


siRNA Knockdown of REDD1 Facilitates Aspirin-Mediated Dephosphorylation of mTORC1 Target 4E-BP1 in MDA-MB-468 Human Breast Cancer Cell Line

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Aistė Savukaitytė¹ 
Greta Gudoitytė¹
Agnė Bartnykaitė¹
Rasa Ugenskienė^{1,2}
Elona Juozaitytė³

¹Oncology Research Laboratory, Institute of Oncology, Lithuanian University of Health Sciences, Kaunas, Lithuania;

²Institute of Biology Systems and Genetic Research, Lithuanian University of Health Sciences, Kaunas, Lithuania; ³Department of Oncology and Hematology, Hospital of Lithuanian University of Health Sciences Kaunas Clinics, Kaunas, Lithuania

Background: Mutations within genes encoding components of the PI3K/AKT/mTOR (phosphoinositide 3-kinase/protein kinase B/mechanistic target of rapamycin) signaling axis frequently activate the pathway in breast cancer, making it an attractive therapeutic target. Inhibition of mTORC1 (mechanistic target of rapamycin complex 1) activity upon aspirin treatment has been reported in breast cancer cells harboring *PI3KCA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) mutation and is considered to account for anticancer action.

Methods: MDA-MB-468 (harbors mutated *PTEN* (phosphatase and TENsin homolog)), MCF-7 (*PI3KCA*-mutated), MDA-MB-231 (no PI3K pathway mutations) cancer cell lines and MCF10A non-cancerous breast epithelial cells were employed for the assessment of modulation of mTORC1 signaling by aspirin. Targeted amplicon-based next-generation sequencing using the Ion Torrent technology was carried out to determine gene expression changes following drug treatment. Western blot was performed to analyze the expression and phosphorylation of proteins. Knockdown by siRNA approach was applied to assess the role of REDD1/DDIT4 (DNA damage-inducible transcript 4) in mTORC1 inhibition by aspirin.

Results: We show a decline in phosphorylation of mTORC1 downstream substrate 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) in response to treatment with aspirin and its metabolite salicylic acid in MDA-MB-468, MCF-7, MDA-MB-231, and MCF10A cell lines. We further demonstrate a novel molecular response to aspirin in breast cancer cells. Specifically, we found that aspirin and salicylic acid increase the expression of REDD1 protein, that is known for its suppressive function towards mTORC1. Unexpectedly, we observed that siRNA knockdown of REDD1 expression facilitated aspirin-mediated suppression of mTORC1 downstream substrate 4E-BP1 phosphorylation in the MDA-MB-468 cell line. REDD1 downregulation slightly encouraged reduction in 4E-BP1 phosphorylation by aspirin in MCF-7 cells but did not elicit a reproducible effect in the MDA-MB-231 cell line. siRNA knockdown of REDD1 did not affect the expression of phosphorylated form of 4E-BP1 following aspirin treatment in MCF10A non-cancerous breast epithelial cells.

Conclusion: The current findings suggest that REDD1 downregulation might improve the anticancer activity of aspirin in a subset of breast tumors.

Keywords: aspirin, breast cancer, REDD1, mTORC1 signaling, 4E-BP1

Introduction

While functioning in two distinct complexes, mTORC1 (mechanistic target of rapamycin complex 1) and mTORC2 (mechanistic target of rapamycin complex 2),

Correspondence: Aistė Savukaitytė
Email aiste.savukaityte@lsmuni.lt

mTOR kinase regulates cell growth through a number of cellular processes¹ and the activation of mTOR signaling is involved in cancer development.² mTOR signaling is often upregulated in breast cancer due to the genetic alterations of the genes encoding upstream components of the pathway, including *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), *PTEN* (phosphatase and TENsin homolog), and *AKT1* (AKT serine/threonine kinase 1).³ Recent studies^{4,5} have shown a prognostic synergy between Anillin-actin binding protein (ANLN), highly expressed in breast cancers and involved in PI3K/AKT signaling, and kinase insert domain receptor (KDR), that is a key receptor mediating cancer angiogenesis/metastasis switch. Activated mTOR signaling has been generally linked with poor prognosis in breast cancer² and resistance to conventional therapies.⁶ However, frequent activation of the pathway makes it an attractive therapeutic target and research has shown that mTOR inhibition is a useful strategy in the treatment of cancers including breast cancer.² Agents targeting the PI3K/AKT/mTOR signaling axis may improve the efficacy of endocrine, HER2-targeted and cytotoxic therapies due to their implication in resistance to these treatment strategies.⁶ Identification of biomarkers which could predict a response to the pathway inhibitors is warranted.⁶

Aspirin (acetylsalicylic acid) has been used in clinical practice for more than a century due to analgesic, antipyretic, and anti-inflammatory properties.⁷ These effects of aspirin are attributed to acetylation-mediated inhibition of COX (cyclooxygenase) enzymes and decreased production of prostaglandins.⁸ Recently, aspirin has attracted attention as an anticancer agent, which is strongly supported by epidemiological data.⁹ However, the underlying mechanisms by which aspirin exerts its antineoplastic effects are not clearly established.⁷ The effect of non-steroidal anti-inflammatory drugs (NSAIDs) is at least partly associated with inhibition of COX-2, which is overexpressed in cancer tissues and is implicated in tumorigenesis.¹⁰ However, since NSAIDs demonstrate antitumor activity in cancer cells, lacking expression of both COX-1 and COX-2, this mechanism of action remains questionable.¹¹ An antithrombotic effect through inhibition of platelet COX-1 is also considered to be relevant.¹² In terms of COX-independent mechanisms, inhibition of NF-kappaB¹³ and Wnt/ β -catenin¹⁴ have been suggested to play a role.

Aspirin-mediated suppression of mTORC1 signaling in colorectal cancer cells has been reported by Din et al¹⁵ and

Sun et al.¹⁶ Observational studies^{17,18} have shown a predictive role for *PIK3CA* mutations in colorectal tumors, which further supports the implication of the PI3K/AKT/mTORC1 pathway in the therapeutic effect of aspirin. Recently, Henry et al¹⁹ have demonstrated that growth suppression mediated by aspirin treatment was more pronounced in breast cancer cells harboring activating *PIK3CA* mutations in comparison with their wild-type counterparts. The anticancer effect was attributed to increased activation of mTORC1 repressor AMPK and the inhibition of mTORC1 signaling described in this report. A decline in mTORC1 signaling was reportedly both dependent and independent of AMPK (AMP-activated protein kinase),^{15,16,19} suggesting that alternative pathways may also inhibit mTORC1 upon aspirin treatment.

In the present study we further explored the modulation of mTORC1 signaling by aspirin in breast cancer cells. We analyzed the expression of genes encoding components of the PI3K/AKT/mTORC1 pathway and found three differentially expressed genes, including *DDIT4/REDD1* (DNA damage-inducible transcript 4; herein after referred as REDD1) following aspirin and salicylic acid treatment. We then demonstrated the drug-induced decrease in phosphorylation of mTORC1 target 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) in various cell lines. Given the ability of REDD1 to suppress mTORC1 signaling,^{20,21} and the lack of data in the literature on the involvement of REDD1 in aspirin anticancer action, we aimed to test the implication of REDD1 in cell response to aspirin treatment. Unexpectedly, we revealed that REDD1 downregulation using siRNA promotes aspirin-mediated dephosphorylation of 4E-BP1 in the MDA-MB-468 cell line. These findings suggest that REDD1 downregulation might improve the anticancer activity of aspirin in certain breast tumors.

Materials and Methods

Cell Lines and Culture Conditions

Human breast cancer cell lines MDA-MB-468 (*PTEN*-mutated), MCF-7 (*PI3KCA*-mutated), and MDA-MB-231 (does not harbor PI3K pathway mutations) were purchased from CLS Cell Line Service (Eppelheim, Germany) and grown as monolayers in *Dulbecco's Modified Eagle's medium* (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA), 100 U/mL penicillin with 100

µg/mL streptomycin (Gibco) and 2 mM L-glutamine (Gibco) at 37°C in humidified 5% CO₂. The MCF10A cell line was kindly provided by dr. Valerija Mikalayeva (Lithuanian University of Health Sciences, Kaunas, Lithuania). MCF10A cells were cultured in DMEM/F-12 (Gibco) with 2.5 mM L-glutamine and 15 mM HEPES supplemented with 100 ng/mL cholera toxin (Sigma-Aldrich), 20 ng/mL epidermal growth factor (Gibco), 10 µg/mL insulin (Gibco), 500 ng/mL hydrocortisone (Sigma-Aldrich), 5% horse serum (Gibco), and 100 U/mL penicillin with 100 µg/mL streptomycin (Gibco) at 37°C in humidified 5% CO₂.

Chemicals and Antibodies

Aspirin and salicylic acid were purchased from Sigma-Aldrich, and 0.5 M stock solutions of both compounds were prepared in water (pH 7) and frozen at -20°C in small quantities to prevent freeze-thaw cycles. Working solutions were prepared before each experiment.

Antibody against 4E-BP1 (#9644) was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-4E-BP1 Ser65 antibody (#MA5-14948) and anti-β-actin (#AM4302) were obtained from Invitrogen (Waltham, MA, USA), while anti-REDD1 (#ab191871) was from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated anti-rabbit (#65-6120) and alkaline phosphatase conjugated anti-mouse (#WP20006) secondary antibodies were bought from Invitrogen.

Cell Treatment

Exponentially growing cells were plated into 40 mm diameter Petri dishes (for RNA expression analysis) or 25 cm² flasks (for protein expression analysis) for 24 hours and then treated with indicated concentrations of aspirin or salicylic acid for 1, 17, or 24 hours. We used 0.5 and 2 mM concentrations of the tested drugs, since these are achievable salicylate plasma concentrations obtained from hydrolysis of anti-inflammatory doses of aspirin.²² Since we²³ and others^{24,25} had previously suggested that salicylic acid contributes to aspirin anticancer action, we performed most of the experiments with salicylic acid in parallel.

RNA Extraction and Quantification

Total RNA was extracted from cell lines using the PureLink™ RNA Mini Kit (Invitrogen) according to manufacturer's instructions. RNA quantity was assessed using a Qubit® 3.0 fluorometer (Life Technologies, Carlsbad,

CA, USA) and the Qubit™ RNA HS Assay Kit (Invitrogen) following manufacturer's recommendations. RNA integrity was assessed by agarose gel electrophoresis.

cDNA Library Preparation

RNA (10 ng) was reverse transcribed using SuperScript™ IV VIL0™ Master Mix with ezDNase™ Enzyme (Invitrogen), as per manufacturer's recommendations. Construction of libraries was carried out using Ion AmpliSeq™ Library Kit Plus (Ion Torrent, Guilford, CT, USA) according to manufacturer's instructions. A custom Ion AmpliSeq™ RNA Panel (Ion Torrent) was used to generate 66 amplicons representing unique targeted genes (the list of genes included in the panel is given in [Supplementary Table 1](#)). Each library was barcoded with the Ion Xpress™ Barcode Adapters 1–16 Kit (Ion Torrent) and subsequently purified using Agencourt™ AMPure™ XP Reagent (Beckman Coulter, Brea, CA, USA) followed by a dilution in Low TE. Library quantification was performed as per Ion Library TaqMan™ Quantitation Kit (Ion Torrent) protocol by quantitative PCR on ABI Fast 7500 System (Applied Biosystems, Foster City, CA, USA). Libraries were then diluted to 30 pM and combined with 12 samples per pool for further processing.

Ion Torrent Sequencing and DEG Analysis

Template preparation and chip loading was carried out using Ion 520™ and Ion 530™ Kit-Chef (Ion Torrent) on Ion Chef™ Instrument (Ion Torrent). Sequencing was performed using Ion 520™ Chip Kit (Ion Torrent) on Ion S5™ System (Ion Torrent). Primary analysis of the sequencing data was performed using ampliSeqRNA plugin in the Torrent Suite™ Software v.5.10.1, as was published before.^{26–28} Further analysis was performed on the EdgeR package from the open-source Bioconductor project. A matrix of gene-wise read counts was used as input. Normalization by trimmed mean of M values (TMM) was performed to adjust for different RNA compositions between libraries and library size. Genes from MCF-7 and MDA-MB-468 cells with less than 21.7 and 36.6 counts per million (CPM) (in any treatment or control group) were excluded from further analysis, respectively. This step was followed by differentially expressed gene (DEG) analysis under the GLM framework. Adjusted *P*-values for multiple testing, using Benjamini-Hochberg to estimate the false discovery rate (FDR), were calculated for the final estimation of DEG significance.²⁶

Genes with a fold change ≥ 1.5 or ≤ 0.67 and an adjusted $P < 0.05$ were considered to be differentially expressed.

Raw sequencing data has been deposited in publicly available Sequence Read Archive (SRA) repository with links to BioProject accession number PRJNA675979 in the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/>).

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Abcam), supplemented with protease (Sigma-Aldrich) and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentration was measured using PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), as per manufacturer's instructions. Fifty micrograms of protein were loaded in each lane for electrophoresis in 12% or 4–12% gradient SDS-PAGE. The resolved proteins were electrophoretically transferred onto PVDF (polyvinylidene fluoride) membranes. Blots were blocked in optimal blocking buffer for each antibody for 45 minutes and incubated overnight at 4°C with phospho-4E-BP1 Ser65 (1:250), 4E-BP-1 (1:1,000), REDD1 (1:1,000), or β -actin (1:2,000) primary antibody. After washing three times using TBST (Tris buffered saline with tween 20) the blots were incubated with HRP (for probing p-4E-BP1, 4E-BP1, and REDD1) or AP-conjugated secondary antibodies (for β -actin). The blots were washed again with TBST three times and the proteins were detected using Super SignalTM West Pico PLUS Chemiluminescent Substrate, SuperSignalTM West Atto Ultimate Sensitivity Substrate, or CDP-Star[®] chemiluminescent substrate from WesternBreeze Chemiluminescent Kit (Invitrogen), respectively. Chemiluminescent reaction was visualized using a CCD imager ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA). Signal intensity of the protein bands was quantified by the ImageLab 6.0.1 Software (Bio-Rad Laboratories, USA). β -actin was used as a loading control.

Cell Transfection

MDA-MB-468, MCF-7, MDA-MB-231, and MCF10A cells were reverse transfected using LipofectamineTM RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's instructions, with Silencer Select siRNA at 8 nM, 8 nM, 35 nM, and 50 nM concentrations, respectively. The following siRNAs were employed: siRNA targeting REDD1 (#s29166; Ambion, Austin, TX, USA), non-targeting siRNA (#4390843; Invitrogen), and positive control siRNA against

GAPDH (#4390849; Invitrogen). Gene knockdown efficiency was evaluated by quantitative reverse transcription-PCR on ABI Fast 7500 System (Applied Biosystems). For this purpose RNA was extracted and quantified as described above. cDNA synthesis was performed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer's instructions. PCR reaction was performed with TaqmanTM Gene Expression Assays (Hs99999905_m1 for GAPDH and Hs01111686_g1 for REDD1 quantification) and TaqmanTM Universal Master Mix II with UNG from Applied Biosystems, following the manufacturer's instructions. Relative gene expression was normalized to β -actin and determined by the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

All experiments were repeated at least three times unless otherwise noted. All data are presented as the means \pm standard deviation (SD). The statistical significance was tested via Student's *t*-test using SPSS software (SPSS Inc, Chicago, IL, USA). The results were considered statistically significant when $P < 0.05$.

Results

Aspirin and Salicylic Acid Induce the Expression of REDD1 in Breast Cancer Cell Lines

Since the literature suggested the mTORC1 pathway to be implicated in aspirin anticancer action, we aimed to further analyze the modulation of mTORC1 signaling. Firstly, we assessed the expression of pathway-related molecules in response to aspirin and its metabolite salicylic acid treatment, using targeted amplicon-based next-generation sequencing. Analysis revealed three differentially expressed genes upon 24-hour exposure to the drugs (Table 1). While a decrease in *IRS1* mRNA and an increase in *DEPTOR* expression were found in the MDA-MB-468 cell line, these changes were not detected in the MCF-7 cell line treated with aspirin or salicylic acid. However, treatment with 2 mM of either drugs elevated the expression of *DDIT4* in both cell lines. All the observed expression changes implied inhibition of mTORC1 signaling by aspirin and salicylic acid, in accordance with the biological function of genes that were differentially expressed upon drug treatment.

Since *DDIT4* was differentially expressed in both cell lines we next examined whether the increase in *DDIT4* mRNA following aspirin and salicylic acid treatment

Table I Regulation of Gene Expression After 24-Hour Treatment with Aspirin and Salicylic Acid

Gene	Function	Positively (↑) or Negatively (↓) Regulates mTORC1	MDA-MB-468				MCF-7			
			0.5 mM ASA	2 mM ASA	0.5 mM SA	2 mM SA	0.5 mM ASA	2 mM ASA	0.5 mM SA	2 mM SA
<i>IRS1</i> (insulin receptor substrate 1)	Docking protein upstream of PI3K	↑	0.59	0.48	NC	0.58	NC	NC	NC	NC
<i>DEPTOR</i> (DEP domain containing mTOR interacting protein)	Component of mTORC1 and mTORC2 complexes; directly interacts with and inhibits mTOR	↓	NC	1.62	NC	1.63	NC	NC	NC	NC
<i>DDIT4</i> (DNA damage-inducible transcript 4)	Inhibits mTORC1 activity in a TSC1/2 - dependent manner	↓	NC	2.58	NC	2.01	NC	1.93	NC	1.91

Notes: Regulation is expressed as fold change (FC) between treatment and control conditions. Changes in gene expression were determined as described under "Materials and methods". FC is given only for DEGs with adjusted $P < 0.05$. ↑ and ↓ indicate how a particular gene regulates (activates or inhibits, respectively) mTORC1 activity regarding the previously reported biological function of the gene.

Abbreviations: ASA, aspirin (acetylsalicylic acid); SA, salicylic acid; NC, no change.

corresponded with elevated protein level in these cell lines. As expected, our data has shown an increase in REDD1 (encoded by *DDIT4* gene) protein amount in both MDA-MB-468 and MCF-7 (Figure 1A) cell lines. We further tested the effect in MDA-MB-231 cancer cell line and MCF10A non-cancerous breast epithelial cells. We found an upregulation of REDD1 in MDA-MB-231 cells and a downregulation in MCF10A cells (Figure 1A). Of note, relatively higher baseline expression of REDD1 was observed in non-cancerous breast epithelial cells in comparison to breast cancer cells (Figure 1B).

Aspirin and Salicylic Acid Treatment Attenuate mTORC1 Signaling

We then investigated whether the observed expression changes upon drug exposure coincide with the expected attenuation of mTORC1 activity. We chose the *PTEN*-mutant MDA-MB-468 cell line over *PIK3CA*-mutated MCF-7 cells for subsequent analysis, primarily because the effects of aspirin on mTOR signaling have not been reported in breast cancer cell lines with this mutation of the pathway. In addition, most gene expression changes were determined in this cell line in the present study. Although eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase (S6K1) are two main targets of mTORC1, we did not detect baseline S6K1 phosphorylation in MDA-MB-468 cells. We used phosphorylation of translational repressor 4E-BP1 as a marker of mTORC1 activity as it is generally accepted to reflect activation of mTORC1²⁹ and predict a response

to AKT/mTOR inhibitors.³⁰ As shown in Figure 2A, aspirin and salicylic acid treatment attenuated the activity of mTORC1. Similarly, aspirin and salicylic acid exposure caused a decrease in 4E-BP1 phosphorylation in MCF-7, MDA-MB-231, and MCF10A cell lines (Figure 2B).

siRNA Knockdown of REDD1 Triggers Aspirin-Mediated Dephosphorylation of 4E-BP1 in the MDA-MB-468 Cell Line

Observation of REDD1 induction in all tested cancer cell lines led us to test what role REDD1 plays in cell response to aspirin treatment. In regards to the above-mentioned REDD1 function in inhibiting mTORC1 signaling, we originally hypothesized that mTORC1 signaling is suppressed through REDD1 induction upon aspirin treatment. To test this hypothesis we used siRNA interference. In consistence with the previously reported ability of REDD1 to inactivate mTORC1, REDD1 downregulation elicited a slight increase in 4E-BP1 phosphorylation, indicating enhancement in mTORC1 activity (Figure 3). Unexpectedly, we found that REDD1 downregulation using siRNA transfection prior to incubation with aspirin promoted the drug-induced dephosphorylation of 4E-BP1 (Figure 3). We next performed analogical experiments in MCF-7, MDA-MB-231, and MCF10A cell lines. Knockdown of REDD1 alone led to a higher increase in phosphorylation of 4E-BP1 in MDA-MB-231 and MCF-7 cells compared to MDA-MB-468 and had no effect in MCF10A cell line (Figure 3). REDD1 downregulation only slightly encouraged reduction in 4E-BP1

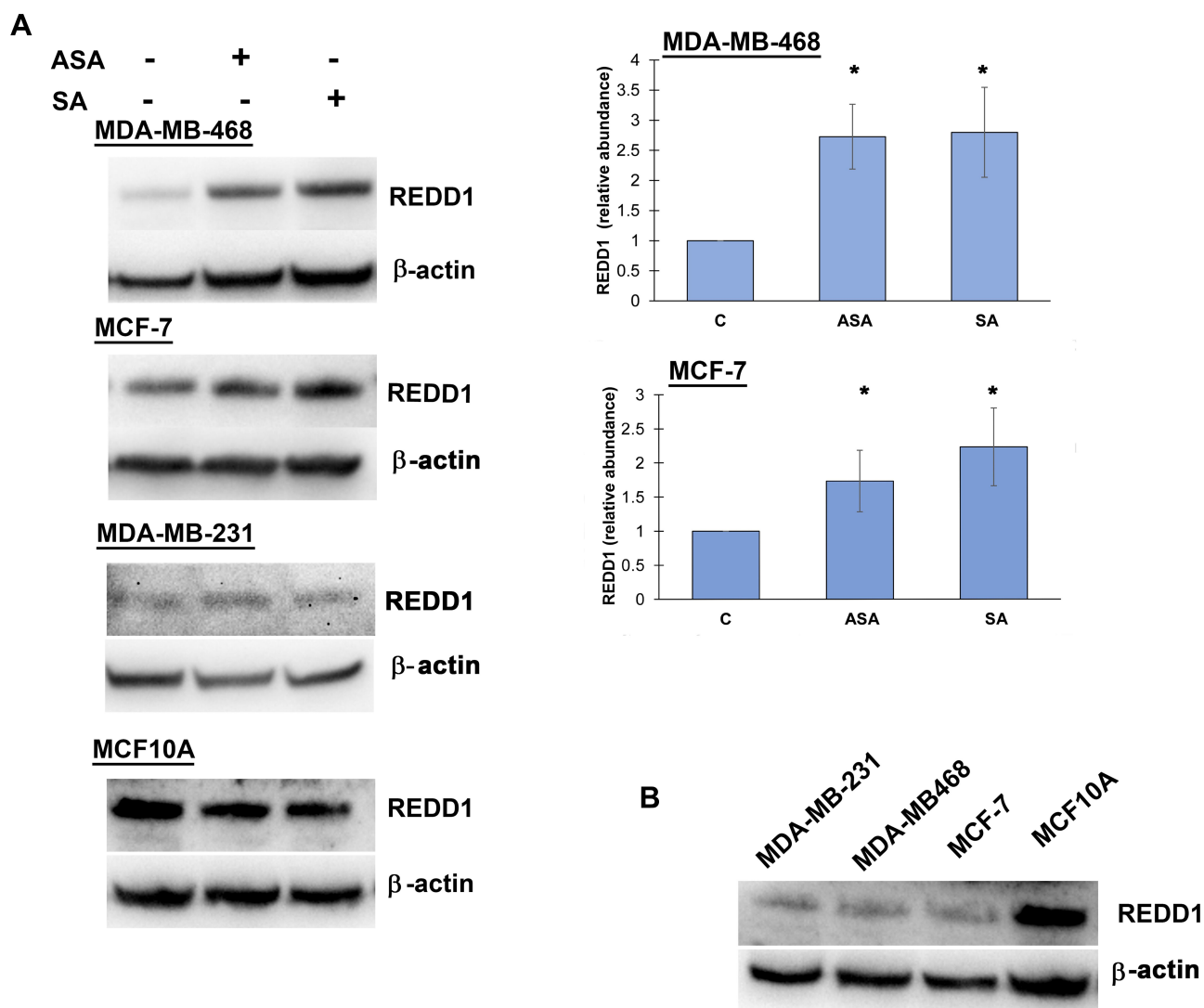


Figure 1 REDD1 expression in various cell lines. **(A)** Effect of aspirin and salicylic acid treatment on REDD1 expression in MDA-MB-468, MCF-7, MDA-MB-231, and MCF10A cell lines. Cells were exposed to 2 mM of aspirin (ASA), salicylic acid (SA), or vehicle control (C) for 24 hours. Equal amounts of proteins (50 μ g) from vehicle or drug-treated cells were loaded on each lane of SDS-PAGE gel for electrophoresis, followed by transfer onto PVDF membranes, which were then probed with anti-REDD1 antibody. β -actin was used as loading control. Densitometric quantification of REDD1 levels were normalized to β -actin for fold change calculations. Data are expressed as means \pm SD of at least three independent experiments. * P <0.05 (vs vehicle control) by Student's *t*-test. **(B)** Baseline levels of REDD1 in non-treated cells.

phosphorylation by aspirin in MCF-7 cells and did not elicit a repetitive effect in MDA-MB-231 cell line (Figure 3). siRNA knockdown of REDD1 did not affect the expression of phosphorylated form of 4E-BP1 following aspirin treatment in MCF10A cells (Figure 3).

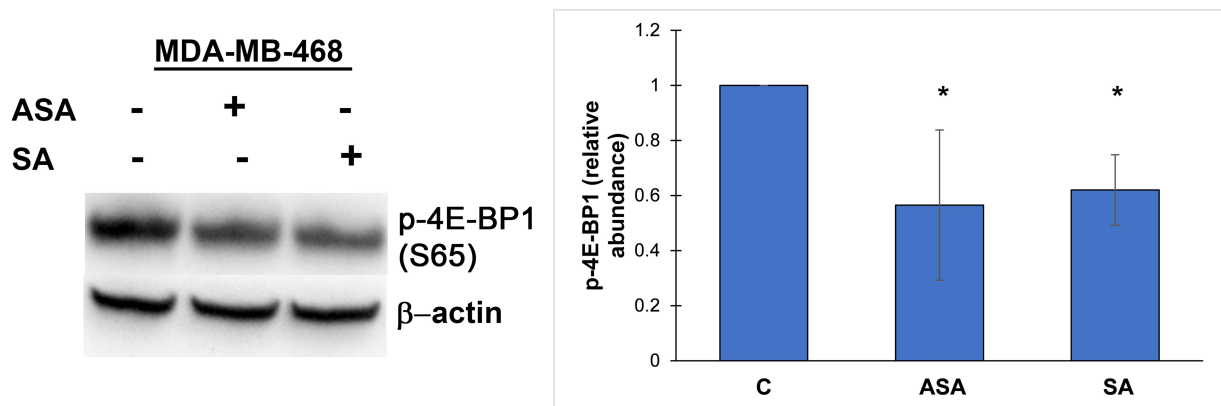
Discussion

In the present study we explored the modulation of mTORC1 signaling by aspirin in breast cancer cells. Here, we show that aspirin and salicylic acid induce REDD1 expression. REDD1 is encoded by *DDIT4* gene. *DDIT4* located on chromosome 10 (10q22.1) is ubiquitously expressed at low levels in most adult tissues³¹ but is

downregulated in a subset of human cancers.^{32,33} REDD1 is induced by various stress conditions, including hypoxia,³¹ energy depletion,³⁴ oxidative stress,³⁵ and endoplasmic reticulum stress.³⁶ REDD1 has been shown to inhibit mTOR signaling^{20,21} in a TSC1/2-dependent manner.³⁷

Little is currently known about the role of REDD1 in breast cancer pathogenesis, and existing studies report contradictory findings. For example, Horak et al³⁸ demonstrated that REDD1 suppressed tumorigenesis in breast cancer. Consistently, Koo and Jung³⁹ reported that REDD1 is downregulated in triple-negative and HER2 overexpression breast cancer subtypes. On the contrary,

A



B

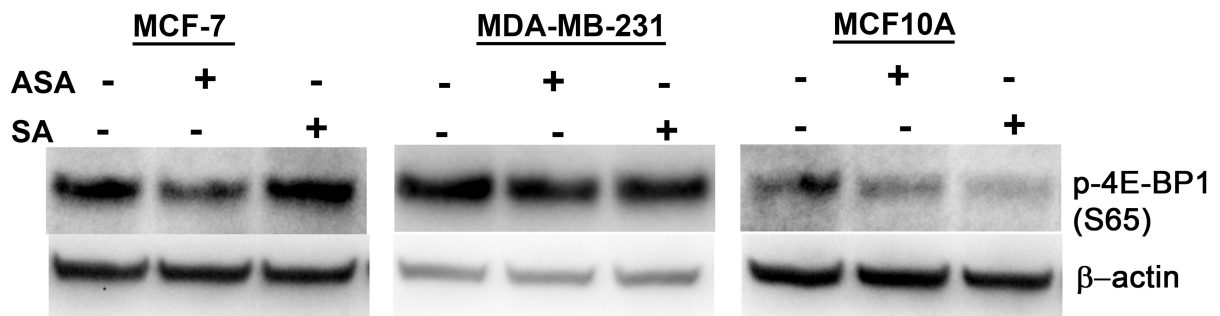


Figure 2 Aspirin and salicylic acid attenuate mTORC1 signaling in breast cancer and non-cancerous breast epithelial cells. Cells were exposed to 2 mM of aspirin (ASA), salicylic acid (SA), or vehicle control (C) for 24 (MDA-MB-468, MCF10A) or 17 (MCF-7, MDA-MB-231) hours. Equal amounts of proteins (50 μ g) from vehicle or drug-treated cells were loaded on each lane of SDS-PAGE gel for electrophoresis, followed by transfer onto PVDF membranes, which were then probed with respective antibody. β -actin was used as loading control. **(A)** Data for MDA-MB-468 cell line are expressed as means \pm SD of four independent experiments. * P <0.05 (vs vehicle control) by Student's *t*-test. **(B)** One experiment was performed in MCF-7, MDA-MB-231, and MCF10A cell lines.

a study by Pinto et al⁴⁰ linked high *DDIT4* expression with tumor aggressiveness in triple-negative breast cancer resistant to neoadjuvant chemotherapy. Our observation of the relatively higher REDD1 expression in non-cancerous breast epithelial cells in comparison to breast cancer cells supports the tumor suppressor role of REDD1.

While REDD1 is known to negatively regulate mTORC1 signaling, the involvement of REDD1 function in anticancer activity of aspirin has not been reported. REDD1 has been implicated in mTORC1 inhibition by metformin,⁴¹ N-butylidenephthalide,⁴² and a combination of melatonin with arsenic trioxide.⁴³ Given the increased expression of REDD1 following aspirin and salicylic acid treatment we raised a hypothesis that REDD1 mediates mTORC1 inhibition upon drug treatment. Surprisingly, siRNA knockdown of REDD1 assisted the aspirin-mediated dephosphorylation of mTORC1 target 4E-BP1 in MDA-MB-468 breast cancer cells. Cap-dependent translational control driven by phosphorylated 4E-BP1 and active downstream eIF4F is essential for tumorigenicity of breast

carcinoma cells.⁴⁴ However, REDD1 downregulation showed a relatively weak benefit in dephosphorylating 4E-BP1 by aspirin in the MCF-7 cell line and did not elicit a reproducible effect in MDA-MB-231 cells. Additionally, REDD1 downregulation itself substantially elevated 4E-BP1 phosphorylation in MCF-7 and MDA-MB-231 cell lines which may abolish the beneficial effect of REDD1 downregulation. Altogether, our results are in contrast with the observations of REDD1 induction-mediated mTORC1 inhibition, where REDD1 downregulation restored dephosphorylation of mTORC1 downstream substrates caused by metformin, N-butylidenephthalide, and a combination of melatonin with arsenic trioxide.

Distinct roles in modulation of mTORC1 activity by REDD1 may depend on the cellular context. Our results in the MDA-MB-468 cell line suggest that REDD1 upregulation might interfere with mTORC1 inhibition by aspirin which contradicts the known biological function of REDD1 to suppress mTORC1 signaling. It is therefore possible that REDD1 induction is a self-defense mechanism of these

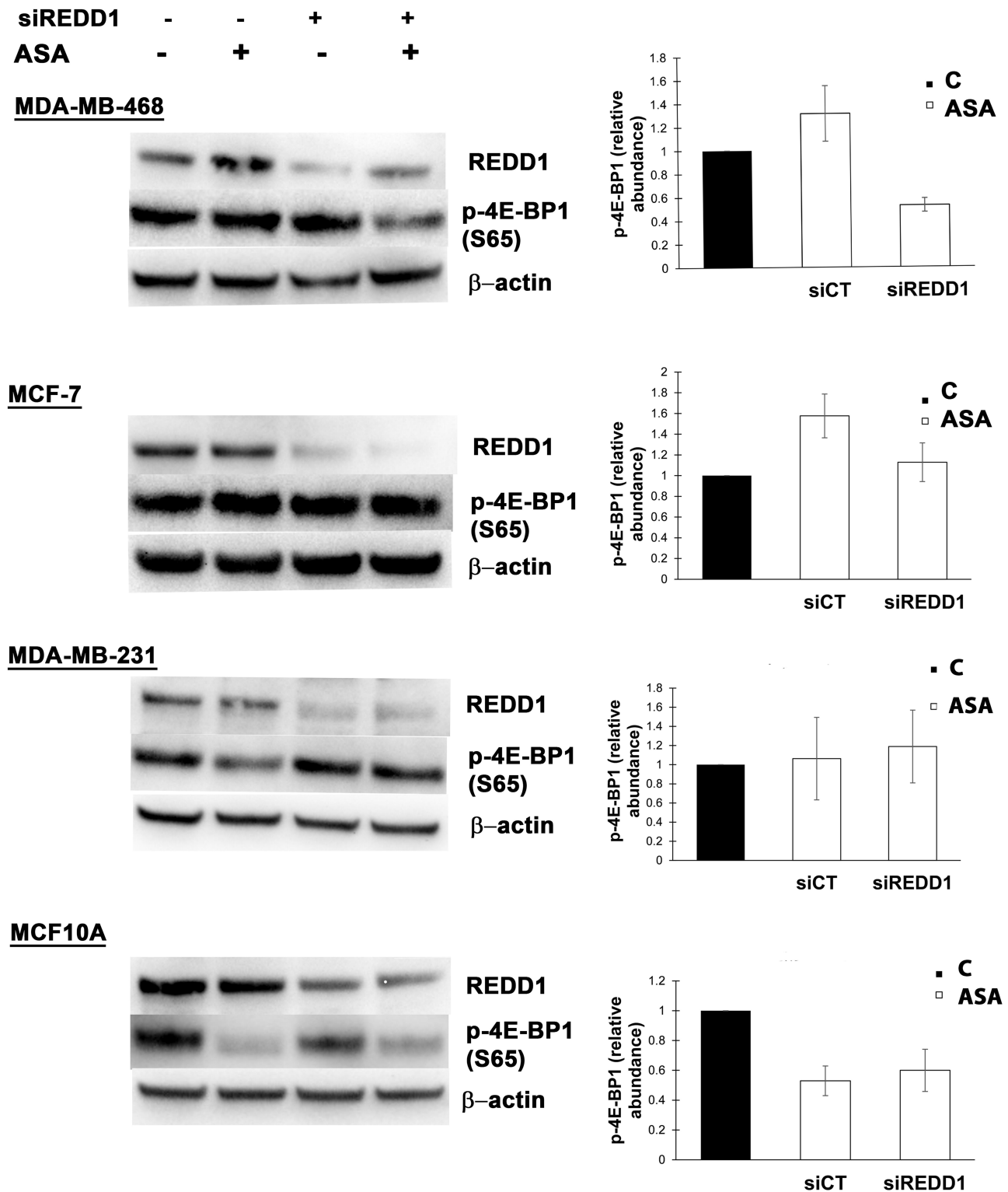


Figure 3 Different effects of REDD1 silencing on aspirin-mediated expression of phosphorylated 4E-BP1. Cells were transfected with non-targeting (siCT) or REDD1 siRNA (siREDD1) for 24 (MDA-MB-468, MCF-7, MDA-MB-231 cell lines) or 48 hours (MCF10A) and treated with vehicle control (C) or 2 mM of aspirin (ASA) for the next 24 hours before cell lysis. Equal amounts of proteins (50 μ g) were loaded on each lane of SDS-PAGE gel for electrophoresis, followed by transfer onto PVDF membranes, which were then probed with respective antibody. β -actin was used as loading control. REDD1 was probed on a different blot than p-4E-BP1. Data are expressed as means \pm SD from three independent experiments for MDA-MB-468, MCF-7, MDA-MB-231, and from two independent experiments for MCF10A.

cancer cells that induces resistance to aspirin treatment. This suggests a modulation of REDD1 activity in cancer treatment strategy, which is supported by several other reports. Vadysirisack et al⁴⁵ demonstrated an increase in sensitivity to doxorubicin and UV radiation determined by REDD1 loss. In a report by Molitoris et al⁴⁶ REDD1 knockdown also elevated dexamethasone-induced cell death in murine lymphocytes. Unlike in our study, downregulation of REDD1 abrogated treatment-related inhibition of mTORC1 activity in these reports. While REDD1 downregulation did not improve dephosphorylation of 4E-BP1 by aspirin in MDA-MB-231 cell line and showed little effect in MCF-7 cells in our study, elucidation of the mechanism through which REDD1 downregulation facilitates aspirin-mediated mTORC1 inhibition in MDA-MB-468 cells is required. Of note, REDD1 downregulation had no effect on aspirin activity towards dephosphorylation of 4E-BP1 in non-cancerous breast epithelial cells. Taken together, downregulation of REDD1 in combination with aspirin use might show specific activity against certain breast cancers while not affecting healthy cells.

As mentioned previously, REDD1 is downregulated in triple negative and HER2 breast tumors³⁹ which may also enhance the efficacy of aspirin in a subset of breast cancers. Thus, a clarification of the molecular basis which determines improved aspirin-induced dephosphorylation of 4E-BP1 by REDD1 downregulation appears promising for the identification of biomarkers that, in combination with intrinsic REDD1 downregulation in tumors, could predict a response to aspirin treatment.

Conclusion

Our results uncover a novel molecular response in breast cancer cells exposed to aspirin. The findings suggest that REDD1 downregulation might improve the anticancer activity of aspirin in certain types of breast tumors.

Abbreviations

PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTORC, mechanistic target of rapamycin; mTORC1, mechanistic target of rapamycin complex 1; mTORC2, mechanistic target of rapamycin complex 2; *PTEN*, phosphatase and TENsin homolog; REDD1/DDIT4, DNA damage-inducible transcript 4; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *AKT1*, AKT serine/threonine kinase 1; ANLN, anillin-actin binding protein; KDR, kinase insert domain receptor; COX,

cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; AMPK, AMP-activated protein kinase; DEG, differentially expressed gene.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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