

Metformin, Independent of AMPK, Induces mTOR Inhibition and Cell-Cycle Arrest through REDD1

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Abstract

Metformin is a widely prescribed antidiabetic drug associated with a reduced risk of cancer. Many studies show that metformin inhibits cancer cell viability through the inhibition of mTOR. We recently showed that antiproliferative action of metformin in prostate cancer cell lines is not mediated by AMP-activated protein kinase (AMPK). We identified REDD1 (also known as DDIT4 and RTP801), a negative regulator of mTOR, as a new molecular target of metformin. We show that metformin increases REDD1 expression in a p53-dependent manner. REDD1 invalidation, using siRNA or REDD1^{-/-} cells, abrogates metformin inhibition of mTOR. Importantly, inhibition of REDD1 reverses metformin-induced cell-cycle arrest and significantly protects from the deleterious effects of metformin on cell transformation. Finally, we show the contribution of p53 in mediating metformin action in prostate cancer cells. These results highlight the p53/REDD1 axis as a new molecular target in anticancer therapy in response to metformin treatment. *Cancer Res*; 71(13): 4366–72. ©2011 AACR.

Introduction

Metformin, a widely used antidiabetic drug, seems to reduce the incidence of cancer in diabetic patients (1) and inhibit cancer cell proliferation and tumor growth in animal models (2–4). Metformin provokes cell-cycle arrest in G₀–G₁ but does not induce apoptosis or autophagy in prostate cancer cells (3). At the molecular level, metformin regulates the AMP-activated protein kinase (AMPK)/mTOR pathway, as it activates AMPK (5) and consequently inhibits the mTOR pathway via tuberous sclerosis 2 protein (TSC2). mTOR upregulates energy-consuming cellular processes and controls cell growth. Because AMPK activation inhibits energy-consuming pathways and protein synthesis (6), metformin could inhibit cell proliferation through AMPK. In agreement, AMPK inhibition reverses the antiproliferative effects of metformin in breast and ovarian cancer cells (7, 8). In contrast, we showed that downregulation of AMPK did not affect metformin action on prostate cancer cell growth and mTOR inhibition (3), suggesting a role for an alternative pathway.

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REDD1 (RTP801/Dig2/DDIT4) was identified as a hypoxia-inducible factor 1 (HIF-1) target gene involved in the regulation of cell survival (9). REDD1 is also regulated in response to DNA damage, nutrient depletion, glucocorticoid, and insulin (10, 11). A great deal of evidence link REDD1 to tumor suppression. First, invalidation of REDD1 potentiates cell proliferation and anchorage-independent growth under hypoxia and REDD1 is downregulated in some human cancers (12). Second, REDD1 is a negative regulator of mTOR (13) and is defined as a key metabolic regulator suppressing tumorigenesis through effects on mTOR activity and mitochondria (14). Finally, REDD1 is implicated in apoptosis (9). These observations led us to study its possible implication in metformin action. We report that REDD1 expression is increased by metformin treatment and can mediate metformin-induced mTOR inhibition. We show that REDD1 is regulated by p53 and is required for metformin effects on cell-cycle arrest. Our study highlights the p53/REDD1 axis as a novel molecular target of metformin.

Materials and Methods

Cell culture

REDD1^{-/-} and p53^{-/-} mouse embryonic fibroblasts (MEF) were obtained from Ellisen and colleagues (10) and P. Roux [Centre National de la Recherche Scientifique (CNRS), Montpellier, France], respectively. Other cell lines were from American Type Culture Collection and maintained according to their recommendations. LNCaP cells were cultured in RPMI 1640 and all other cell lines in Dulbecco's modified Eagle's medium (DMEM; Invitrogen). Both media contain 4,500 mg/L glucose supplemented with

10% FBS, 100 U/mL penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine.

Cell transfection

LNCaP cells were transfected with siRNA targeting REDD1, p53, $\alpha 1$ and $\alpha 2$ AMPK, TSC2, or a control siRNA (Ambion) using Lipofectamine RNAi max (Invitrogen). Metformin (Sigma) was added 48 hours after transfection. DU145 cells were transfected with Flag-REDD1 plasmid using Lipofectamine 2000.

Cell analysis

Cell extracts were prepared using lysis buffer (3). Immunoblotting was carried out with antibodies against REDD1 (ProteinTech); Thr¹⁷²P-AMPK, Phospho-S6 ribosomal protein, S6 ribosomal protein, AMPK, and TSC2 (Cell Signalling technology); p53 and HSP90 (Santa Cruz Biotechnology); cyclin D1 (BD Bioscience); and α tubulin (Sigma). Cell cycle was analyzed by flow cytometry (3).

Real-time quantitative PCR

The relative amount of REDD1 mRNA was quantified by real-time quantitative PCR using ABI Prism 7000 (Applied Biosystems; ref. 11). The primer sequences are available upon request.

Bromodeoxyuridine incorporation assay

Cells were incubated for 24 hours in medium containing 10% FBS and then treated in the presence or absence of metformin (5 or 10 mmol/L) for 24 hours before bromodeoxyuridine (BrdUrd) incorporation assay (3).

In vitro transformation assay

LNCaP cells were transfected with siControl (siCT) or siREDD1 as above, and the soft agar assay was conducted as recommended by the manufacturer using the Fluorometric CytoSelect Cell Transformation Assay (Cell Biolabs). Photographs were acquired with a Zeiss microscope.

Results and Discussion

Metformin increases REDD1 expression in a p53-dependent manner

Effects of metformin on mTOR and cell growth are not mediated by AMPK in prostate cancer cells (3). Because of its role in mTOR regulation, we asked whether REDD1 could mediate metformin effects. REDD1 expression markedly increased from 4 to 24 hours after metformin treatment in LNCaP cells and REDD1 mRNA levels increased after 4 hours (Fig. 1B). A similar effect of metformin occurred in breast (MCF7) and lung cancer (A549) cells (Fig. 1C).

Because REDD1 is a direct p53 transcriptional target (10), we asked whether p53 regulates REDD1 expression upon metformin treatment. LNCaP cells harbor wild-type (WT) p53, whereas DU145 and PC3 cells are p53 mutated and null, respectively. Whereas REDD1 expression increased in LNCaP cells, it remained unchanged in cells lacking a functional p53 (Fig. 2A). In LNCaP cells, metformin slightly increased AMPK phosphorylation, whereas it induced a stronger activation in DU145 and PC3 cells. Conversely, it strongly decreased the phosphorylation of S6 ribosomal protein in LNCaP cells and only moderately in DU145 and PC3 cells (Fig. 2A). In agreement, REDD1 did not increase anymore in response to metformin following p53 invalidation using siRNA in

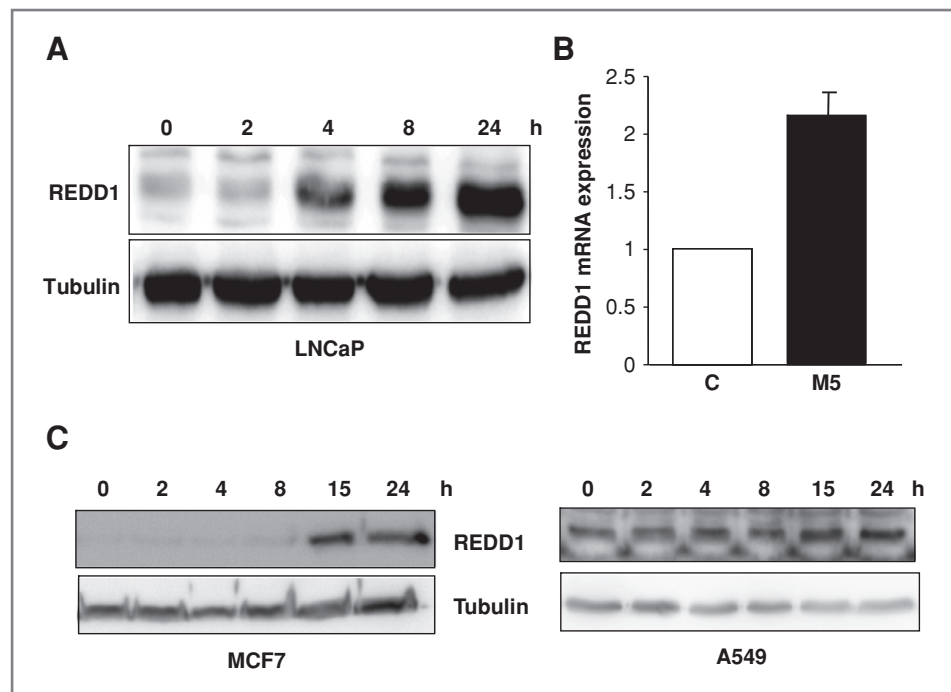


Figure 1. Metformin increases REDD1 expression in different cancer cell lines. LNCaP (prostate cancer; A and B), MCF7 (breast cancer), and A549 (lung cancer) cells (C) were treated with 5 mmol/L metformin as indicated before immunoblotting with REDD1 or mRNA quantification. C, control; M5, 5 mmol/L metformin.

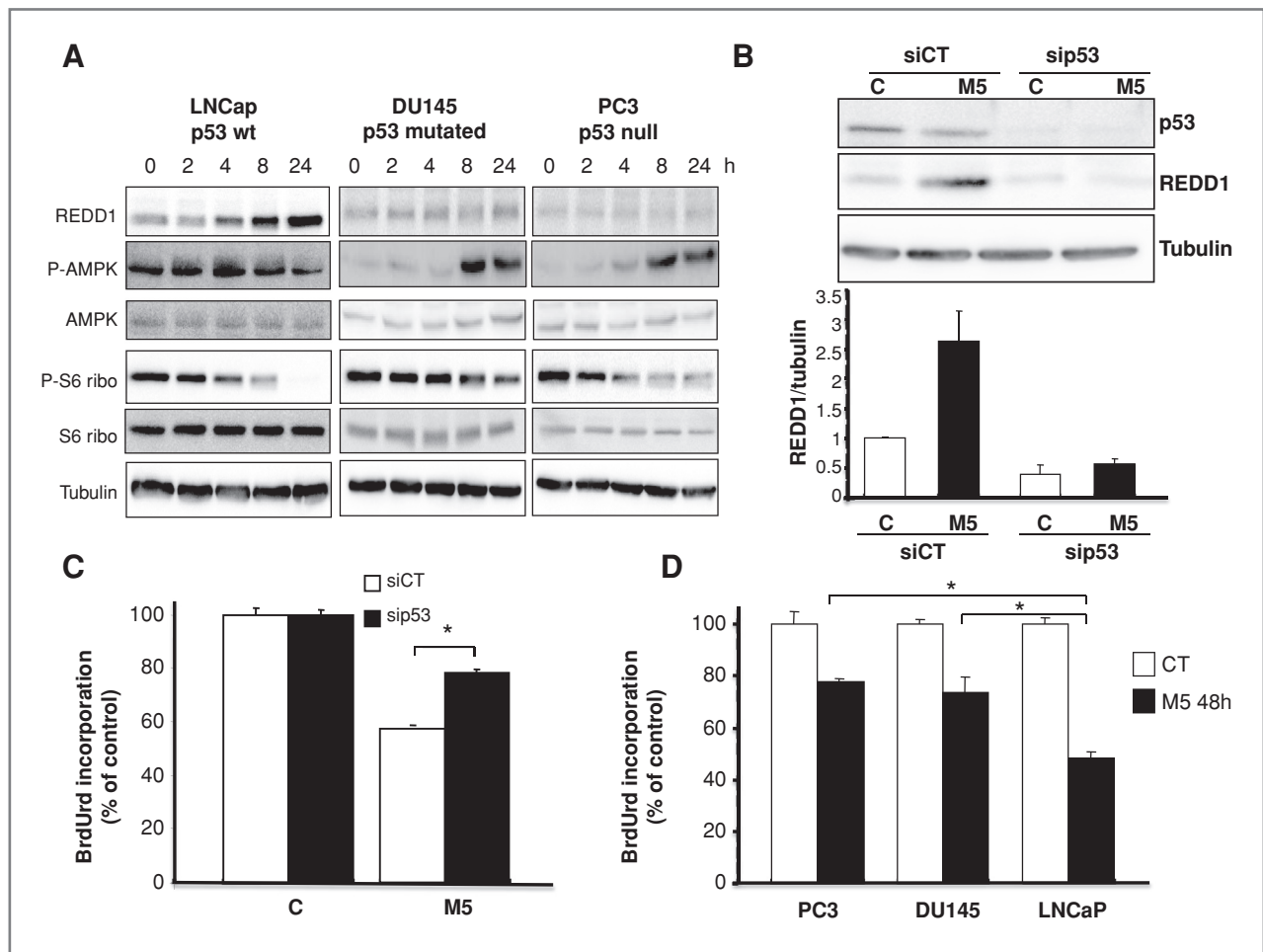


Figure 2. p53 regulates metformin-induced REDD1 expression and metformin antiproliferative action. A, LNCaP, DU145, and PC3 cells were treated with 5 mmol/L metformin and the indicated proteins were immunoblotted. B, LNCaP cells were transfected with p53 siRNA over 48 hours and treated with 5 mmol/L metformin for 24 hours before immunoblotting with p53, REDD1, and tubulin. Three independent experiments were quantified. C, BrdU incorporation carried out in LNCaP cells transfected with siCT or p53 siRNA and treated with metformin. *, $P < 0.05$. D, BrdUrd incorporation in cells treated with 5 mmol/L metformin.

LNCaP cells (Fig. 2B). Therefore, REDD1 appears as a p53-mediated molecular target of metformin. Similarly, REDD1 is a p53 target upon DNA damage but is regulated independently of p53 in response to alkylating agents (10). We have shown that HIF-1 controls REDD1 expression in response to insulin and hypoxia in adipocytes (11). However, HIF does not appear to be implicated in metformin-induced REDD1 expression, as HIF-1 expression was not affected in LNCaP cells (data not shown).

We then determined whether p53 is implicated in metformin antiproliferative action in LNCaP cells. As shown in Fig. 2C, metformin decreased BrdUrd incorporation by 45% in cells transfected with a control siRNA. Transfection with a p53 siRNA partially prevented the inhibitory effect of metformin with a 20% decrease. Similarly, metformin was not effective in p53^{-/-} MEFs (Supplementary Fig. S1). BrdUrd incorporation was then compared in PC3, DU145, and LNCaP cells. Metformin was significantly more powerful to inhibit cell proliferation

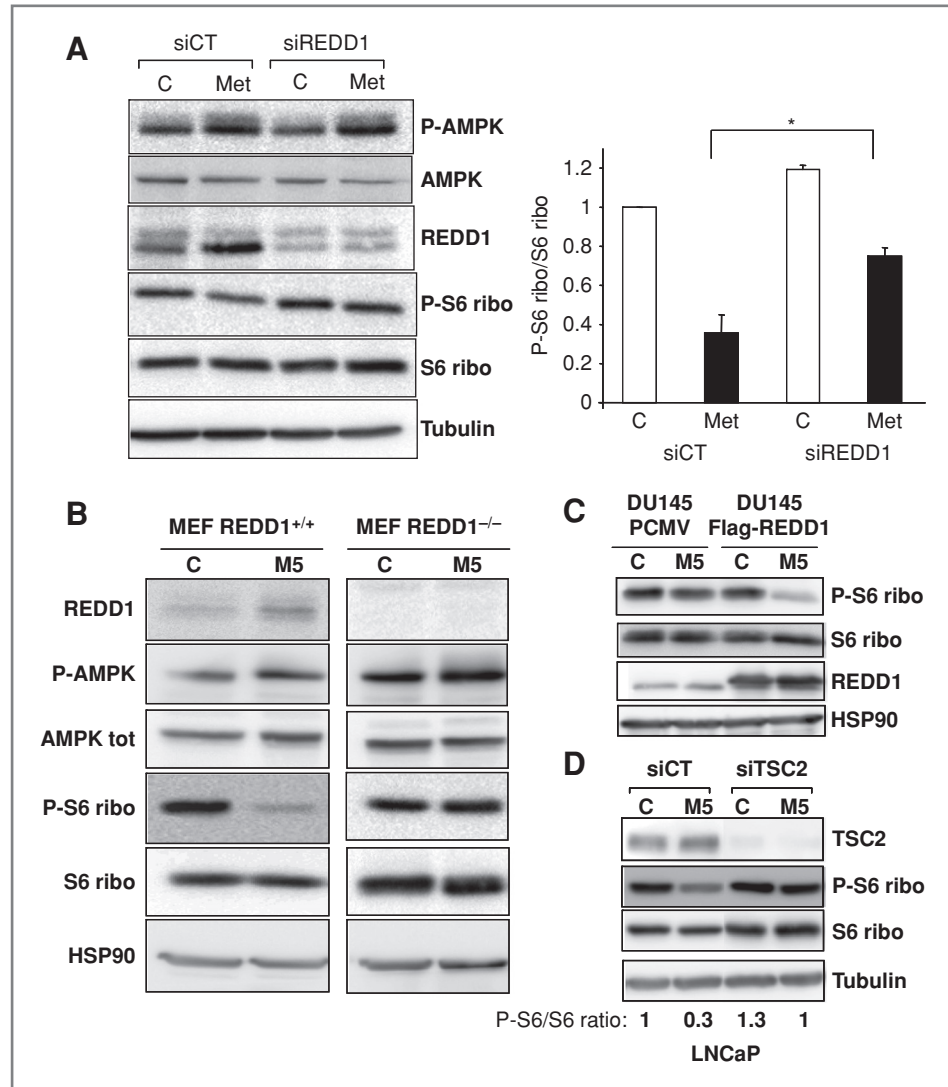
in LNCaP cells (52% decrease) than in DU145 (26%) and PC3 (22%), two p53-deficient cell lines (Fig. 2D). We highlight here the important role of p53 in the antiproliferative effect of metformin in prostate cancer cells.

Our results are at variance with those from Buzzai and colleagues, who have shown that metformin selectively impairs the growth of HCT116 colon cancer cells, which are deficient for p53 (4). This discrepancy may depend on cancer cell types; indeed, metformin induces autophagy in a p53-dependent manner in colon cancer cells (4), whereas it inhibits 2-deoxyglucose-induced autophagy in prostate cancer cells (15). Finally, our observation shows that the p53 status may influence the efficiency of metformin in cancer therapy.

REDD1 mediates mTOR inhibition in response to metformin in LNCaP cells

We then asked whether REDD1 mediates effects of metformin on mTOR activity in LNCaP cells. When REDD1 was

Figure 3. Metformin mediates mTOR inhibition through REDD1. **A**, LNCaP cells were transfected with REDD1 siRNA and treated with 5 mmol/L metformin for 8 hours before immunoblotting. Phospho-S6 (P-S6) was quantified in 3 experiments. *, $P < 0.04$. **B**, REDD1^{+/+} and REDD1^{-/-} MEFs were treated for 8 hours with 5 mmol/L metformin before immunoblotting. **C**, DU145 cells overexpressing REDD1 or transfected with the empty vector (PCMV) were treated with 5 mmol/L metformin before immunoblotting. **D**, LNCaP cells transfected with siCT or siTSC2 were treated with 5 mmol/L metformin before immunoblotting.



downregulated, using siRNA, S6 phosphorylation was significantly less decreased by metformin treatment than in control cells (siCT), whereas the phosphorylation of AMPK was not affected (Fig. 3A). In agreement, metformin did not affect S6 phosphorylation in REDD1^{-/-} MEFs compared with REDD1^{+/+} MEFs (Fig. 3B). Whatever the status of REDD1, metformin activated AMPK. These data show that REDD1 negatively regulates mTOR activity in prostate cancer cells and show for the first time that metformin inhibits mTOR via REDD1.

To reinforce the role of REDD1, we overexpressed REDD1 in DU145 cells, which harbor a nonfunctional p53. We observed a stronger inhibition of S6 phosphorylation in cells overexpressing REDD1 and treated with metformin compared with control conditions (Fig. 3C). Similarly, the re-expression of REDD1 in REDD1^{-/-} cells restored the metformin-induced inhibition of mTOR (Supplementary Fig. S2). Altogether, these results show that REDD1 potentiates the inhibitory effect of

metformin on mTOR. The TSC2 negatively regulates mTOR when it is phosphorylated on T¹²²⁷ and S¹³⁴⁵ by AMPK (16). To determine its role in response to metformin, we used TSC2 siRNA. Following TSC2 invalidation, metformin no longer inhibited S6 phosphorylation (Fig. 3D).

Our results are in accordance with studies showing that REDD1 regulates mTOR in response to hypoxia, 2-deoxyglucose, and nutrient depletion (13). It is noteworthy that the downregulation of S6 phosphorylation is less important in DU145 and PC3 than in LNCaP cells (Fig. 2A), and overexpression of REDD1 in DU145 cells potentiates the effect of metformin on mTOR. These results suggest that REDD1 plays an important role in mTOR inhibition. They also show that mTOR inhibition by metformin is mediated by TSC2 and is not dependent on REDD1 in p53-deficient cells. Because the effects of metformin on mTOR were not mediated by AMPK in prostate cancer cells (3), REDD1 may represent one alternative route that downregulates mTOR.

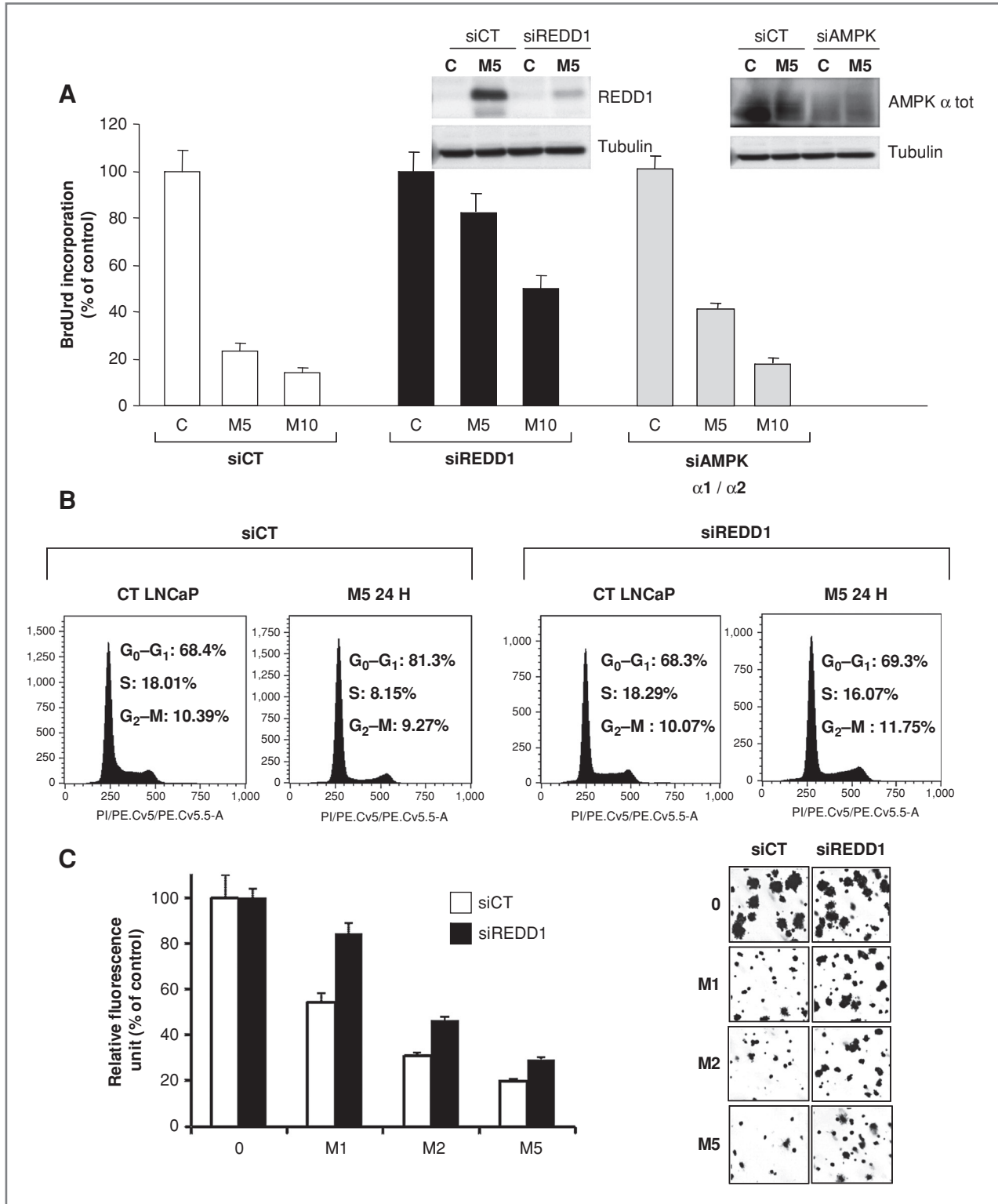


Figure 4. REDD1, but not AMPK, is required for metformin antiproliferative effects. **A**, LNCaP cells transfected with control, REDD1, or AMPK ($\alpha 1 / \alpha 2$) siRNA and treated with 5 or 10 mmol/L metformin were used for BrdUrd incorporation assay. **B**, flow cytometry of LNCaP cells transfected with control or REDD1 siRNA and treated with 5 mmol/L metformin. **C**, soft agar assay in LNCaP cells transfected with CT or REDD1 siRNA and treated for 7 days with metformin (1, 2, and 5 mmol/L; left); photographs of colonies stained with MTT (magnification $\times 100$). M1, 1 mmol/L metformin; M2, 2 mmol/L metformin; M5, 5 mmol/L metformin; M10, 10 mmol/L metformin.

Similarly, acadesine (AICAR or aminoimidazole carboxamide ribonucleotide), another activator of AMPK, downregulates mTOR independently of AMPK in chronic myelogenous leukemia cells (17). In contrast, Schneider and colleagues reported that AMPK regulates REDD1, which inhibits mTOR in response to energy stress induced by prolonged hypoxia. However, they also observed an early AMPK-independent increase of REDD1 in response to hypoxia and suggested that REDD1 regulation can be AMPK dependent or independent depending on the duration and the nature of the stress (18). For example, AMPK starts to be activated 18 hours after hypoxia in HNSCC, whereas it is transiently activated 4 hours after metformin in LNCaP cells (Fig. 1A). We suggest that the discrepancy observed with the study conducted by Schneider and colleagues may be related to the intensity, the duration, and the nature of the stress. It was recently reported that metformin can act independently of AMPK to downregulate mTOR and, instead, signals through Rag GTPase (19). Whether or not REDD1 and this GTPase interact remains to be determined.

REDD1 regulates metformin antiproliferative effect and cyclin D1 decrease independently of AMPK

To determine whether REDD1 is implicated in metformin-induced inhibition of cell viability, LNCaP cells were transfected with REDD1 siRNA and treated with metformin. BrdUrd incorporation was affected by only 18% in cells transfected with REDD1 siRNA compared with 76% in cells transfected with control siRNA (Fig. 4A) in the presence of 5 mmol/L metformin. In parallel, the inhibition of AMPK had a minor impact on metformin effects with a reduction of BrdUrd incorporation of 60% and 80% compared with 76% and 82% with control siRNA in the presence of 5 and 10 mmol/L of metformin, respectively (Fig. 4A). These results show that REDD1 but not AMPK mediates the antiproliferative effects of metformin in LNCaP cells. Metformin-induced cell-cycle arrest and cyclin D1 decrease are not dependent on AMPK in LNCaP cells (3). To determine the implication of REDD1 in this effect, we analyzed cyclin D1 expression and cell cycle in cells transfected with siREDD1. Cyclin D1 was no longer decreased, and S6 phosphorylation was significantly less affected after metformin treatment compared with control siRNA conditions (Supplementary Fig. S3). Furthermore, the cell cycle was not blocked in G₀-G₁ in LNCaP cells treated

with metformin and transfected with REDD1 siRNA (Fig. 4B). To search for a biological consequence of these observations, we conducted an *in vitro* transformation assay in LNCaP cells treated with increasing concentrations of metformin and transfected or not with REDD1 siRNA. Metformin inhibited growth in soft agar in a dose-dependent manner, an inhibition which was significantly less important in cells invalidated for REDD1 (Fig. 4C). Our observations show that REDD1 is a mediator of metformin-induced cell-cycle arrest in prostate cancer cells and is one of the components of a critical pathway for human tumor suppression (12, 13). Indeed, we show for the first time that metformin upregulates REDD1, and that REDD1 participates to its anticancerous effect. We and others showed that metformin alters mitochondrial metabolism (4, 15) and REDD1 regulates mitochondrial reactive oxygen species (ROS) production in MEFs (14). Although metformin reduces ROS in normal endothelial cells (20), it did not affect ROS production in prostate cancer cells (data not shown), suggesting that metformin-induced REDD1 expression does not regulate ROS formation in these cells. However, whether or not REDD1 could participate in the other observed effects of metformin on cancer cell metabolism (i.e., increased glycolysis and inhibition of complex I) remains to be elucidated. In summary, elucidating the mechanism by which metformin inhibits mTOR could be beneficial to the treatment of cancer and other human pathologies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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