

RBBP6 Interaction with the p53–MDM2 Complex Supports Plerixafor-Based Anticancer Targeting

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Abstract

The Retinoblastoma Binding Protein 6 (RBBP6) is a multidomain protein with an E3 ubiquitin ligase motif (RING) and negatively regulates p53 stability. While its role in cell proliferation, cellular signalling, and RNA processing is well established, the subcellular distribution and functional relevance of its association with p53 in cancer remain incompletely understood. In this study, we investigated the subcellular localization and interactions of RBBP6, p53, and MDM2 in a normal human (HEK293) and cancer cell lines. Confocal microscopy revealed that in HEK293 cells, RBBP6 localizes predominantly to nuclear speckles, whereas p53 is largely cytoplasmic, suggesting compartmental separation under basal conditions. In lung adenocarcinoma A549 cells, RBBP6 exhibited increased cytoplasmic localization and p53 accumulated in the nucleus, with some co-localization. Co-immunoprecipitation experiments performed in HEK293 and MCF-7 breast cancer cells confirmed the formation of a tripartite RBBP6/p53/MDM2 complex, demonstrating that this interaction occurs in normal and malignant cells. We further examined the functional consequences of targeting this axis using plerixafor, an FDA-approved CXCR4 antagonist previously predicted *in silico* to bind the RBBP6 RING finger domain. Plerixafor induced apoptosis in A549 cells at concentrations not toxic to HEK293 cells. The apoptotic effect was coupled with downregulation of p21, without changes in RBBP6 or ERK expression. Since the RBBP6/p53/MDM2 interaction influences p53 tumor suppressor activity, disrupting this axis could restore p53 function and inhibit tumor growth. The p21 perturbation suggests that plerixafor may directly or indirectly interfere with the p53 pathway, providing a basis for potential therapeutic repurposing in cancers with dysregulated p53 pathways. This work provides mechanistic insight into RBBP6-mediated p53 regulation and supports the potential repurposing of plerixafor for cancers characterized by dysregulation of the p53/MDM2/RBBP6 axis.

1. Introduction

Cancer results from a series of events leading to abnormal cell proliferation that has resulted from the cell bypassing cell cycle checkpoints [1], [2]. The tumour suppressor protein p53 is a master regulator of cellular stress responses, with well-characterised roles in DNA repair, cell cycle arrest, apoptosis, senescence, and autophagy [3], [4], [5], [6]. Under normal physiological conditions, p53 is maintained at low intracellular levels through ubiquitin-dependent degradation, largely mediated by the Murine Double Minute 2 (MDM2) protein. MDM2 is an E3 ubiquitin ligase that directly binds p53 and targets it for proteasomal degradation [7], [8], [9], [10]. In many cancers, p53 is either mutated or functionally inactivated by overexpression of its negative regulators, notably MDM2 and related proteins [11], [12], [13].

Emerging evidence implicates Retinoblastoma Binding Protein 6 (RBBP6) as an important co-regulator of the p53/MDM2 axis. RBBP6 is a multi-domain protein with E3 ligase through a RING finger domain. It also possesses the ubiquitin-like domain DWNN (Domain With No Name), Zinc Knuckles, and binding domains for two tumour suppressor genes, namely p53 and Rb (retinoblastoma). RBBP6 plays critical roles in mRNA processing, cell cycle regulation, and cell proliferation [14], [15], [16], [17], [18], [19].

Several isoforms of RBBP6 have been identified, including the pro-apoptotic cytoplasmic isoform 3 (~ 17 kDa), which is made up of the DWNN domain, and the full-length isoform 1 (~ 200 kDa), which is active in nuclear processes and cancer progression [20], [21], [22], [23], [24], [25]. RBBP6 has been found to be overexpressed in several cancers, is often linked to a poor prognosis, and has been identified as a potential cancer biomarker [21], [26], [27], [28], [29]. However, whether the degradation of p53 is due to direct interaction with the RBBP6's own RING finger domain or indirectly through interaction with MDM2 or a combination of both still needs to be determined. Nevertheless, the subcellular localization and colocalization dynamics of RBBP6 and p53 in normal versus cancer cells have not been thoroughly investigated.

Despite extensive characterization of the p53/MDM2 interaction, the existence of a tripartite RBBP6/MDM2/p53 complex in human cells has not been rigorously validated. While animal and yeast studies support functional cooperation, co-immunoprecipitation and *in situ* imaging data in human cancer cell lines are lacking [14], [18]. This represents a critical gap in understanding how post-translational modifiers like RBBP6 regulate p53 availability and function especially in tumours with wild-type *TP53* but suppressed p53 activity.

Plerixafor (AMD3100), an FDA-approved small molecule that inhibits the binding of chemokine stromal cell-derived factor 1 α to CXCR4, its cognate receptor, was shown, through molecular modelling [30]. Plerixafor is used to mobilize haematopoietic stem cells for autologous transplantation in patients with multiple myeloma and non-Hodgkin lymphoma [31], [32], [33], [34]. Plerixafor blocks the CXCL12–CXCR4 signalling axis, which is implicated in tumour-stroma interaction, metastasis, angiogenesis, and drug resistance [35], [36]. Twala showed that plerixafor binds to the RING finger domain of RBBP6 with high affinity [30]. Since RBBP6 interacts with and negatively regulates p53, and enhances the enzymatic activity of Mdm2, we reasoned that inhibiting its E3 ligase activity could stabilise and reactivate p53 in cancer cells where RBBP6 and/or Mdm2 are overexpressed in a favourable p53 genetic background. The pharmacological reactivation of p53 can occur in a background of wild-type p53 or in the presence of certain mutational variants whose conformation can be restored to wild type [37]. This computational hypothesis, if validated, would significantly expand the therapeutic repertoire of plerixafor, transforming it from a stem cell mobilizing agent to a dual-function anticancer compound. To date, no biological studies have investigated plerixafor's impact on RBBP6 function.

2. Methods and Materials

2.1. Cell lines

A549 (Cat# A54-FL/C/T/V) (flask) and MDA-MB-231 (Flask) cell lines were purchased from Cellonex (South Africa). HEK293 cells were kindly donated by Dr. Jitcy Joseph from the National Institute for Occupational Health (NIOH) in Johannesburg, South Africa, and MCF-7 by Prof. Raymond Motadi from the Department of Biochemistry at the University of Johannesburg, South Africa.

2.2 Immunocytochemistry

Cells were cultured at 37°C, with 5% CO₂ in 89% DMEM, 10% FBS and 1% pen/strep media. Cells were seeded using trypsin-EDTA onto sterilised coverslips and grown until they reached a confluency of between 70 and 80%. The cells were fixed with 3% formaldehyde and permeabilised by 0.25% Triton X-100 in phosphate buffered saline (PBS) and blocked for 1 hour 30 minutes using 10% normal goat serum with 0.1% bovine serum albumin (BSA) before the primary antibody was added. The cells were then incubated at 4°C for 12–16 hours. After washing five times with PBS containing 0.1% Tween the cells were incubated in secondary antibody for one hour 30 minutes at 37°C in the dark and washed five times with PBS with 0.1% Tween. The procedure was repeated for the second set of antibodies for double-stained cells. A range of concentrations from 1:50 to 1:5000 of primary and secondary antibodies was used. Prolong Gold Anti-fade reagent with DAPI (Invitrogen) (10P36935) was used as mounting media and the nucleus stain. A Zeiss LSM 710 series confocal fluorescence microscope with Zen Black imaging software was used to analyse the stained cells. ImageJ software [38] with the JACoP plugin [39] was used to quantify colocalization analysis.

2.3. Coimmunoprecipitation

Cells were sonicated on ice for five cycles of 10 seconds at 60 Amps. The lysed cells were then centrifuged at 12,100 x g for 20 minutes at 4°C before adding 50 µl/ml of protease inhibitor. After adding the primary antibody, the mixture was incubated for 5 hours at 4°C, with rotation. Next, Protein A agarose was added to the cell lysate/antibody mixture and incubated for another 12–16 hours at 4°C with rotation. Any unbound protein was removed, and the beads were washed five times with PBS and centrifuged at 1000xg for 5 minutes. Proteins were eluted off the beads by boiling the beads with SDS-PAGE sample buffer. Samples were then analysed using SDS-PAGE and Western blot.

2.4. Western blot

Western blot analysis was conducted either according [40], [41] or by using the automated system, Jess™ Automated Protein Immunodetection and Quantification (Biotechne). Protein samples were run on 10 % SDS-PAGE and then transferred onto PVDF membranes using transfer sandwiches. The transfer occurred overnight at 40 V at 4°C. Next, membranes were blocked with Sigma casein blocking buffer for 1 hour at room temperature. Next, membranes were incubated in primary antibody (1:10000 for RBBP6 and p53, 5:10000 for Mdm2) overnight at 4°C with shaking. Membranes were washed with TBST before being incubated in HRP conjugated secondary antibody (1:10000) for 1 hour 30 minutes at room temperature with shaking. Membranes were again washed with TBST before being stained with Chemiluminescent substrate (Thermo Scientific, Massachusetts, United States).

2.5. Cell viability assays

Cell viability was assessed using the Cell Proliferation Kit I (MTT; Roche, Cat. No. 11465007001). A549 and HEK293 cells were seeded at 10 000 cells per well in 96-well plates (Corning, Cat. No. 3596) and allowed to adhere overnight. Plerixafor was added at concentrations of 0, 0.1, 1, 10, and 25 µM in triplicates. Cells were incubated for 48 hours. After treatment, 10 µl of MTT solution (0.5 mg/ml in phosphate-buffered saline, PBS) was added to each well. The plates were incubated at 37°C for 4 hours,

allowing the formation of formazan crystals by metabolically active cells. Following incubation, the insoluble formazan crystals were dissolved by 100 µl of dimethyl sulfoxide (DMSO). The plates were gently shaken for 15 minutes to ensure complete dissolution of the formazan. Absorbance was measured at 570 nm using a Varioskan Lux Multimode Microplate Reader (Thermo Scientific). Cell viability was expressed as a percentage of the control, which was considered 100% viable. All experiments were performed in triplicate, and data were presented as mean ± standard deviation (SD). Differences between treated and control groups were analysed using one-way ANOVA. A p-value of < 0.05 was considered statistically significant. Statistical analysis was performed using the GraphPad Prism version 5.5. (2015). IC₅₀ values were calculated using the AAT Bioquest IC50 calculator.

Plerixafor (AMD3100; Sigma-Aldrich, Cat. No. A5602) was dissolved in sterile distilled water to prepare a 10 mM stock solution and stored at – 20°C. Working concentrations ranging from 0 to 25 µM were freshly prepared in complete medium.

2.6. Apoptosis analysis following plerixafor treatment

Apoptotic cell death was evaluated using the Annexin V-FITC Apoptosis Detection Kit (Invitrogen, Cat. No. V13242). A549 and HEK293 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and treated with Plerixafor at 3 µM and 30 µM for 48 hours. Cells were harvested by trypsinization, washed twice with cold phosphate-buffered saline (PBS), and resuspended in 100 µL of binding buffer. Each sample was stained with 5 µL of Annexin V-FITC and 5 µL of Propidium Iodide (PI) for 15 minutes at room temperature in the dark. After adding 400 µL of binding buffer, samples were analysed using a BD FACS Aria III flow cytometer. A total of at least 10 000 events per sample were recorded, and data were analysed using FACSDiva software to quantify the proportions of viable, early apoptotic, late apoptotic and necrotic cells.

2.7. Automated western blot analysis for cell cycle-related protein expression

Western blotting was performed to evaluate the expression levels of p21, MDM2, RBBP6 and ERK1 proteins. Cells were treated with plerixafor at 3 µM and 30 µM for 48 hours, followed by protein extraction using CellLytic M Cell Lysis Reagent (Sigma-Aldrich, Cat. No. C2978) supplemented with Protease Inhibitor Cocktail (Roche, Cat. No. 11697498001). Protein concentrations were determined using the Qubit™ Protein Broad Range (BR) Assay Kit (A50668, Thermo Fisher Scientific, United States), measured by spectrophotometry with the Qubit™ Flex Fluorometer (Thermo Fisher Scientific, United States).

Protein samples were mixed with the ProteinSimple master mix (4 parts protein: 1 part master mix) and denatured by heating at 95°C for 10 minutes. Ten microliters of the denatured protein samples were loaded onto a 25-well plate/module pre-loaded with buffers, part of the 12–230 kDa Separation Module (SM-W001, ProteinSimple). Antibodies used were anti-p21 (Abcam, ab109520), anti-MDM2 (Abcam, ab38618), anti-RBBP6 (Abcam, ab95909), and anti-ERK1 (ProteinSimple, SP-003-152). The Western Blot

analysis was executed using the Jess™ Automated Western Blot System (ProteinSimple, Bio-Techne), employing a 25-well chemiluminescence cartridge for protein separation and immunodetection. The automated system ran for approximately 3 hours, with data analysis performed using Compass software version 6.2.0 (ProteinSimple, Bio-Techne). Protein separation, immunodetection and chemiluminescence signal capture were performed automatically, and band quantification was carried out using Compass software.

3. Results

3.1. RBBP6 and p53 exhibit distinct subcellular localization patterns in normal and cancer cell lines

In the current study, we find that p53 and RBBP6 have an inverse relationship in a normal cell line (HEK293) where RBBP6 is expressed, almost exclusively in nuclear speckles while p53 is distributed throughout the cell but more prominently in the cytoplasm (Fig. 1).. Early studies show that p53 has nuclear and cytoplasmic functions, as a transcription factor in the nucleus and as cytoplasmic signalling [42], [43], [44], [45]. A RBBP6 primary antibody was used that recognizes isoforms 1, 2, and 4 based on the epitope specified by the manufacturer. This means this antibody cannot recognize isoform 3, the shortest isoform of RBBP6, known as DWNN. Isoform 3 has been found to be localised in the cytoplasm and to be pro-apoptotic (Mbita et al., 2012). In contrast, RBBP6 is evidently detectable in the cytoplasm of the cancer line MCF7 in addition to nuclear speckles and p53 is expressed more prominently in the nuclei of the cancer cell lines compared to the normal cell line. In the A549 cell line RBBP6 and p53 have distinct inverse subcellular localization and concentration profiles. In MDA-MB-231 cells whose p53, is mutated in the DNA binding domain, both RBBP6 and p53 are more highly expressed in the cytoplasm than in the nucleus.

To determine if there was subcellular colocalization between p53 and RBBP6 cells were dually stained with antibodies that recognise RBBP6 and p53 in MCF7 cells and A549 cells (Fig. 2). Quantitative analysis was undertaken on the colocalization images using ImageJ colocalization JACoP plugin software [46]. Manders and Pearson's coefficients were generated and are displayed in Table 1. Colocalization was calculated for RBBP6 to p53 as well as p53 to RBBP6. The background threshold was set using the Costes method [47] to prevent bias. Manders overlap coefficient measures the co-occurrence, and Pearson's correlation coefficient measures if there is a correlation. Colocalization analysis of the Pearson Coefficient (r) indicates that a portion of the two molecules colocalizes ($r = 0.759$). Alternatively, colocalized expression of RBBP6 and p53 is confined in discreet regions within the nucleus.

The measurements in Table 1 show positive colocalization occurring in both cell lines, with the Manders coefficient values indicating a higher colocalization of RBBP6 to p53 than p53 to RBBP6 in both MCF7 and A549 cells. The values do not indicate a perfect colocalization value of 1; however, this is expected

as both p53 and RBBP6 are involved in several biological functions within the cell that do not relate to their Interaction. On basic visual inspection, p53 and RBBP6 do not colocalise in the nuclear speckles.

Table 1
Quantitative co-localisation measurements. Manders and Pearson's coefficients were calculated using the JACoP plugin of ImageJ [46] for MCF7 and A549 cells.

Measurement	MCF7	A549
Mean Manders coefficient of RBBP6 to p53	0.904	0.834
Mean Manders coefficient of p53 to RBBP6	0.761	0.784
Mean Pearson's coefficient	0.759	0.630

3.2. RBBP6 forms a tripartite complex with MDM2 and p53 in normal and cancer cells.

We explored the potential p53/MDM2/RBBP6 complex formation using coimmunoprecipitation on lysates of normal (HEK293) and cancer (MCF7) cell lines. Briefly, the lysate was mixed with either an anti-p53 or anti-RBBP6 antibody, and complexes were pulled using Protein A. These complexes were visualized by Western blot analysis. Bands corresponding with p53, MDM2, and RBBP6 were detected in complexes derived from cancer and normal cells (Figs. 3 and 4). The physical interaction between Mdm2, p53, and RBBP6 is demonstrated by Western blot analysis.

3.3. Plerixafor selectively reduces viability in A549 lung cancer cells but not in HEK293 normal cells

To investigate the selective cytotoxicity of plerixafor, we performed MTT assays on A549 and HEK293 cells following 48 hours of treatment across a concentration range of 0 to 25 μ M. All experiments were conducted in biological triplicates. In A549 cells, treatment with plerixafor led to a clear dose-dependent reduction in cell viability as shown in Fig. 5 (left panel). At 0.1 μ M, cell viability remained indistinguishable from the untreated control indicating no cytotoxic effect at this concentration. However, at 1.0 μ M, viability decreased to approximately 60% marking the onset of cytotoxicity. Increasing the concentration to 10 μ M and 25 μ M resulted in further reduction in viability to ~ 50%, reflecting a strong antiproliferative effect of the drug at higher doses.

The observed trend was consistent across all replicates. Based on these results, the IC₅₀ of plerixafor in A549 cells was calculated using a four-parameter logistic regression model (AAT Bioquest IC₅₀ calculator), yielding a value of 2.4196 μ M. Consequently, a concentration of 3 μ M was selected for subsequent mechanistic studies, representing a pharmacologically relevant dose just above the IC₅₀ threshold.

In contrast to A549 cells, HEK293 cells exhibited strong resistance to plerixafor treatment. As shown in Fig. 5 (right panel), cell viability remained close to 100% across all tested concentrations. No significant differences in viability were observed when compared to the untreated control. Even at the highest dose of 25 μ M, HEK293 cells showed no reduction in viability, indicating that plerixafor is well tolerated by non-cancerous epithelial cells under these conditions. This differential response underscores Plerixafor's potential as a cancer-selective cytotoxic agent with a favourable therapeutic index.

3.4. Plerixafor induces apoptosis in A549 cells and not in HEK293 cells

Having demonstrated the dose-dependent cytotoxicity of plerixafor in A549 cells, we next investigated whether this reduction in cell viability was mediated by apoptosis. To this end, Annexin V/Propidium Iodide (PI) staining was performed followed by flow cytometry to distinguish viable, early apoptotic, late apoptotic and necrotic cells. Both A549 and HEK293 cells were treated with plerixafor at concentrations of 3 μ M (near the IC_{50}) and 30 μ M (supraphysiological dose) for 48 hours, with untreated cells serving as controls. The Annexin V⁻/PI⁻ population represented viable cells, Annexin V⁺/PI⁻ indicated early apoptosis, Annexin V⁺/PI⁺ represented late apoptosis, and Annexin V⁻/PI⁺ corresponded to necrotic/dead cells.

In untreated A549 cells, the majority (~ 85%) were viable, with approximately 10% early apoptotic and 5% late apoptotic cells, consistent with normal cellular turnover. Upon treatment with 3 μ M plerixafor, cell viability decreased to ~ 55% with concurrent increases in early (~ 25%) and late (~ 20%) apoptotic cells (Fig. 6A). Treatment with 30 μ M plerixafor produced a similar apoptotic profile with ~ 50% viable, ~ 30% early apoptotic and ~ 20% late apoptotic cells.

Quantitative analysis (Fig. 6B) confirmed that both treatment concentrations led to statistically significant increases in apoptotic populations and decreases in viable cells compared to controls. Notably, no additional apoptotic benefit was observed at 30 μ M compared to 3 μ M, suggesting that plerixafor induces apoptosis maximally at concentrations near its IC_{50} as previously determined by MTT assay.

In contrast to A549 cells, HEK293 cells displayed minimal apoptotic response to plerixafor. As shown in Fig. 7A, viability remained above 60% on average. Quantification of apoptotic fractions (Fig. 7B) demonstrated no significant differences between untreated and treated groups at either concentration.

3.5. Western blot analysis of p21, MDM2, RBBP6 and ERK expression in plerixafor treated A549 and HEK293 cell lines

To gain mechanistic insight into the selective cytotoxic and pro-apoptotic effects of plerixafor observed in A549 cells, we performed Western blot analysis to assess changes in the expression of four key regulatory proteins: RBBP6, p21, MDM2, and ERK1/2. Both A549 and HEK293 cells were treated with

Plerixafor at 3 μ M (IC_{50}) and 30 μ M for 48 hours. The experiments were conducted using the Jess Simple Western™ system, allowing precise multiplexed detection of target proteins.

RBBP6 is a multifunctional E3 ubiquitin ligase that facilitates p53 degradation via MDM2 and plays essential roles in cell cycle progression and apoptosis regulation. Previous *in silico* docking studies by Twala, 2017 suggested that plerixafor may bind to the RING finger domain of RBBP6, potentially inhibiting its E3 ligase function. However, whether this affects RBBP6 protein stability remained unknown. Western blot analysis (Fig. 8A) revealed a single immunoreactive band near $\sim$ 100 kDa in both A549 and HEK293 cells which is distinct from the canonical full-length isoforms ($\sim$ 200 kDa). This may indicate detection of an alternative isoform or a proteolytically cleaved fragment, particularly under stress or drug treatment conditions.

In contrast to expectations, plerixafor treatment led to a decrease in p21 ($\sim$ 21 kDa) expression in A549 cells. Untreated A549 cells (Lane 2) exhibited higher basal levels of p21, but treatment with 3 μ M (Lane 3) and 30 μ M (Lane 4) resulted in a marked reduction in p21 levels as shown in Fig. 8A. This suggests that plerixafor may promote apoptosis in A549 cells through a p21-independent mechanism, potentially involving alternative regulators of cell cycle progression and apoptosis. Interestingly, p21 was undetectable in HEK293 cells (Lanes 5 and 6), suggesting either that p21 expression is inherently low or that it is below the detection threshold of the assay. This further supports the notion that HEK293 cells are minimally affected by Plerixafor in terms of p21-mediated cell cycle regulation.

A strong ERK expression ($\sim$ 42–44 kDa) was detected in untreated A549 cells (Lane 2) as shown in Fig. 8B, consistent with its well-established role in promoting cell survival and proliferation in cancer cells. However, despite expectations, plerixafor treatment at 3 μ M (Lane 3) and 30 μ M (Lane 4) did not significantly alter ERK expression, suggesting that plerixafor does not exert its effects by directly modulating ERK-mediated survival pathways in these cells. Notably, ERK was undetectable in HEK293 cells (Lanes 5 and 6), suggesting that its expression is either inherently low or absent under the tested conditions. Consistent with Fig. 8A, p21 levels in Fig. 8B were significantly reduced in A549 cells following plerixafor treatment, while both p21 and ERK were undetectable in HEK293 cells.

4. Discussion

The primary concern of this study is to understand the molecular underpinnings of the drugability of the p53 pathway. A further interest is testing the validity of plerixafor as an anticancer agent. We have found that p53, its prototypical negative regulator, Mdm2, and RBBP6 form a complex, and that RBBP6 and p53 exhibit dynamic subcellular localisation patterns. Furthermore, plerixafor induces apoptosis in cancer cell lines at concentrations that are not toxic to normal cells, and it appears to impact the expression pattern of RBBP6 and p53.

The tumor suppressor protein p53 is dysregulated in many cancers, and we report here, for the first time, that p53 forms a tripartite complex with Mdm2 and RBBP6. This tripartite complex is a critical checkpoint in oncogenesis, as its disruption can stabilise p53, producing an anticancer effect, or

degrade p53, leading to cell proliferation or an oncogenic effect. Therefore, targeting this complex could yield insights into novel therapeutics for cancers in which the p53 pathway is compromised. Confocal microscopy reveals dynamic spatial localization of RBBP6 and p53 across different cellular contexts. In the normal cell line, RBBP6 is localized almost exclusively in nuclear speckles, while p53 is distributed throughout the cell but more prominently in the cytoplasm. These observations indicate that RBBP6 and p53 sometimes function independently. The localization of RBBP6 in nuclear speckles is consistent with its roles in RNA processing, gene regulation, and the coordination of transcription with splicing [48]. Although there are some gaps to be addressed, this study helps understand the dynamics of the tripartite relationship and carcinogenesis and informs anticancer drug mechanisms targeting it. Taken together, our data indicate that this complex functions in the nucleus in some instances and in the cytoplasm in others. Accordingly, based on our observations, plerixafor likely affects p53 at the cytoplasmic level, and we have constructed a conceptual schematic of this scenario (Fig. 9). It is noteworthy that the antibody used in these studies recognizes the antiapoptotic isoforms of p53, namely isoforms 1, 2, and 4. The antibody does not detect the short and proapoptotic isoform 3. Previously, Isoform 3 was shown to be localized to the cytoplasm and deregulated in carcinogenesis [49].

In contrast, the current study shows that p53 is prominently expressed in the nucleus, and RBBP6 localization in the cytoplasm increases in A549 and MCF7 cells. Only isoforms 1 and 2 have the SR domain, which is characteristic of proteins in nuclear speckles. It is interesting that both RBBP6 and p53 are prominently localized in the cytoplasm in the p53 mutant cell line MDA-MB-231, as this suggests that RBBP6 may be involved in p53's cytoplasmic functions, which include mitochondria-dependent apoptosis, metabolic regulation, and stress signalling [44], [52].

Plerixafor was initially identified as a nontoxic drug targeting HIV but was serendipitously found to be a viable agent for the mobilization of CD34⁺ hematopoietic stem cells [53]. It was found to be an antagonist of the chemokine receptor CXCR4 and to be strictly specific for this receptor compared with other chemokine receptors [53]. Recently, plerixafor was reviewed as a potential agent for non-small cell lung carcinoma (NSCLC) and small cell lung cancer (SCLC), given that the CXCR4 receptor is highly expressed in both. It has therefore been tested in in vivo preclinical trials as a potential component of specifically targeted combination anticancer therapy [54]. The findings of the current work add another dimension to the potential of plerixafor as an anticancer agent, given its binding to RBBP6.

The *in silico* study identifying plerixafor binding to the RING finger of RBBP6 has not been experimentally verified to date [30]. We needed to demonstrate the formation of the tripartite p3/Mdm2/RBBP6 complex, as this supports the pharmacodynamic rationale for repurposing plerixafor as an anticancer agent. The RING domain of RBBP6 was shown to have three potential drug-binding pockets. In the current study, plerixafor is effective against A549 cancer cells, with an IC₅₀ of 3 μ M at concentrations that are not toxic to the normal cell line. The cancer cells die by early apoptosis, indicating a death pathway that occurs before DNA repair, or, conversely, cell death mediated by mitochondrial signals. Molecular analysis by Western blot indicates that plerixafor increases proteins that cross-react with RBBP6 antibodies and downregulates the p21-effector pathway. The ERK, a component of the Mitogen-

Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK/ERK) pathway, known as a regulator of cell proliferation, survival, and differentiation, was not altered by plerixafor treatment. Since plerixafor is predicted to interact with the catalytic domain of RBBP6, we would expect its inhibition to lead to p53 accumulation and apoptosis. CXCR4 is moderately expressed and is a marker and driver of stem-like behavior in A549/GR cells [55]. However, our findings suggest that plerixafor's proapoptotic activity in A549 cells may not be solely due to CXCR4 inhibition. First, the downregulation of p21 and unchanged ERK expression upon plerixafor treatment do not align with canonical downstream effects of CXCR4 blockade, which would typically affect MAPK/ERK signalling pathways and potentially increase rather than decrease p21 levels. Second, previous *in silico* docking studies indicate that plerixafor may directly bind to the RING finger domain of RBBP6, potentially inhibiting its E3 ligase activity and altering p53 regulation. Our apoptosis and viability data are consistent with this alternative mechanism.

Functionally, plerixafor treatment induced robust apoptosis in A549 cells at concentrations around the IC_{50} ($\sim 3 \mu M$) with no further increase at higher doses, suggesting a saturation effect similar to that reported in osteosarcoma models [36]. The significant downregulation of p21, a canonical p53 transcriptional target, represents a particularly intriguing finding that supports the RBBP6-mediated mechanism. If plerixafor were acting primarily through CXCR4 blockade, we would expect an increase in p21 expression, as CXCR4 signalling typically promotes cell survival partly through PI3K/AKT-mediated phosphorylation and cytoplasmic sequestration of p21. Instead, the observed decrease in total p21 protein suggests that plerixafor induced cell death in A549 cells independently of p21 activation. There is evidence that these three molecules act together, although they also exhibit independent activities.

5. Conclusion

RBBP6 is generally considered a scaffolding protein on which many biological reactions may be anchored and exists as four isoforms, each with distinct roles, owing to the distinct configurations of functional domains. The current findings of a tripartite complex with two essential negative regulators of p53 indicate that inducible spatial and temporal signals drive its assembly, as the components exhibit dynamic subcellular expression patterns. We have shown that plerixafor is toxic to cancer cells at concentrations that are tolerated by the normal cell line. It is now necessary to investigate the role of RBBP6 and to support the proposed RBBP6-dependent mechanism of action. WE can do this using RBBP6 null mutants or gene knockdown experiments. These findings, in the context of plerixafor's use and *in vivo* studies as a therapeutic agent, suggest that its delivery and targeting require agility and creativity.

Declarations

Author contributions: MMN conceptualised the research. STM and BLR performed the experiment. All authors wrote and reviewed the manuscript.

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Conflict of Interests

The authors declare no conflicts of interest.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

This study generated original data using cell lines and did not utilise material from humans, animals, or plants. Therefore, ethical approval is not applicable.

Consent to participate

Not applicable

Consent to publish

Not applicable

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Figures

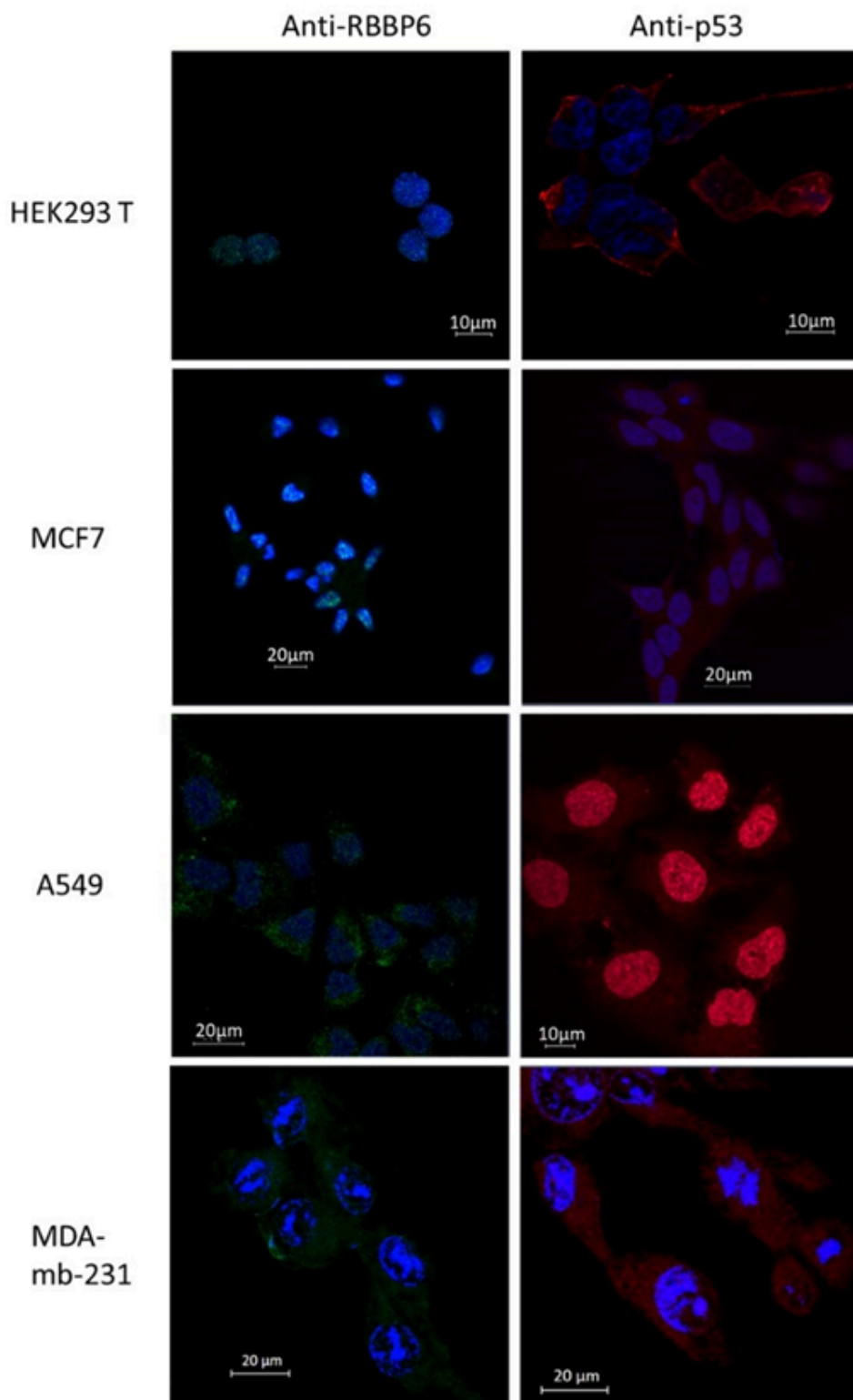


Figure 1

Localisation of RBBP6 and p53 in several cell lines. HEK293 T, MCF7, A549 and MDA-mb-231 cell lines were stained with polyclonal anti-RBBP6 (green) and monoclonal anti-p53 (red). DAPI (blue) shows the stained cell nucleus. The scale bar is indicated for each cell line. Images were captured by confocal microscopy, using optimised gain and laser power settings as determined by controls.

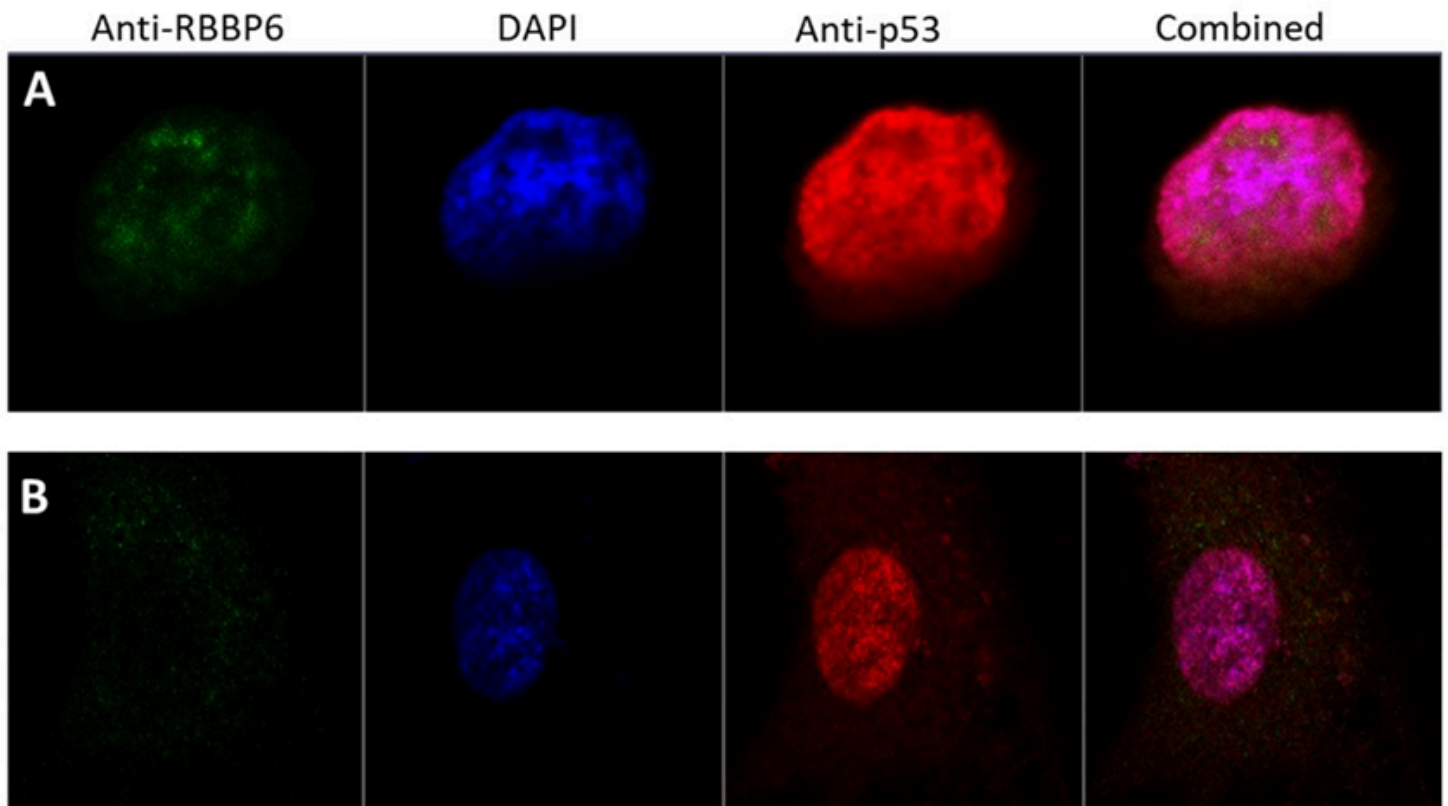


Figure 2

Co-localisation of p53 and RBBP6 in MCF7 cells and A549 cells. (A) MCF7 cells were stained with polyclonal anti-RBBP6 (green) and monoclonal anti-p53 (red). The scale bar is 5 μ m. (B) A549 cells were stained with polyclonal anti-RBBP6 (green) and monoclonal anti-p53 (red). The scale bar is 10 μ m. DAPI (blue) shows the stained cell nucleus.



Figure 3

Western blot analysis on samples collected from Co-IP assays performed using RBBP6 and p53 antibodies on HEK293 cells. Three independent Western blots were performed to investigate the presence of p53, RBBP6, and MDM2 in Co-IP assays performed using crude cell lysate, Protein agarose A and anti-RBBP6 (lanes 1 and 2) and anti-p53 (lanes 4 and 5), respectively. Lanes 1 and 4 show the flow-through samples, which contain the proteins in the crude cell lysate that did not interact with the Protein A agarose-bound antibody. Lanes 2 and 5 contain the immuno-complexes isolated during the Co-IP assays. Lane 3 is the HEK293 T crude cell lysate used in Co-IP assays. Full-length blot in supplementary file **Figure S1**.



Figure 4

Western blot analysis on samples collected from Co-IP assays performed using p53, MDM2, and RBBP6 antibodies on MCF7 cells. Three independent Western blots were performed to investigate the presence of p53, RBBP6, and MDM2 in Co-IP assays performed using crude cell lysate, Protein A agarose, and anti-p53 (lanes 1 and 2), anti-MDM2 (lanes 3 and 4), and anti-RBBP6 (lanes 5 and 6) antibodies, respectively. Lanes 1, 3, and 5 show the flow-through samples, which contain the proteins in the crude cell lysate that did not interact with the Protein A agarose-bound antibody. Lanes 2, 4, and 6 contain the immuno-complexes isolated during each Co-IP assay, respectively. Full-length blot in supplementary file **Figure S2**.

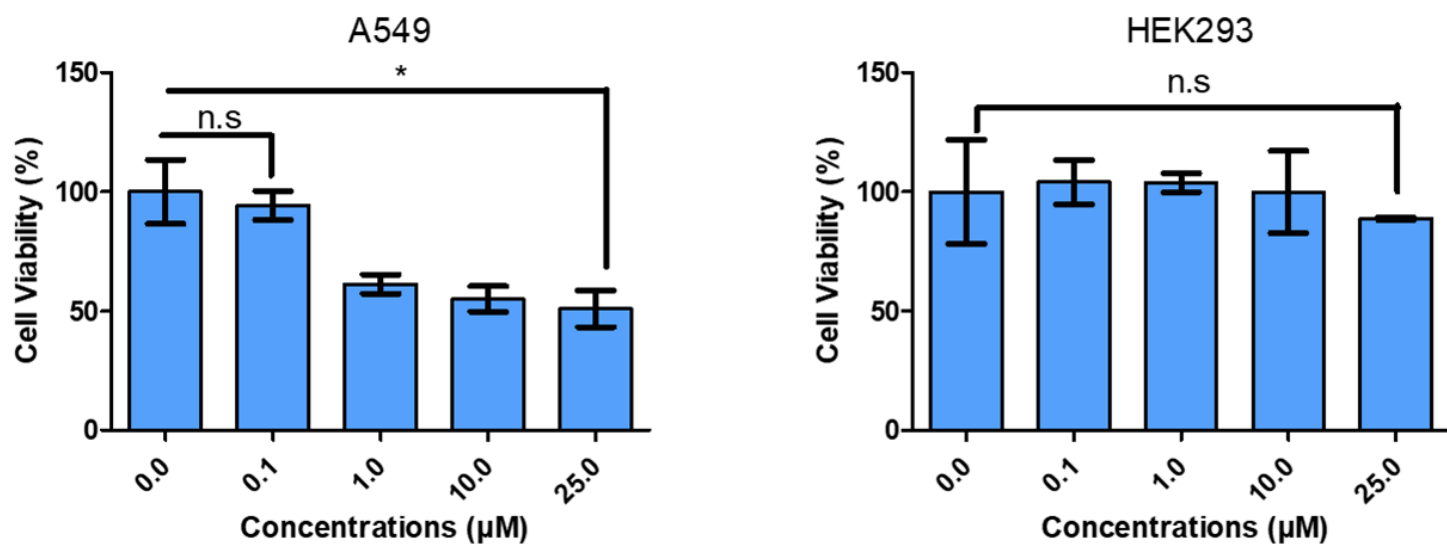


Figure 5

Effect of plerixafor on A549 and HEK293 cell viability after 48 hours of treatment. Cell viability was assessed using the MTT assay across a range of concentrations (0 μM to 25 μM). The left panel represents A549 lung adenocarcinoma cells, while the right panel represents HEK293 normal human embryonic kidney cells. Data are presented as mean ± standard deviation (SD) from three independent experiments. (* = $p < 0.05$; ns = non significant).

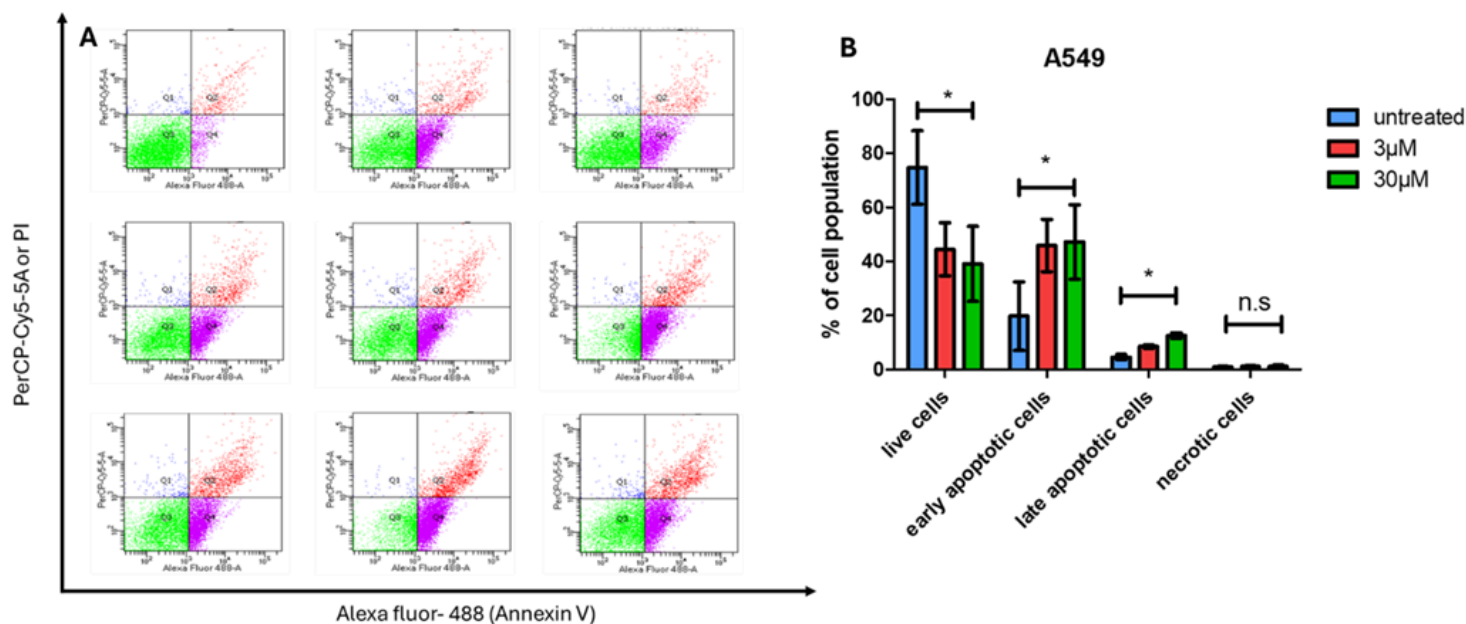


Figure 6

Plerixafor induces apoptosis in A549 Cells. (A) Representative flow cytometry dot plots of Annexin V/PI staining in A549 cells after 48-hour treatment with Plerixafor at 3 μM and 30 μM. Each quadrant represents a different cell population: viable cells (Annexin V-/PI-, lower left-Q3), early apoptotic cells (Annexin V+/PI-,

lower right -Q4), late apoptotic cells (Annexin V⁺/PI⁺, upper right -Q2), and necrotic/dead cells (Annexin V⁻/PI⁺, upper left -Q1). **(B)** Quantification of cell populations, expressed as mean $\pm$ SD from three independent experiments. Statistical analysis using one-way ANOVA (* $p < 0.05$) indicates a significant increase; ns = non significant.

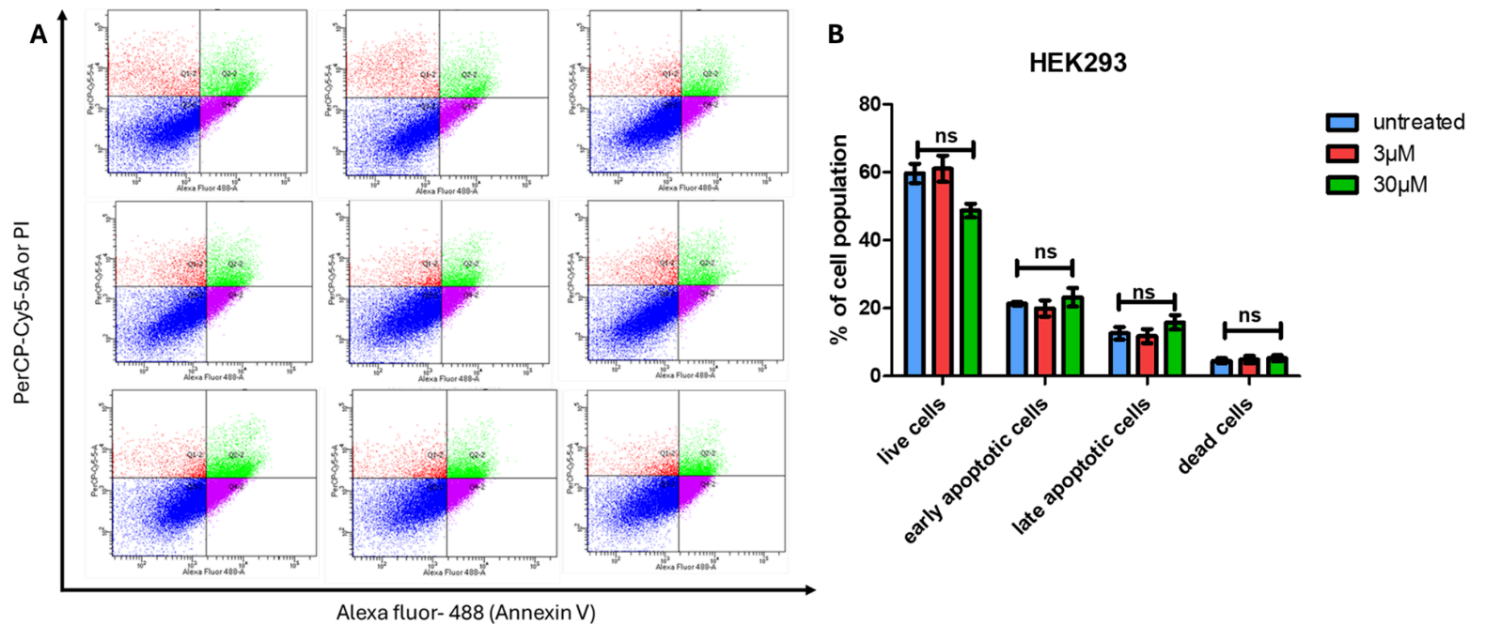


Figure 7

HEK293 cells exhibit minimal apoptotic response to plerixafor. **(A)** Representative flow cytometry dot plots of Annexin V/PI staining in HEK293 cells after 48-hour treatment with Plerixafor at 3 μ M and 30 μ M. Each quadrant represents a different cell population: viable cells (Annexin V⁻/PI⁻, lower left-Q3), early apoptotic cells (Annexin V⁺/PI⁻, lower right -Q4), late apoptotic cells (Annexin V⁺/PI⁺, upper right -Q2), and necrotic/dead cells (Annexin V⁻/PI⁺, upper left -Q1). **(B)** Quantification of apoptotic and necrotic cell populations, expressed as mean $\pm$ SD from three independent experiments. Statistical analysis indicates no significant differences (ns).

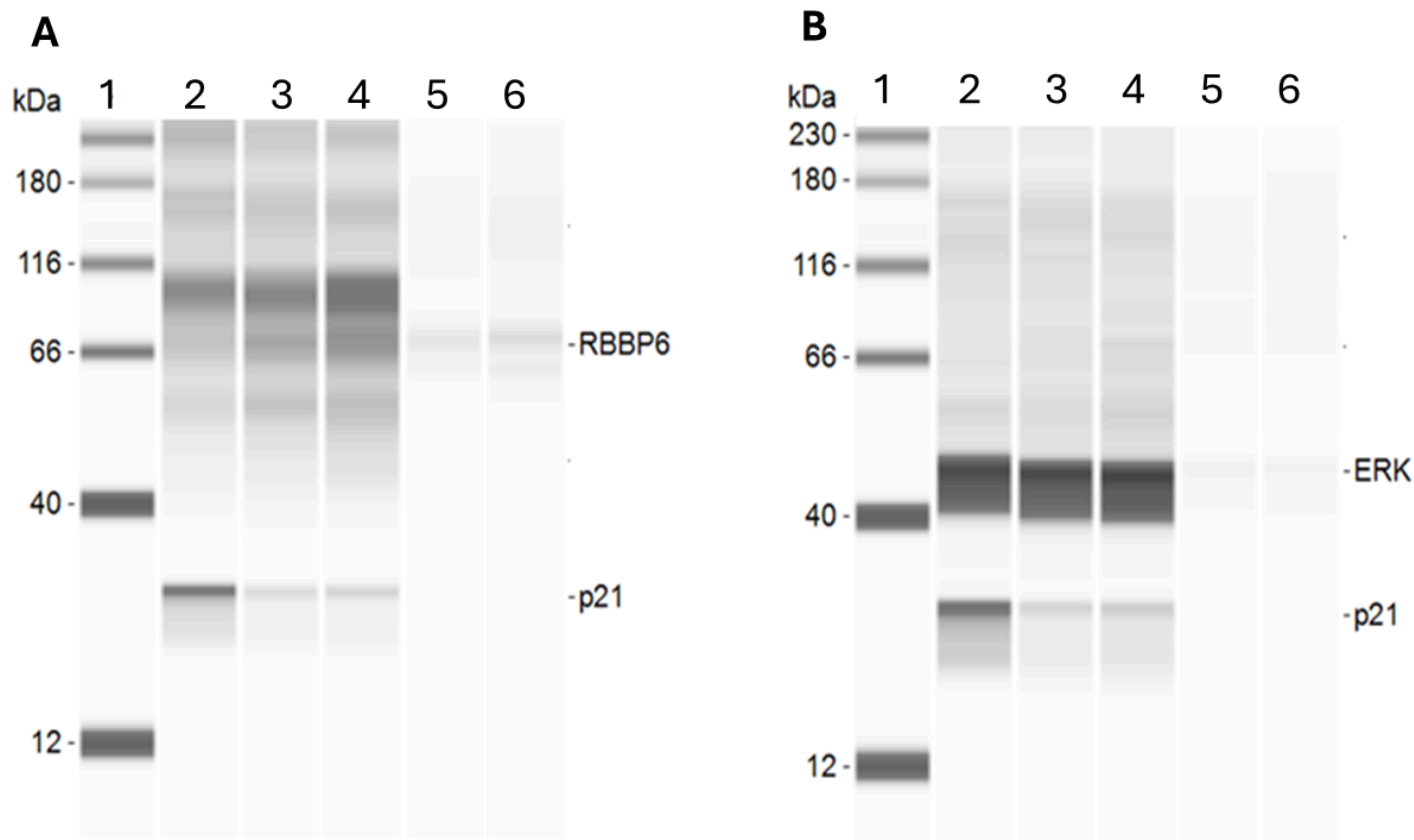


Figure 8

Western blot analysis of RBBP6, p21, and ERK Expression in A549 and HEK293 cells following plerixafor treatment. **(A)** Multiplexed detection of RBBP6 and p21. Only a 100 kDa RBBP6 band was detected, with no significant changes following treatment. p21 expression decreased in A549 cells but was undetectable in HEK293 cells. **(B)** Multiplexed detection of ERK and p21. plerixafor had no effect on ERK levels in A549 cells, while ERK was undetectable in HEK293 cells. Lanes: 1 – Molecular weight marker (12–230 kDa), 2 – A549 untreated, 3 – A549 3 μ M plerixafor, 4 – A549 30 μ M plerixafor, 5 – HEK293 untreated, 6 – HEK293 3 μ M plerixafor. Comprehensive blot from the Jess automated capillary Western system in the supplementary file **Figure S3**.

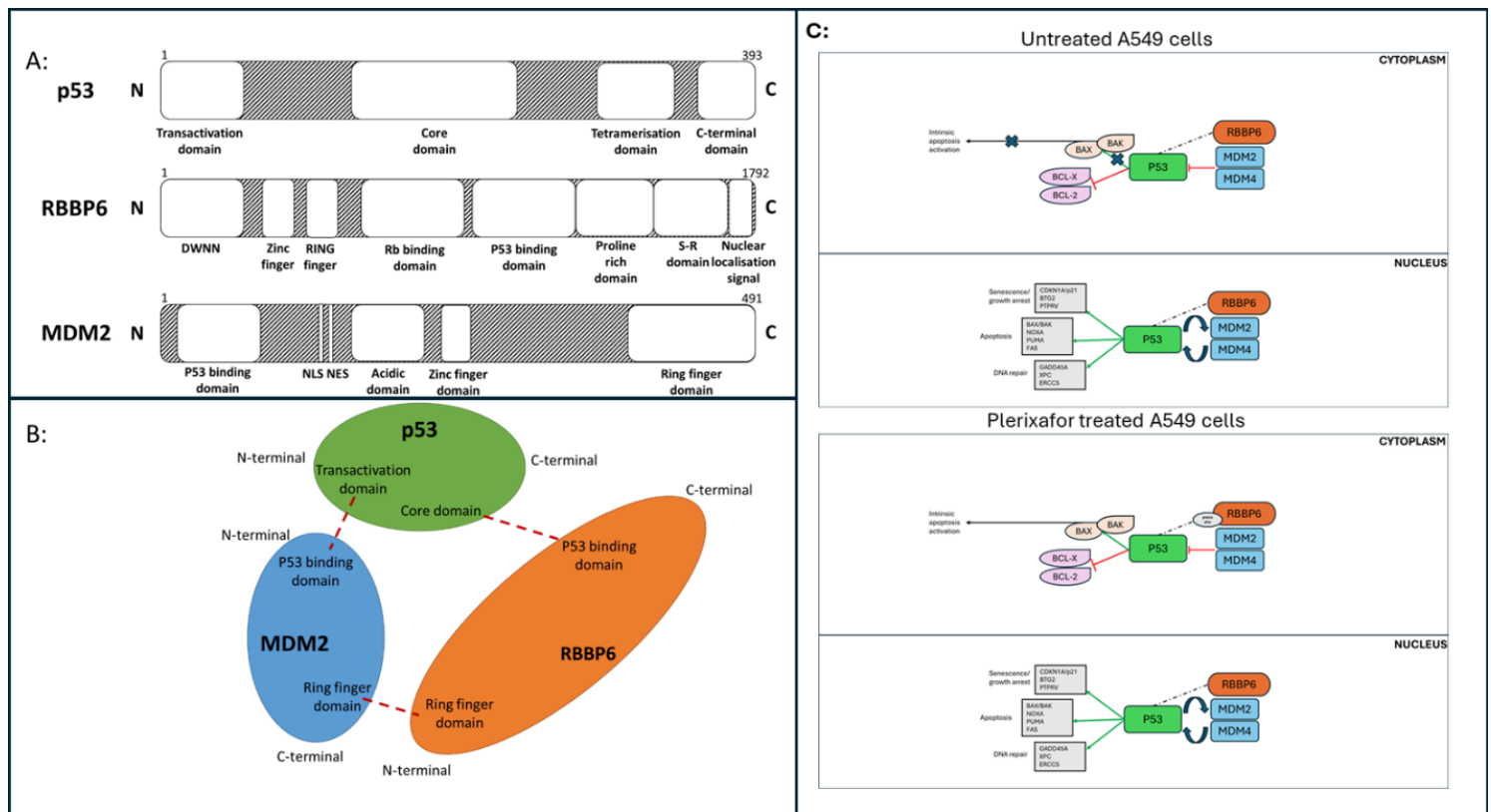


Figure 9

Domain structure and interaction model of the tripartite complex.

A, P53, RBBP6, and Mdm2 are all multidomain proteins. The basic structural organisation of p53, RBBP6, and MDM2 is shown. The core DNA-binding domain of p53 interacts with the C-terminal p53-binding domain of RBBP6 [50]. It has also been suggested that the RING finger domains of RBBP6 and MDM2 can interact [14]. **B**. The transactivation domain of p53 has been shown to bind with the N-terminal p53 binding domain on MDM2 [51], and p53-associated cellular protein-testes derived (PACT), the mouse orthologue of RBBP6. PACT was shown to enhance Mdm2's E3 ligase activity, but it is unclear whether it directly catalyzes p53 ubiquitination. **C. Proposed model of p53 pathway regulation in A549 cells with and without plerixafor.** Schematic representation of p53 signalling in untreated (Panel A) versus plerixafor-treated (Panel B) A549 lung adenocarcinoma cells, shown separately for the cytoplasm (upper) and nucleus (lower). In the cytoplasm, p53 interfaces with BCL-2 family regulators: anti-apoptotic BCL-2/BCL-XL and pro-apoptotic BAX/BAK, thereby controlling mitochondrial (intrinsic) apoptosis. In untreated cells, intrinsic apoptosis is suppressed. In the nucleus, p53 transcriptionally regulates target gene programs associated with cell-cycle arrest/senescence (e.g., CDKN1A/p21, BTG2, PTPRV), apoptosis (e.g., BAX/BAK, NOXA, PUMA, FAS), and DNA repair (e.g., GADD45A, XPC, ERCC5). p53 is negatively regulated by MDM2 and MDM4 and interacts with RBBP6 (dashed line), but it is unclear whether it directly catalyzes p53 ubiquitination. In plerixafor-treated cells (Panel B), plerixafor is proposed to interact with/perturb RBBP6, altering the regulation of the p53 network and favouring intrinsic apoptotic signalling. Green arrows indicate activation/positive regulation; red blunt lines indicate inhibition/negative regulation; dashed lines indicate proposed/indirect interactions; blue "X"

marks inhibition of the pathway. MDM4 interaction with p53-MDM2 is a bit complex/nuanced. Its interaction with p53 is contextual and MDM4 is known to exist in several conformations [37].

Supplementary Files

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