle profile. (A) Cells were seeded in 6-well plates and at different time points the cells were trypsinized, stained with trypan blue and those negative were counted. Growth of both cell lines fit

(r^2>0.95) to exponential curves with doubling times of 18 and 32 h for CAL51 and CALDOX cells, respectively. Data represent the average ± SD of at least three experiments. (B) Cell cycle profiles (*left panel*) indicate CALDOX cells have an increased proportion of cells at G1, with less proportion of cells at S and G2/M (*right panel*). Experiments were repeated at least twice, and one representative experiment is shown.

Figure 3. Higher concentrations of doxorubicin are necessary to trigger a p53-driven DNA damage response in CALDOX than in CAL51 cells. Cells were treated with increasing concentrations of doxorubicin for 24 h. (A) Cell cycle profile of CAL51 (*upper panels*) and CALDOX (*lower panels*) cells. Doxorubicin induces an increase in the proportion of cells at G1 and sub-G1 (apoptotic). Conversely, the profile of CALDOX cells only shows apoptotic cells at very high doxorubicin concentration. (B) Western blot analysis of p53 and p21 *(left panel)* and quantification of at least two blots using Image J (*right panels*). ~~and~~ (C) *CDKN1A* mRNA expression analysis upon doxorubicin treatment. Doxorubicin can trigger a p53 response in CALDOX cells but at higher concentrations than in CAL51 cells. Note the presence of p21 in CALDOX cells in the absence of drug. Experiments were repeated at least twice, and one representative experiment is shown in (A) and (B, *left panel*). RNA expression data (C) is shown using two normalizers and represent the average ± SD of three independent replicates.

Figure 4. The p53-DNA damage response pathway is not responsible for the drug resistant phenotype of CALDOX cells. CALDOX cells were retrovirally transduced to express a small hairpin targeting *TP53* mRNA. (A) Down-regulation of *TP53* and the p53 target *CDKN1A* by RT-QPCR. Two normalizers were used and data represent the average ± SD of three independent replicates. (B) sh*TP53* CALDOX cells have a cell cycle profile similar to that of

CAL51 cells. (C) Doxorubicin response curves obtained by sulphorhodamine B staining after three cell doublings indicates that knocking down p53 does not change drug resistance. sh*TP53* CALDOX cells (*straight line*); empty vector CALDOX cells (*dashed line*). Data represent the mean ± SD of at least four independent experiments.

Figure 5. Down-regulation of topoisomerase II leads to drug resistance.(A) *TOP2A* mRNA expression by RT-QPCR of drug naïve (CAL51), drug resistant (CALDOX) and CAL51 cells retrovirally transfected with a small hairpin targeting *TOP2A* mRNA. Control cells were transfected with empty vector (EV). Data represent the mean ± SD or three independent replicates. (B) Clonogenic assay for drug resistance. Cells (2.5×10^4 / 25 cm^2) were treated with 0.1 μM doxorubicin and resistant clones were left to grow for 4 weeks. Flasks were stained with crystal violet. Pictures shown are representative of at least two independent experiments.

Figure 6. The *MGMT* promoter is hypermethylated in CALDOX cells. (A) O^6-methylguanine-DNA methyltransferase is not expressed in CALDOX cells. Expression data obtained by by RT-QPCR normalized to *RPS6* and *RPS14* showed a complete absence of *MGMT* transcripts. Data represent the mean ± SD or three independent replicates. (B) Pyrosequencing analysis of methylation of CpG sites within the *TOP2A* (4 sites) and *MGMT* (8 sites) gene promoters in CAL and CALDOX cells as well as normal human genomic DNA. Standard *LINE1* methylation (4 sites) analysis was performed to assess the global methylation levels of the sample. Data represent the mean ± SD of three cytosine methylation measurements.

Figure 7. Collateral sensitivity to carmustine can be rescued by over-expresion of MGMT.

(A) Retrovirally transfected CALDOX cells overexpressing MGMT (*CALDOX-MGMT*) increased its mRNA levels nearly to those found in CAL51 cells. An empty vector was used to generate control cells (*CALDOX-EV*). Expression was determined by RT-QPCR using two normalizers. Data represent the mean ± SD of two independent experiments. (B-D) Overexpression of MGMT reversed the carmustine sensitive phenotype to that of parental CAL51 cells but did not change the resistance to topoisomerase II inhibitor~~poison~~s. Sulphorhodamine B staining was used as a surrogate for cell density after cells were treated for 6 days with 1 µM doxorubicin (B), 50 µM etoposide (C) and 30 µM carmustine (D). Data represent the average ± SD of at least four replicates.

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Loss of O^6-methylguanine-DNA methyltransferase confers collateral sensitivity to carmustine in topoisomerase II-mediated doxorubicin resistant triple negative breast cancer cells

Selina Raguz[a], Caroline Adams[b], Nahal Masrour[b], Sabeena Rasul[b], Panagiotis Papoutsoglou[b], Yunhui Hu[c,b], Giulia Cazzanelli[b], Yuan Zhou[1b], Naina Patel[b], Charles Coombes[b], Ernesto Yagüe[b*]

[a] *Clinical Sciences Centre, Medical Research Council, London, UK*

[b] *Division of Cancer, Imperial College London, UK*

[c] *Institute of Hematology and Blood Diseases Hospital, Tianjin, China*

* Corresponding author: Dr Ernesto Yagüe, Division of Cancer, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, Great Britain. Tel: +442075942802. Email: ernesto.yague@imperial.ac.uk

[1]Present address: State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Science and Peking Union Medical College,288 Nanjing Road, Tianjin 300020, P. R. China

Email addresses: s.raguz@csc.mrc.ac.uk (S.Raguz); adamsce@umich.edu (C.Adams); n.masrour@imperial.ac.uk (N.Masrour); s.rasul@imperial.ac.uk (S.Rasul); p.papoutsoglou11@imperial.ac.uk (P. Papoutsoglou); y.hu@imperial.ac.uk (Y.Hu); giulia.cazzanelli11@imperial.ac.uk (G.Cazzanelli); cams_yuan@hotmail.com (Y.Zhou); naina.patel@imperial.ac.uk (N.Patel); c.coombes@imperial.ac.uk (C.Coombes).

Abstract

Triple-negative breast cancer is characterized by aggressive tumors whose cells lack estrogen and progesterone receptors and do not over-express HER2 . It accounts for approximately 10-15% of breast cancer cases. We sought to generate a cellular model of chemotherapy drug resistance for this type of disease to provide the tools for the development of new therapies. Doxorubicin is a component of some chemotherapy regimes used to treat this form of cancer but resistance preventing disease eradication frequently occurs, mainly due to over-expression of drug transporters such as P-glycoprotein. CALDOX cells were generated by exposure of CAL51 to doxorubicin. Resistance to doxorubicin did not involve drug transporters, as the both parental and resistant cells accumulated doxorubicin to comparable levels. CALDOX cells had slower proliferation rate and an extended G1 cell cycle stage than the parental line, mainly due to an intrinsic activation of *CDNK1* (p21), but this cell cycle block was not involved in the mechanism of resistance. CALDOX cells had reduced levels of *TOP2A* (topoisomerase IIα) and were cross resistant to the topoisomerase II inhibitorss etoposide and mitoxantrone. CALDOX cells showed collateral sensitivity to carmustine due to the lack of O^6 -methylguanine-DNA-methyltransferase (MGMT) expression, related to the hypermethylation of its promoter. The collateral sensitivity of CALDOX cells to carmustine provides the rationale to evaluate *MGMT* promoter methylation status to design better therapeutic strategies for triple negative breast cancer.

Keywords

Triple negative breast cancer, collateral sensitivity, TOP2A, MGMT

1. Introduction

Cancer chemotherapeutic treatment was introduced into clinical practice at the end of the Second World War when Gilman and colleagues used nitrogen mustard to treat a patient with advanced lymphoma. Since then, chemotherapy has been one of the main therapeutic strategies in cancer treatment. However, in a significant proportion of cases, and after an initial response, relapse occurs. This is accompanied by phenotypic changes giving the chemotherapy-surviving cancer cells the capacity to invade other organs and become resistant to further treatment, normally with fatal consequences. Standard chemotherapeutic strategies have used compounds such as alkylating agents, antimetabolites, antibiotics, as well as synthetic and hormonal agents that interfere with the basic machinery of the cell. However, the unravelling at the molecular level of many of the alterations present in the cancer cell [1] has enabled the development of targeted therapies for some forms of cancer such as tamoxifen [2] or imatinib [3].

Phenotypic changes associated with drug resistance *in vitro* often involve the development of collateral sensitivity to other drugs and have been reported since the first cellular models of drug resistance were generated [4],[5]. Drug resistance due to P-glycoprotein up-regulation has been unequivocally demonstrated to predict the chemotherapy response in leukaemia patients and good evidence of its involvement in breast cancer exists [6],[7], [8]. P-glycoprotein is a member of a large group of membrane proteins, ABC transporters, which use the energy provided by ATP hydrolysis to pump out of the cell a variety of cytotoxic compounds [9], although other ABC transporters have also been associated with drug resistance. However compounds, generally inhibitors, targeting P-glycoprotein have not passed multiple clinical trials [10]. Importantly, resistance appears not only to traditional chemotherapy (through P-glycoprotein-dependent or –independent mechanisms), but also to targeted therapy such as tamoxifen, that targets the estrogen receptor

(ER) in breast cancer [11], or imatinib, that targets the kinase activity from the translocated *BCR-ABL* in chronic myelogenous leukemia [12]. For these, aromatase inhibitors or second generation inhibitors targeting imatinib-insensitive ABL kinase mutations, such as dasatinib, are in use. However, due to the genetic flexibility of the cancer cell [1], the capacity to develop resistance is intrinsic to cancer development, as the environmental pressure of drug treatment selects for cells no longer constrained by molecular and cellular checkpoints [13]. Collateral sensitivity might offer another therapeutic strategy for cancer treatment, as tumours which have developed resistance to one or several drugs could be effectively treated with the drug to which cancer cells have acquired sensitivity. Although such a therapeutic strategy has not been applied as such, the combined use of several drugs to treat many cancers may have fortuitously, and in an empirical fashion, made use of the collateral sensitivity phenotype [14]. With the advent of personalized medicine it is envisaged that cancer phenotyping and patient tailored treatment will be mainstream in the not-so-distant future [15]. Thus, an understanding of the mechanisms involved in collateral senstivity will be important in the development of future cancer therapeutics.

Treatment of breast cancer patients is currently stratified according to the presence of several markers. ER-positive patients receive, in the first instance tamoxifen and later aromatase inhibitors, whereas those HER2-positive are treated with Herceptin. Triple negative (ER, progesterone receptor and HER2) patients, who represent between 10-15% of cases, receive standard systemic chemotherapy, as well as all patients who develop progressive disease [16],[17]. Most cellular models of breast cancer have traditionally used the ER-positive cell line MCF7. Although this has been important for many preclinical studies, other cellular models, especially of ER-negative breast cancer are necessary to study mechanism of drug resistance and collateral sensitivity before initiating clinical studies. Here, we describe the generation of a new model of drug resistance using the triple negative cell line CAL51,

which has a normal karyotype [18]. Resistance to doxorubicin, etoposide and mitoxantrone was associated with down-regulation of topoisomerase II. We found that collateral sensitivity to carmustine, an alkylating agent, was due to lack of O^6-methylguanine-DNA methyl transferase (MGMT) expression by methylation of its promoter.

2. Material and Methods

2.1 Cell culture, transduction and transfection

The breast cancer CAL51 cell line (German Resource Centre for Biological Material, DSMZ, Braunschweig, Germany) was grown in low glucose (1 g/L) DMEM-glutamax (Invitrogen, Carlsbad, California, USA) supplemented with 10% foetal calf serum [18]. CAL51 authentication by short tandem repeat profiling was performed by LGC Standards (Teddington, UK). For the generation of CALDOX cells, 0.4 μM doxorubicin (Tocris Bioscience, Bristol, UK) was added to CAL51 cultures until a stable population of cells emerged that could proliferate in the presence of doxorubicin. Thereafter, CALDOX cells were routinely maintained in low glucose DMEM-glutamax supplemented with 10% foetal calf serum and 0.4 μM doxorubicin. The multidrug resistant cell line NCI/ADR-Res [19] was cultured in the presence of 1 μM doxorubicin. GP2-293 pantropic retroviral packaging cells (Clontech Laboratories, Mountain View, California, USA) and HEK293T cells (American Type Culture Collection, LGC Standards, Teddington, UK) were maintained in DMEM supplemented with 4.5 g/L glucose, 10% foetal calf serum and 4 mM L-glutamine (Invitrogen). All cells were routinely tested for the presence of mycoplasma using the Lonza MycoAlert kit (Basel, Switzerland).

2.2 DNA procedures

Retroviral plasmids pRetroSuper-puromycin (pRSp) expressing short hairpins targeting *TP53* (*5′*-CTACATGTGTAACAGTTCC-3') and non-targeting scrambled (5'-TAGCGACTAAACACATCAA -3') have been described [20]. Lentiviral vector pGIPZ

targeting *TOP2A* (5'-CAGTAGAGAATACAAGAAA-3') was obtained from Thermo Fisher Scientific (Whaltham, Massachussets). A commercial plasmid carrying the *MGMT* coding sequence (Origene, Rockville, Maryland) was digested with *Eco*RI and *Not*I and the approximately 900-bp fragment was ligated into a modified MSCVpuro (Clontech) retroviral vector digested with the same restriction enzymes, transformed in *E. coli* and the recombinant sequence verified. This plasmid was used to transfect CALDOX cells to generate CALDOX-MGMT and CALDOX-EV (carrying only empty vector).

2.3 Viral transduction

Viral transductions were essentially as described [20]. Briefly, retroviral transfections were performed using 20 μg retroviral plasmid, 2 μg pVSV-G (Clontech) and 55 μg polyethylenimine 25000 (Polysciences, Warrington, Philadelphia, USA) prior to cotransfecting into GP2-293 pantropic retroviral packaging cells. Lentiviral transfections were performed using 20 μg lentiviral plasmid, 2 μg pVSV-G, 8 μg pPAX2 (Addgene, Cambridge, Massachusetts, USA) and 75 μg polyethylenimine (Sigma-Aldrich, St. Louis, Missouri, USA) 25000 prior to cotransfecting into HEK293T cells. Viral supernatants were collected 24-48 hours after transfections, filtered through 0.45 μm cellulose acetetate filters and supplemented with 8 μg/mL Polybrene (Sigma-Aldrich) prior to adding to recipient cells. A maximum of 3 rounds of infections were carried out. Puromycin (1 μg/ml; Sigma-Aldrich) was added 96 hours after first infection to recipient cells for selection of stable transgene expression.

2.4 Antibodies

Antibodies for immunodetection following standard immunoblotting procedures were DO-I for p53 (Santa Cruz Biotechnology, Santa Cruz, CA), EA10 for p21 (EMD Biosciences, San Diego, CA) and AC-15 for β-actin (Sigma). Appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma) were detected by enhanced chemiluminescence (GE Healthcare Life Sciences, Pittsburgh, Philadelphia, USA). Immunoblots were quantified using Image J [21].

2.5 Gene expression analysis

Total RNA (isolated using RNAzol B, Biogenesys, Poole, UK) was reverse transcribed with avian myeloblastis virus reverse transcriptase (Roche, Burgess Hill, UK) and real-time quantitative PCR was performed using SYBR-Green (Bioline Reagents, London, UK) and gene specific primers (Table 1) on an ABI Prism 7700 detection system (PerkinElmer Life Sciences, Waltham, Massachusetts, USA). A comparative threshold cycle was used to determine the relative gene expression as previously described [22]. PCR reactions followed the MIQE guidelines [23] and included three normalizers (*RPS6*, R*PS14* and *RPLP0*). The two showing the least variability were used normalization.

2.6 IC_{50}

A sulphorhodamine B (Sigma-Aldrich) assay [24] was used to screen for drug cytotoxicity essentially as previously described [25]. IC_{50} was considered as the drug concentration that killed 50% of cells in a time equivalent to three cell doublings.

2.7 Flow cytometry

Cell surface P-glycoprotein was determined by flow cytometry using the phycoerythrin-conjugated UIC2 antibody (Immunotech, Marseille, France) or the corresponding isotype control (Sigma-Aldrich) essentially as described [22]. Functional drug efflux was performed using 0.1 μM BODIPY-taxol (Invitrogen) in the presence or absence of 1 μM cyclosporin A (Sigma-Aldrich) essentially as described [13]. For the analysis of intracellular doxorubicin accumulation, cells were incubated with 0.4 μM doxorubicin (Tocris) for 15 min and detected in the flow cytometer red channel. For cell cycle analysis, cells were stained with propidium iodide (Sigma-Aldrich) after fixation with ice-cold 70% methanol and the DNA content estimated determined by flow cytometry essentially as described [25].

2.8 Pyrosequencing

The methylation status of specific cytosine residues in the *TOP2A* and *MGMT* gene promoters was determined following bisulphite modification of DNA essentially as described [26]. Briefly, genomic DNA was extracted from CAL51 and CALDOX cells with Genomic DNA extraction kit (Sigma-Aldrich), bisulphite treated using EpiTect Bisulfite kit (Qiagen, Crawley, UK) and PCR amplified with FastStart Taq DNA Polymerase (Roche) using annealing temperature of 59°C for *TOP2A,* 53^{o}C for *LINE1* and 57°C for *MGMT* (Table 2). The biotinylated strand of the amplicons was purified with streptavidin-coated Sepharose beads (GE Healthcare, Chalfont St Giles, UK) and Vacuum Prep Tool (Qiagen) and subsequently annealed to the sequencing primer. Pyrosequencing was performed using PyroGold Reagent kit (Qiagen) with Pyromark Q96 MD instrument. The percentage methylation at individual CG sites for each gene was analysed using Pyro Q-CpG software (Qiagen). Each assay was performed in triplicate for each sample. All runs contained a

standard curve, prepared by diluting hypermethylated DNA (CpGenome Universal Methylated DNA, Millipore, Whatford, UK) in normal human DNA (Promega, Madison, Wisconsin, USA). Whole genome amplified DNA (GE Healthcare) was used as unmethylated standard. *LINE1* methylation analysis was performed to assess the global methylation levels of the sample [27].

3. Results

3.1 Doxorubicin resistance in CALDOX cells is not mediated by membrane transporters

Cell sensitivity assays indicated that CALDOX cells were approximately 30 fold more resistant to doxorubicin than CAL51 cells with IC_{50} values of 0.6 μM and 0.02 μM, respectively (Fig. 1A). The drug resistance phenotype was stable, as CALDOX cells grown in the absence of doxorubicin for up to 20 passages where able to grow again in the presence of 0.4 μM doxorubicin (data not shown). Since ABC transporters are frequently up-regulated in drug resistant cells [6], we asked whether that would also be the case in CALDOX cells. P-glycoprotein, which is responsible for doxorubicin, among many other drugs, efflux was not up-regulated in CALDOX cells (Fig.1B). In addition, we used an efflux assay with a fluorescent substrate (BODIPY-taxol) which is pumped by P-glycoprotein and some other ABC transporters, again with negative results (Fig. 1B). Since doxorubicin is fluorescent we also tested whether CALDOX cells could pump out doxorubicin and thus were not fluorescent. Results showed that both CAL51 and CALDOX cells accumulated doxorubicin to the same degree, whereas NCI-ADR/RES cells, which express high levels of ABC transporters, did not (Fig. 1B). Thus, doxorubicin resistance in CALDOX cells in independent of membrane transporters.

3.2 p53 and the doxorubicin response in CALDOX cells

While drug naïve CAL51 cells showed a doubling time of 18 h, CALDOX cells showed a slower proliferation rate, with a doubling time of approximately 32 h (Fig. 2A). This slow proliferation rate was accompained by an increase in the percentage of cells at the G1 stage of the cell cycle (approximately 50% in CAL51cells and 75% in CALDOX cells) and a concomital decrease in the percentage of cells in the S and G2/M phases (Fig. 2B).

However, this extended G1 period in CALDOX cells did not affect cell viability (absence of sub-G1 population, Fig 2B).

Next, we asked whether p53 might have a role in blocking cells at this stage of the cell cycle by up-regulating p21. For this, we treated first both cell lines with increasing doses of doxorubicin. Drug sensitive CAL51 cells, as expected, responded with a G1 arrest and apoptosis, which was clear even at 0.1 µM doxorubicin and increased with increasing drug concentrations (Fig. 3A). However, there was no noticeable alteration in the cell cycle in response to up to 1 µM doxorubicin, and only a small percentage (5-10%) of cells underwent apoptosis at 10 µM doxorubin in CALDOX cells. This resistance of CALDOX cells to doxorubicin was confirmed when the p53 and p21 levels were monitored. As p53 transcriptionally activates *CDKN1A* (coding for p21), we used p21 levels as a surrogate of p53 activation due to genotoxic stress. As expected, p21, both at mRNA and protein levels, increased in response to doxorubicin in CAL51 cells (Fig. 3B,C). However, CALDOX cells showed an intrinsic activation of p21 in the absence of doxorubicin which only increased very slightly with higher doxorubicin doses (Fig. 3B,C). Thus, higher concentrations of doxorubicin are necessary to trigger a p53-driven DNA damage response in drug resistant CALDOX cells than in sensitivy CAL51 cells.

We hypothesized that a p53-p21 controlled cell cycle regulation at the G1 stage could be responsible for maintaining the cells for longer at G1 and this would allow cells to repair DNA before entering into the S phase of the cell cycle, contributing thus to the drug resistant phenotype. To test this hypothesis, we knocked-down *TP53* (coding for p53) expression *via* stable expression of short hairpins in CALDOX cells. As before, we used changes in *CDKN1A* mRNA levels as a surrogate for p53 activity. A high efficient knock-down of *TP53* mRNA was obtained (approximately 95%) and, as expected, this led to down-regulation of *CDKN1A* mRNA levels by approximately 80% (Fig. 4A). This decrease in the p21 levels

restored the CALDOX cell cycle profile to that of the parental, drug sensitive CAL51 cells (Fig. 4B) without altering their sensitivity to doxorubicin (Fig. 4C) or their doubling time (data not shown). Thus, although the cell cycle profile of CALDOX cells is controlled by p53 *via* p21, the p53-DNA damage response pathway is not responsible for the drug resistance phenotype.

3.3 CALDOX cells are resistant to topoisomerase II inhibitors and show collateral sensitivity to carmustine

Mechanistic insight into the effectors of drug resistance can be gained by performing drug sensitivity screens, as different drugs have different target molecules and modes of action. Doxorubicin cytotoxicity is multifactorial, and formation of free radicals, membrane damage as well as DNA intercalation has been described as some of its mechanisms of action [28]. However, doxorubicin also binds topoisomerase II leading to an increase in the levels of topoisomerase II:DNA complexes, formation of DNA strand breaks and triggering of the apoptotic cascade [29]. Thus, to gain insight into the effectors of drug resistance in CALDOX cells, we screened a small number of drugs with different modes of action to doxorubicin, as well as another two topoisomerase IIα inhibitors, etoposide and mitoxanthrone. Doxorubicin-resistant CALDOX cells were cross-resistant to the other two topoisomerase II inhibitors used, showing greater resistance index to mitoxantrone (166 fold) and etoposide (greater than 150 fold) than to doxorubicin (45 fold). However, CALDOX cells were not resistant to camptothecin, a topoisomerase I inhibitor (Table 1). CALDOX cells did not display cross resistance to the DNA cross linking agent cisplatin and were marginally cross resistant to the microtubule depolymerizing agent, taxol (2.6 fold). Interestingly, CALDOX cells showed collateral sensitivity to carmustine, a drug forming O^6-guanine alkyl adducts (with a resistance index of less than 0.16, that is CAL51 cells were more than 6 fold resistant to

carmustine than CALDOX cells). Thus, CALDOX cells are cross resistant to topoisomerase IIα inhibitors with collateral sensitivity to the alkylating agent carmustine.

3.4 Down-regulation of TOP2A leads to drug resistance

Down-regulation of topoisomerase II causes resistance to drugs such as doxorubicin and etoposide in a variety of cell systems [30],[31],[29]. In CALDOX cells we also found that expression of *TOP2A* was down-regulated by approximately four fold (Fig. 5A). Initial attempts to over-express *TOP2A* in CALDOX cells in order to rescue the drug resistance phenotype were unsuccessful (data not shown), as it is well established that experimental up-regulation of *TOP2A* has deleterious effects [32]. Therefore, we decided to down-regulate *TOP2A* levels in CAL51 by RNA interference using a lentiviral vector. CAL51-shTOP2A cells expressed approximately 50% less *TOP2A* mRNA than the corresponding control cells transfected with the empty vector (Fig. 5A). Down-regulation of *TOP2A* led to a notable increase in the number of doxorubicin resistant colonies (Fig. 5B). Thus, down-regulation of *TOP2A* leads to drug resistance in CALDOX cells.

3.5 Methylation of MGMT promoter in doxorubicin-resistant cells leads to collateral sensitivity to carmustine

Carmustine, and similar alkylating agents, cause cytotoxicity through the formation of O^6-methylguanine, genotoxicity and apoptosis. These adducts are removed from the O^6-guanine by O^6-methylguanine-DNA methyl transferase (MGMT; E.C. 2.1.1.63) in a reaction that transfers the alkyl group from the DNA to an internal cysteine residue in the MGMT, thus restoring the integrity of DNA [33]. Since it is well established that cells with low levels of MGMT are sensitive to alkylating agents [34],[35], we asked whether the collateral sensitivity of CALDOX cells to carmustine could be due to MGMT down-regulation. Indeed,

MGMT mRNA was undetectable in CALDOX cells, while expressed at abundant levels in CAL51 cells (Fig. 6A). Epigenetic modifications in *MGMT* have been described in colorectal [36] and more recently in triple negative breast cancer [37]. We, thus, asked whether *MGMT* promoter was methylated in CALDOX cells. We analyzed methylation at 8 CpG sites in the CpG island on the *MGMT* promoter in both CAL51 and CALDOX cells. Hypermethylation of these 8 sites was found in CALDOX cells (approximately 75%) whereas CAL51 cells only showed background levels (around 5%) as these cells express *MGMT* (Fig. 6B). In contrast, *TOP2A* promoter remained unmethylated in both cell lines. Methylation values of *LINE1*, which have been reported as a surrogate for global methylation [27], did not change either between cell lines and when compared to normal human genomic DNA. This indicates that during the generation of CALDOX cells there has been no major change in global methylation and that the changes found are specific for *MGMT.* Thus, the collateral sensitivity to carmustine in doxorubicin-resistant cells is due epigenetic changes involving hypermethylation at the *MGMT* locus.

In order to confirm the role of MGMT in the collateral sensitivity of CALDOX cells to carmustine, we generated a stably transfected CALDOX derivative, CALDOX-MGMT, overexpressing MGMT by retroviral transduction. These cells expressed approximately 70% *MGMT* mRNA levels than those found in the parental CAL51 cells (Fig. 7A). Reintroduction of MGMT resulted in the reversal of carmustine sensitivity (Fig. 7D), without altering resistances to etoposide (Fig. 7C) or doxorubicin (Fig. 7B). Thus, down-regulation of *MGMT* is responsible for the collateral sensitivity to carmustine in doxorubicin-resistant CALDOX cells.

4. Discussion

The use of drug resistant derivatives from cell lines is of paramount importance for the characterization of the molecular mechanisms involved in cancer drug resistance. Many of the traditional drug resistant lines were generated by exposition to increasing concentration of the chemotherapeutic agent in a step-wise fashion, generating drug resistant derivatives with several-hundred-fold greater resistance to the drug relative to the parental cell line [38],[39], and is a method that is still in use these days [40]. These drug resistant lines were very important before the techniques to ectopically express foreign DNA were of routine used, since they over-expressed one or several of the molecular effectors conferring drug resistance, such as P-glycoprotein, and biochemical assays could be easily performed with them [41],[42],[43]. However, such drug resistant cell lines are far from ideal when studying regulatory mechanisms controlling drug resistance since the concentration of drug used vastly exceeds that found by cancer cells. Current protocols for the generation of resistant sublines involve the continuous use of lower doses of drug than those used in the above studies [22],[13] or the treatment with single, or repeated, doses of drug for a very short period (hours), mimicking the burst of drug in the serum of chemotherapy-treated patients [44]. It has been argued that the protocol used in the generation of resistant cells influences the mechanisms of resistance selected in such cells [45], being this, in the majority of cases, up-regulation of P-glycoprotein. We have used a moderate concentration of doxorubicin (0.4 µM) to generate doxorubicin resistant derivatives from CAL51, a breast cancer cell line with a normal diploid karyotype and luminal phenotype negative for ER, PgR and ERBB2 [18], although subtle genomic alterations, as expected in a cancer cell, have been described [46],[47]. Very few cellular models, if any, for drug resistance in triple negative breast cancer are available. As this form of cancer is particularly aggressive and currently lacking any

targeted therapy, the development of cellular models is of paramount importance to unravel molecular mechanisms of drug resistance and to test novel drugs. Surprisingly CALDOX cells have not up-regulated P-glycoprotein expression, or other drug transporter, being thus an excellent model for P-glycoprotein-independent triple negative breast cancer.

Drug sensitivity assays confirmed the lack of involvement of ABC transporters, as CAL51 and CALDOX cells were equally sensitive to the P-glycoprotein substrate camptothecin [48]. However, cross-resistance to topoisomerase II, but no topoisomerase I, inhibitors was observed. Topoisomerase II performs essential DNA topologic changes in proliferating eukaryotic cells decatenating the newly replicated sister DNA molecules. Doxorubicin and mitoxantrone intercalate in DNA and trap topoisomerase II as a covalent complex on DNA, whereas etoposide interaction is weaker and other protein-drug interactions have been suggested to play key roles in the formation of topoisomerase II trapped complexes [49],[29]. As this process ultimately leads to DNA damage, activation of apoptosis and cell death, it is clear that the overall level of topoisomerase II is a determinant of drug sensitivity. Decreased levels of the enzyme have been found to cause doxorubicin resistance in cell systems and mouse tumour models [50] due to differential expression during the cell cycle [51] and promoter methylation [52]. In addition, mutations in the coding region in both cell lines and clinical samples resulting in drecreased activity lead to drug resistance [30]. Conversely, high levels of topoisomerase II, due to gene amplification, positively correlates with response to anthracyclin neo-adjuvant chemotherapy in locally advance primary breast cancer [53]. In the triple negative breast cancer model presented here, down-regulation of *TOP2A* mRNA is a determinant for the generation of doxorubicin resistant cells. The mechanism by which *TOP2A* expression is reduced in this drug resistance model is not due to either mutations in the coding region (data not shown) or altered CpG methylation in the -200

to -166 promoter region, although methylation at other sites or mutations affecting gene expression could have arisen in an as yet unidentified region of the locus.

Collateral sensitivity in drug resistant cells has been considered a case of "synthetic lethality" in which the genetic alterations that confer resistance to a drug sensitizes it to other drugs [5]. Doxorubicin-resistant cells expressing P-glycoprotein have been described to show collateral sensitivity to cisplatin, cytarabine or gemcitabine [54],[55]. In addition, other drug resistant cells by down-regulation of topoisomerase II have been described to show collateral sensitivity to gemcitabin, but not to alkylating agents [54]. No systematic studies screening drug resistant cells for collateral sensitivity have yet been reported. There are reports in the literature describing colateral sensitivity in a variety of drug resistant models, but none include sensitivity to carmustine [4].

Although alkylating agents, such as cyclophosphamide, form part of routine breast cancer chemotherapy, those producing O^6-methylguanine or O^4-methylthymine (which are inactivated by MGMT) are used in the treatment of glioblastoma and to a lesser extent in treating malignant melanoma, gastointestinal and pancreatic cancer, Hodgkin's and non-Hodgkin's lymphoma [33]. However, some trials using temozolomide have been reported [56], and carmustine has been used in stage II-III and metastatic breast cancer [57],[58]. Loss of MGMT has been found in breast tumours recurring locally after operation, indicating that MGMT is a positive predictive factor for relapse-free survival [59], and *MGMT* promoter methylation has been recently reported in breast cancer [37]. Our data highlights the link between doxorubicin resistance and colateral sensitivity to carmustine and offers the potential new avenue of evaluating of *MGMT* promoter methylation status to design better therapeutic strategies for triple negative breast cancer.

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Figure captions

Figure 1. Doxorubicin resistance in CALDOX cells is not mediated by membrane transporters**.** (A) CALDOX cells (IC_{50} 0.6 μM) are 30 fold more resistant to doxorubicin than CAL51 cells (IC_{50} 0.02 μM). Sulphorhodamine B staining after 3 cell doublings. (B) Absence of membrane pumps in CALDOX cells. *Left panels*, Flow cytometric analysis of P-glycoprotein expression using the UIC2-phycoerythrin-conjugated antibody (*filled peaks*) and the corresponding IgG isotype control (*clear peak*). For CAL51 and CALDOX cells, the IgG and UIC2 peaks overlap, indicating that active P-glycoprotein is not present at the cell surface. NCI/ADR-Res cells are used as a positive control. *Middle panels*, Flow cytometric analysis of BODIPY-taxol efflux. The incubation of cells in the presence of a membrane pump inhibitor (cyclosporin A, *filled peaks*) produces an increase in the fluorescence of the cells since they cannot extrude the fluorescent dye out of the cell. *AF*, autofluorescence. For CAL51 and CALDOX cells, the fluorescence peaks in the presence and absence of cyclosporin do overlap, indicating absence of cyclosporin-inhibitable membrane pumps. NCI/ADR-Res cells are used as a positive control. *Right panels*, Flow cytometric analysis of intracellular doxorubicin accumulation. Cells were incubated with 0.4 μM doxorubicin for 15 min and detected in the flow cytometer red channel (*filled peaks*); *AF*, autofluorescence. In both CAL51 and CALDOX cells doxorubicin accumulates intracellularly and the cells fluoresce. Cells with active membrane pumps (NCI/ADR-Res) do not accumulate doxorubicin intracellularly. Experiments were repeated at least twice, and one representative experiment is shown.

Figure 2. CALDOX cells have a slow proliferation rate and abnormal cell cycle profile. (A) Cells were seeded in 6-well plates and at different time points the cells were trypsinized, stained with trypan blue and those negative were counted. Growth of both cell lines fit

(r^2>0.95) to exponential curves with doubling times of 18 and 32 h for CAL51 and CALDOX cells, respectively. Data represent the average ± SD of at least three experiments. (B) Cell cycle profiles (*left panel*) indicate CALDOX cells have an increased proportion of cells at G1, with less proportion of cells at S and G2/M (*right panel*). Experiments were repeated at least twice, and one representative experiment is shown.

Figure 3. Higher concentrations of doxorubicin are necessary to trigger a p53-driven DNA damage response in CALDOX than in CAL51 cells. Cells were treated with increasing concentrations of doxorubicin for 24 h. (A) Cell cycle profile of CAL51 (*upper panels*) and CALDOX (*lower panels*) cells. Doxorubicin induces an increase in the proportion of cells at G1 and sub-G1 (apoptotic). Conversely, the profile of CALDOX cells only shows apoptotic cells at very high doxorubicin concentration. (B) Western blot analysis of p53 and p21 (*left panel)* and quantification of at least two blots using Image J (*right panels*). (C) *CDKN1A* mRNA expression analysis upon doxorubicin treatment. Doxorubicin can trigger a p53 response in CALDOX cells but at higher concentrations than in CAL51 cells. Note the presence of p21 in CALDOX cells in the absence of drug. Experiments were repeated at least twice, and one representative experiment is shown in (A) and (B, *left panel*). RNA expression data (C) is shown using two normalizers and represent the average ± SD of three independent replicates.

Figure 4. The p53-DNA damage response pathway is not responsible for the drug resistant phenotype of CALDOX cells. CALDOX cells were retrovirally transduced to express a small hairpin targeting *TP53* mRNA. (A) Down-regulation of *TP53* and the p53 target *CDKN1A* by RT-QPCR. Two normalizers were used and data represent the average ± SD of three independent replicates. (B) sh*TP53* CALDOX cells have a cell cycle profile similar to that of

CAL51 cells. (C) Doxorubicin response curves obtained by sulphorhodamine B staining after three cell doublings indicate that knocking down p53 does not change drug resistance. sh*TP53* CALDOX cells (*straight line*); empty vector CALDOX cells (*dashed line*). Data represent the mean ± SD of at least four independent experiments.

Figure 5. Down-regulation of topoisomerase II leads to drug resistance.(A) *TOP2A* mRNA expression by RT-QPCR of drug naïve (CAL51), drug resistant (CALDOX) and CAL51 cells retrovirally transfected with a small hairpin targeting *TOP2A* mRNA. Control cells were transfected with empty vector (EV). Data represent the mean ± SD or three independent replicates. (B) Clonogenic assay for drug resistance. Cells (2.5×10^4 / 25 cm^2) were treated with 0.1 μM doxorubicin and resistant clones were left to grow for 4 weeks. Flasks were stained with crystal violet. Pictures shown are representative of at least two independent experiments.

Figure 6. The *MGMT* promoter is hypermethylated in CALDOX cells. (A) O^6-methylguanine-DNA methyltransferase is not expressed in CALDOX cells. Expression data obtained by by RT-QPCR normalized to *RPS6* and *RPS14* showed a complete absence of *MGMT* transcripts. Data represent the mean ± SD or three independent replicates. (B) Pyrosequencing analysis of methylation of CpG sites within the *TOP2A* (4 sites) and *MGMT* (8 sites) gene promoters in CAL and CALDOX cells as well as normal human genomic DNA. Standard *LINE1* methylation (4 sites) analysis was performed to assess the global methylation levels of the sample. Data represent the mean ± SD of three cytosine methylation measurements.

Figure 7. Collateral sensitivity to carmustine can be rescued by over-expresion of MGMT.

(A) Retrovirally transfected CALDOX cells overexpressing MGMT (*CALDOX-MGMT*) increased its mRNA levels nearly to those found in CAL51 cells. An empty vector was used to generate control cells (*CALDOX-EV*). Expression was determined by RT-QPCR using two normalizers. Data represent the mean ± SD of two independent experiments. (B-D) Overexpression of MGMT reversed the carmustine sensitive phenotype to that of parental CAL51 cells but did not change the resistance to topoisomerase II inhibitors. Sulphorhodamine B staining was used as a surrogate for cell density after cells were treated for 6 days with 1 µM doxorubicin (B), 50 µM etoposide (C) and 30 µM carmustine (D). Data represent the average ± SD of at least four replicates.

Table 1

Table 1. Oligonucleotides used for quantitative PCR

Gene	Orientation	Name	Sequence
MGMT	sense	OLEY337	5'-GGTCTGCACGAAATAAAGCTCC-3'
	antisense	OLEY338	5'-GCACGGGGAACTCTTCGATA-3'
RPS14	sense	OLEY373	5’-TCACCGCCCTACACATCAAACT-3’
	Antisense	OLEY374	5’-CTGCGAGTGCTGTCAGAGG-3’
RPS6	sense	OLEY375	5’-AGGGTTATGTGGTCCGAATCA-3’
	antisense	OLEY376	5’-TGCCCCTTACTCAGTAGCAGG-3’
RPLP0	sense	OLEY377	5’-GGCGACCTGGAAGTCCAACT-3’
	antisense	OLEY378	5’-CCATCAGCACCACAGCCTTC-3’
TOP2A	sense	OLEY309	5’-TCATCAAGATTGTGGGTCTTCAG -3’
	antisense	OLEY310	5’- CCTCCAGAAAACGATGTCGCA-3’
TP53	sense	OLEY295	5’-CCGCAGTCAGATCCTAGCG-3’
	antisense	OLEY296	5’-AATCATCCATTGCTTGGGACG-3’
CDKN1A	sense	OLEY297	5’-CCTGTCACTGTCTTGTACCCT-3’
	antisense	OLEY298	5’-GCGTTTGGAGTGGTAGAAATCT-3’

Table 2

Table 2. Oligonucleotides used for pyrosequencing

Gene	Orientation	Use	Sequence
TOP2A	sense	PCR	5’-AGTGAGTTTTTTTTATTGGTTAGATTT-3’
	antisense	PCR	5’-Biotin-CAAACCAACCAATCCCTAACT-3’
	sense	sequencing	5’-GGTGGATATAAAAGGTAAGTT-3’
MGMT	sense	PCR	5'-GTTTAGGATATGTTGGGATAGT-3'
	antisense	PCR	5'-Biotin-ACCCAAACACTCACCAAATC-3'
	sense	sequencing	5’-GGATATGTTGGGATAGTT-3’
LINE1	sense	PCR	5’-GGATTTTTTGAGTTAGGTGTGGG-3’
	antisense	PCR	5’-Biotin-CAAAAATCAAAAAATTCCCTTTCC-3’
	sense	sequencing	5'-AGGTGTGGGATATAGT-3'

Table 3

Table 3. CALDOX sensitivity to different drugs

Drug	Target / mode of action	IC_{50}		CALDOX resistance ratio
		CAL51	CALDOX	
Doxorubicin	Topoisomerase IIα	0.015 7μM	0.6 μM	45
Etoposide	Topoisomerase IIα	0.65 μM	>100 μM	>150
Mitoxantrone	Topoisomerase IIα	0.0015 μM	0.25 μM	166
Camptothecin	Topoisomerase I	0.07 μM	0.08 μM	1.1
Cisplatin	DNA crosslinking	20.7 μM	30.5 μM	1.4
Taxol	Microtubule depolymerization	3.5 nM	9.2 nM	2.6
Carmustine	Alkyl adduct formation (O^6 guanine)	>80 μM	13 μM	0.16[†]

[†]CALDOX cells are more sensitive to carmustine than CAL51 cells

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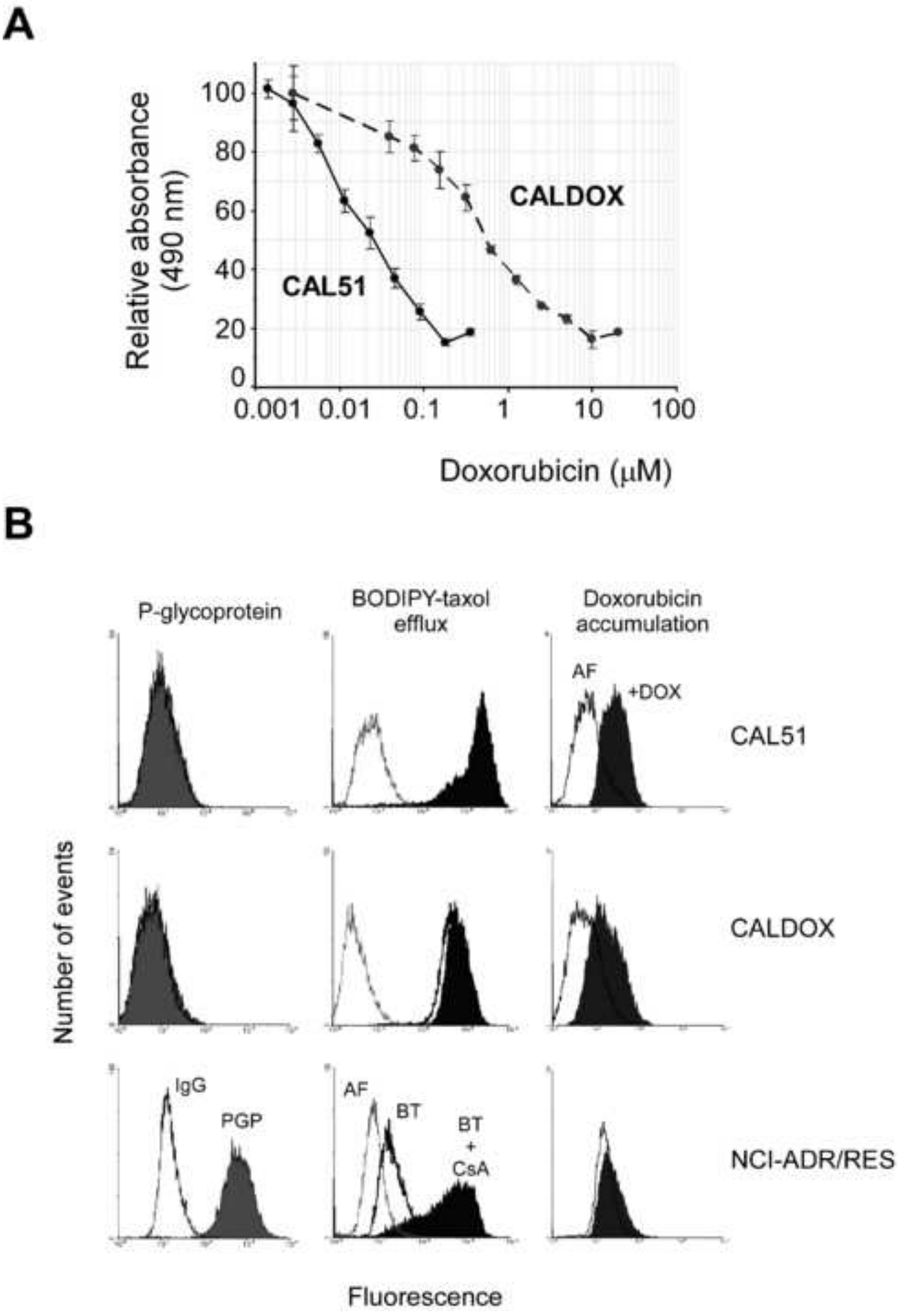


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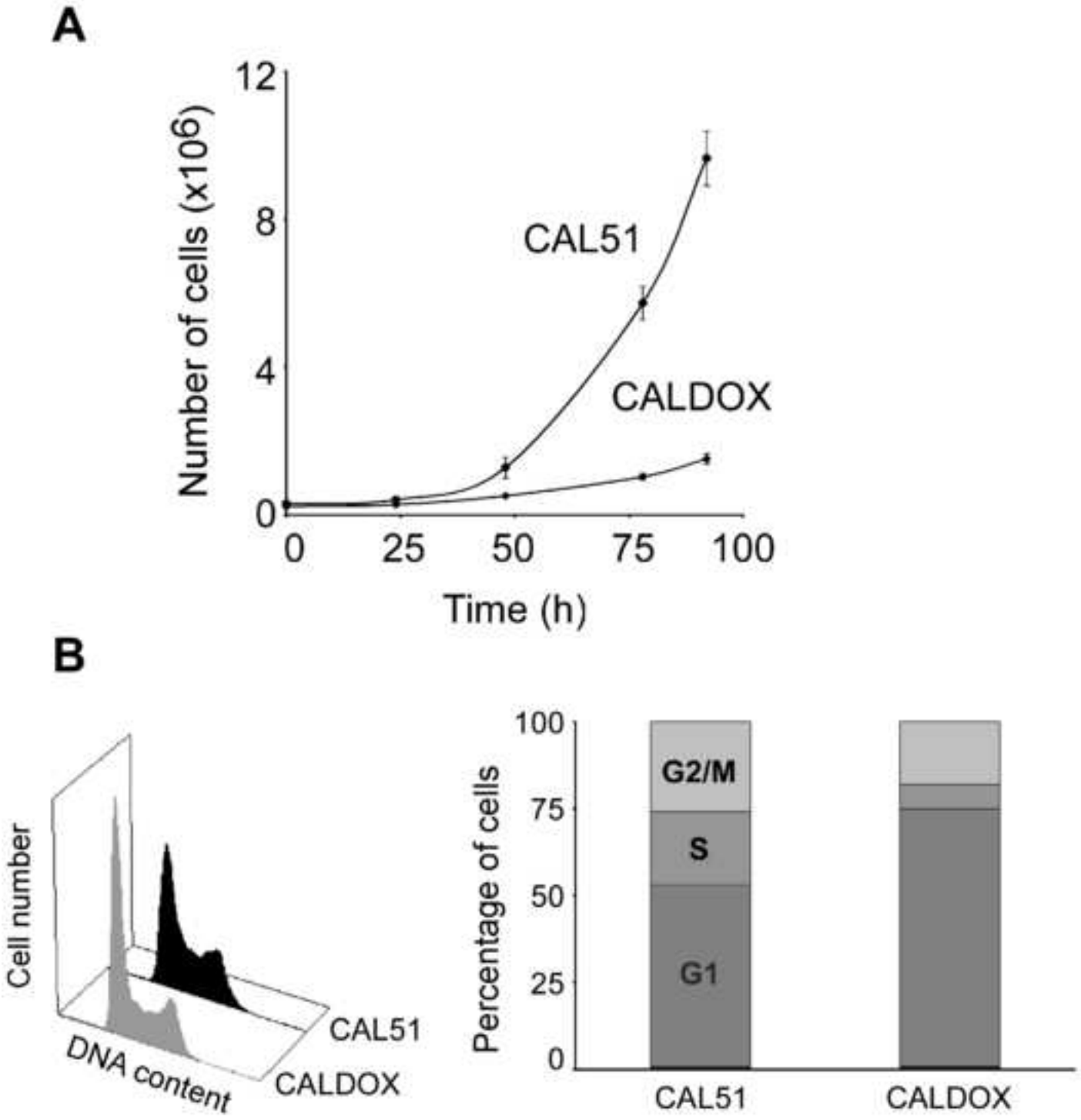


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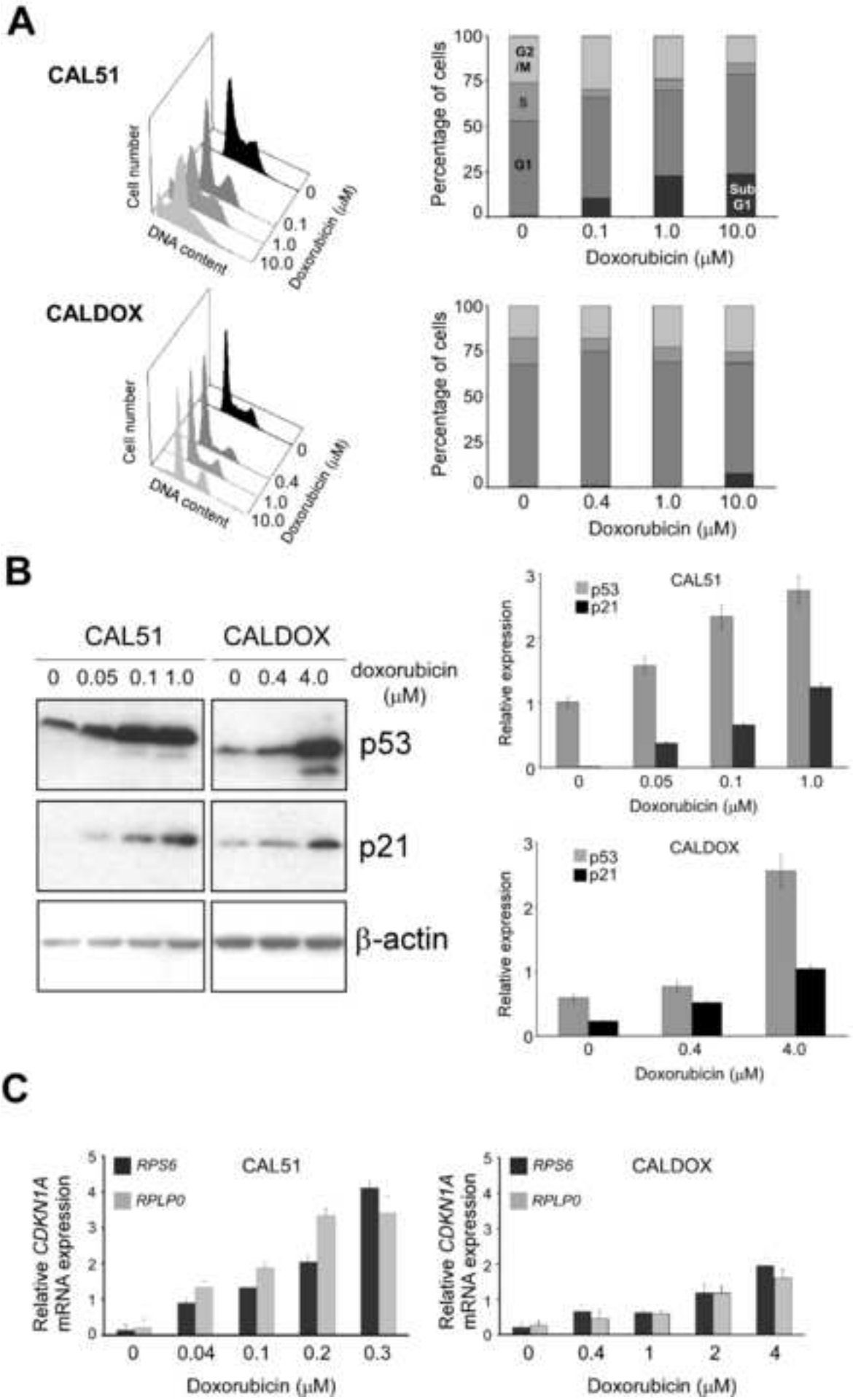


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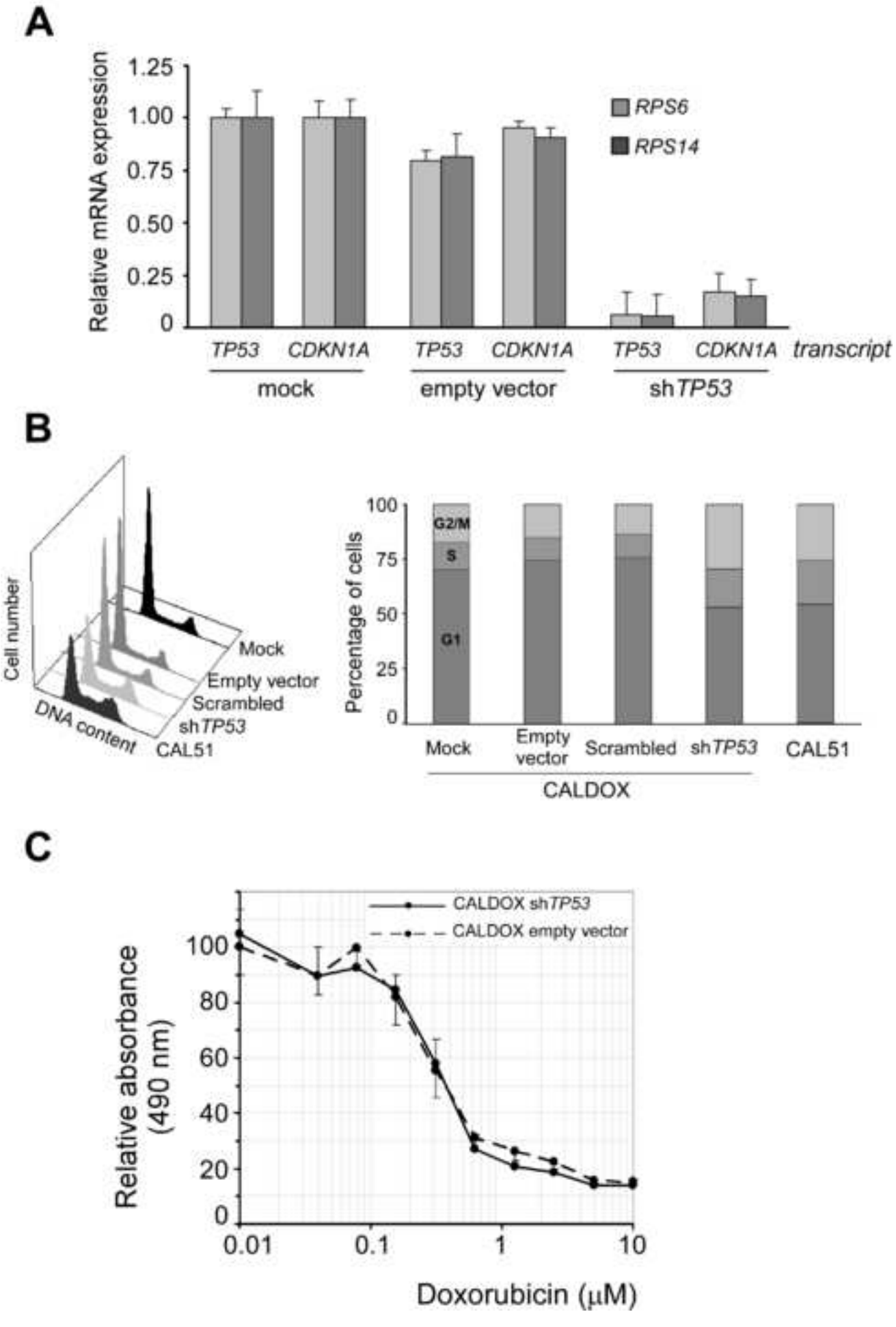


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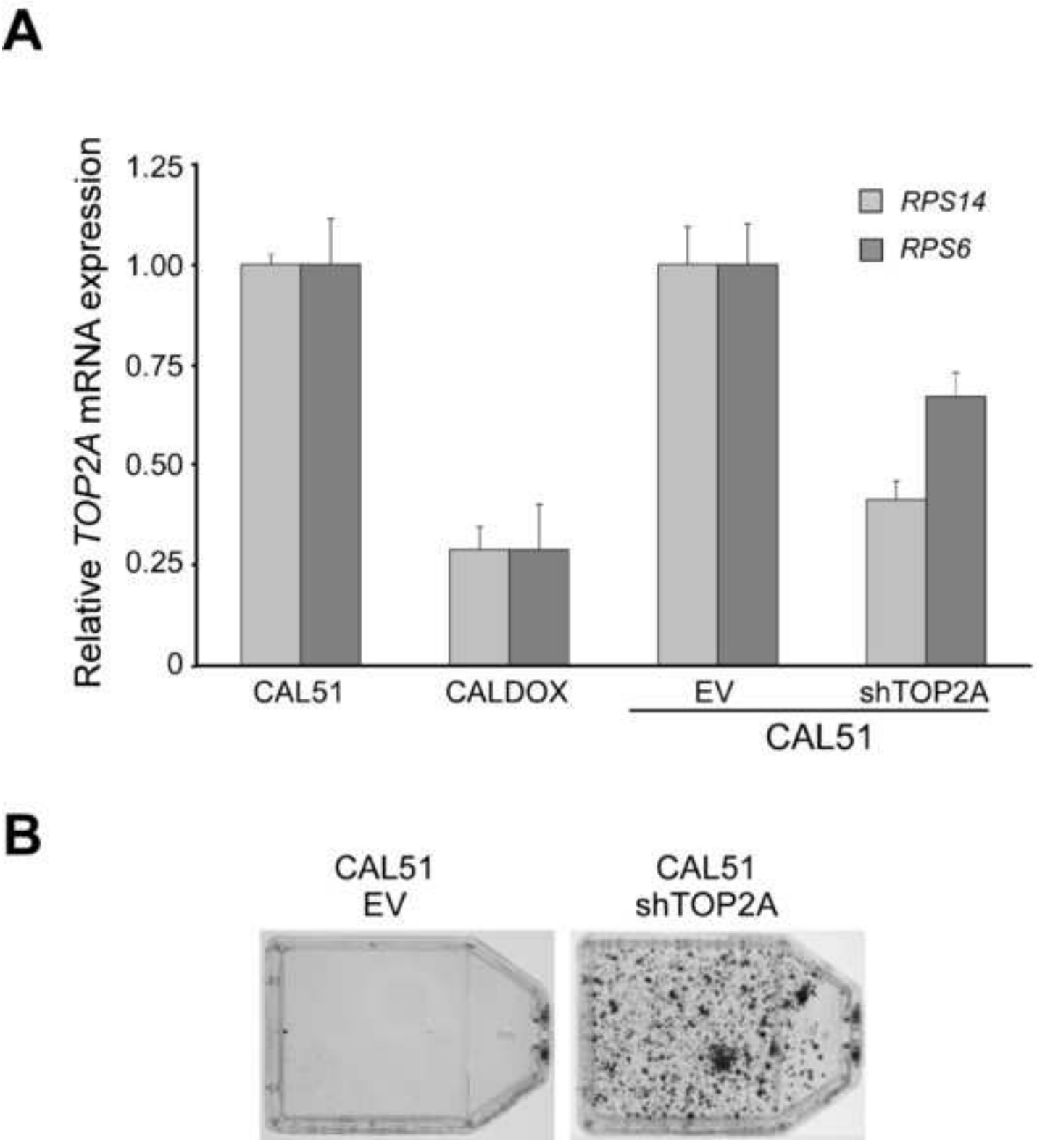


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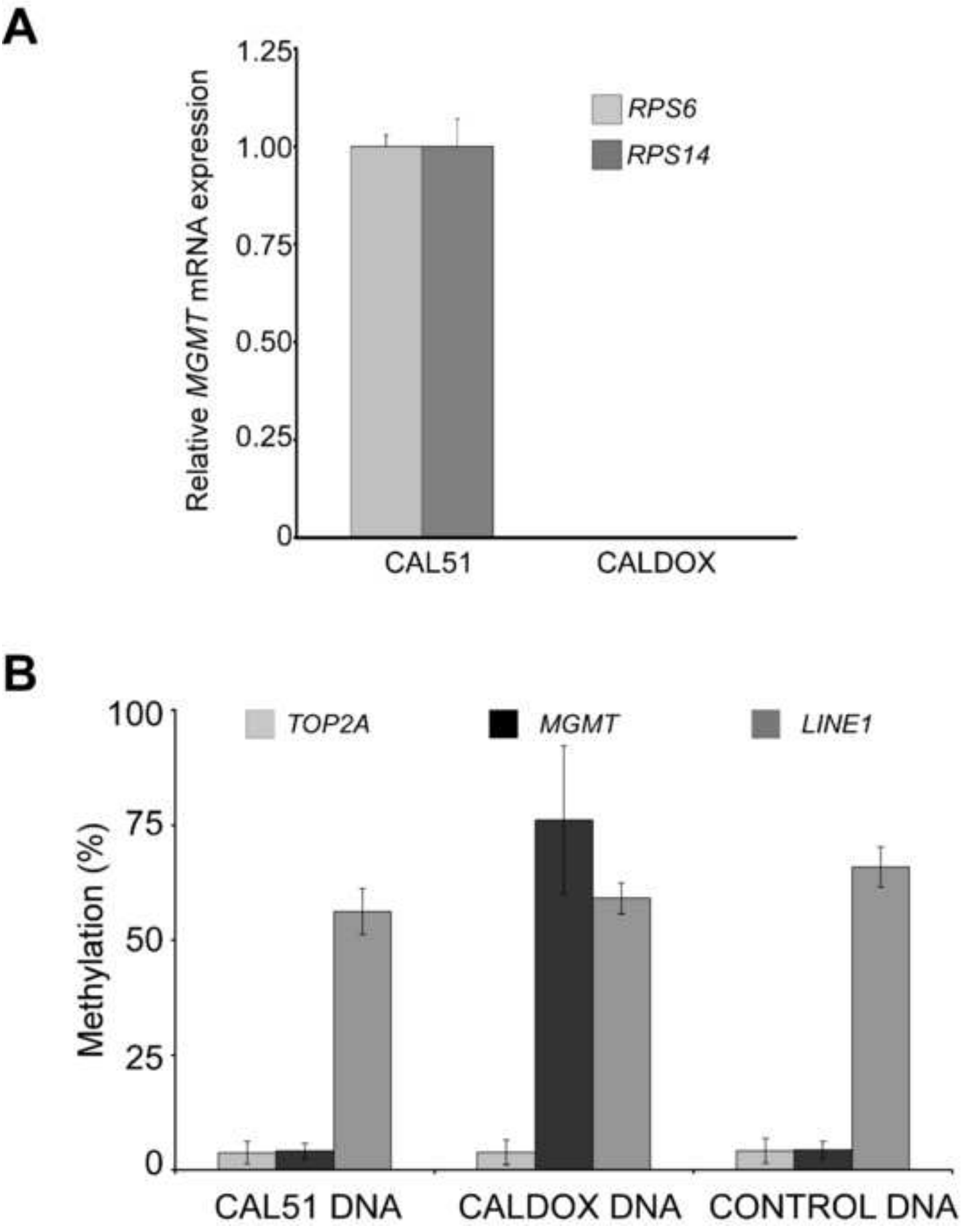
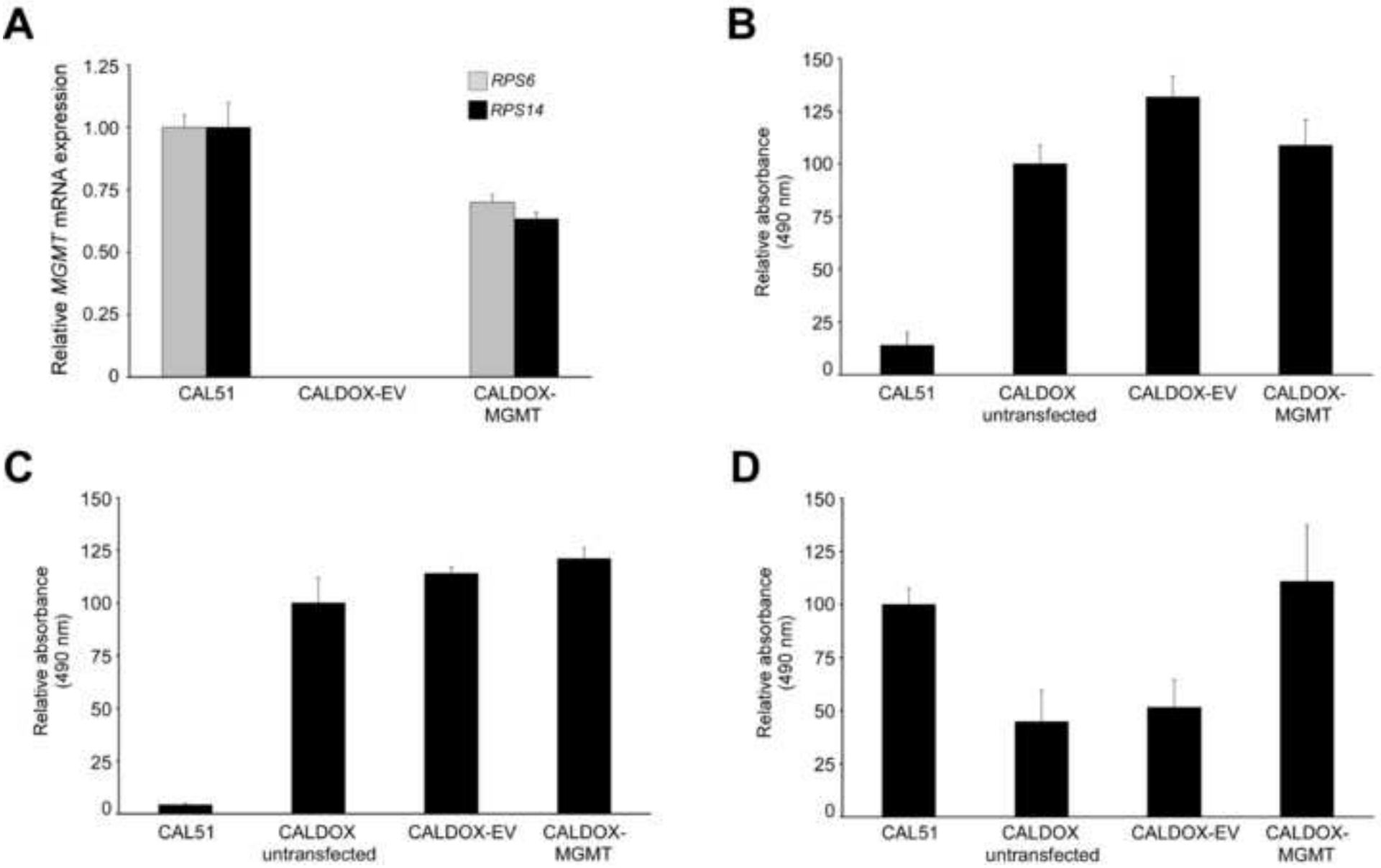


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*Graphical Abstract (for review)

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