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P90 ribosomal S6 kinase confers cancer cell survival by mediating checkpoint kinase 1 degradation in response to glucose stress

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Abstract

In solid tumors, cancer cells have devised multiple approaches to survival and proliferate in response to glucose starvation that is often observed in solid tumor microenvironments. However, the precise mechanisms are far less known. Herein, we report that glucose deprivation activates 90-kDa ribosomal S6 kinase (p90 RSK), a highly conserved Ser/Thr kinase, and activated p90 RSK promotes cancer cell survival. Mechanistically, activated p90 RSK by glucose deprivation phosphorylates checkpoint kinase 1 (CHK1), a key transducer in checkpoint signaling pathways, at Ser280 and triggers CHK1 ubiquitination mediated by $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase and proteasomal degradation, subsequently suppressing cancer cell apoptosis induced by glucose deprivation. Importantly, we identified an inverse correlation between p90 RSK activity and CHK1 levels within the solid tumor mass, with lower levels of CHK1 and higher activity of p90 RSK in the center of the tumor where low glucose concentrations are often observed. Thus, our study indicates that p90 RSK promotes CHK1 phosphorylation at Ser280 and its subsequent degradation, which allows cancer cells to escape from checkpoint signals under the stress of glucose deprivation, leading to cell survival and thus contributing to tumorigenesis.

Abbreviations: AMPK, AMP-activated protein kinase; ATR, ataxia telangiectasia-mutated and Rad3-related kinase; BAD, BCL2 associated agonist of cell death; CHX, cycloheximide; DKO, double knockout; GSK3β, glycogen synthase kinase 3β; MEF, mouse embryonic fibroblast; p90 RSK, 90-kDa ribosomal S6 kinase; PARP, poly(ADP-ribose) polymerase.

Ying Ma, Danrui Cui, and Linchen Wang contributed equally to this work.

KEYWORDS
CHK1, glucose deprivation, P90 RSK, phosphorylation, β-TrCP

1 | INTRODUCTION

In solid tumors, cancer cells often suffer glucose starvation in the harsh tumor microenvironment, caused by increased glucose consumption and insufficient vascularization. Therefore, cancer cells have devised mechanisms by which they are able to survive and proliferate in conditions of low glucose. For example, transient glucose starvation increases the invasive ability of tumor cells, thus facilitating tumor progression.[1] Although increasing evidence has shown that solid tumor cells are exposed to adverse endogenous and exogenous stresses that directly cause mutations and genomic instability,[2] far less is known about how cells escape from checkpoint signals, particularly under low-glucose conditions.

The p90 RSK family members, including p90 RSK1-4, are highly conserved Ser/Thr kinases belonging to AGC kinase family. Activated by the canonical Ras/ERK signaling cascade, p90 RSK regulates diverse cellular processes, such as cell growth, proliferation, survival, and motility by phosphorylating its various substrates, such as GSK3β, BAD, procaspase-8, tuberous sclerosis complex 2 , and c-Fos.[3,4] For example, p90 RSK2 phosphorylates GSK3β at Ser9 and inhibits its kinase activity, and thereby releases the inhibition of translation initiation factor eIF2B, subsequently facilitating mRNA translation.[5,6] P90 RSK1/2 phosphorylate proapoptotic protein BAD at Ser112 and confer cell survival by suppressing BAD-mediated apoptosis.[7,8] Recently, it was reported that p90 RSK phosphorylates procaspase-8 at Thr265 in the necrosome, and subsequently releases the blockade of necroptosis.[9] However, it is totally unknown whether and how p90 RSK regulates cell survival under low-glucose conditions in solid tumors.

The ATR-CHK1 pathway constitutes the core signaling network in cell cycle checkpoint pathways, which play a key surveillance role to ensure genomic integrity when cells are threatened by both endogenous and exogenous stresses.[10] In response to replication blocks or DNA damage, CHK1, phosphorylated on Ser317 and Ser345 by activated ATR, triggers a series of signaling cascades to induce cell cycle arrest or delay, DNA repair, and apoptosis.[11,12] In addition to the response to DNA damage, CHK1 has been shown to be phosphorylated on Ser280/Ser284 by AMPK to promote cell survival in response to glucose deprivation.[13]

In this study, we reported that p90 RSK is essential for cancer cell survival by promoting CHK1 degradation under glucose deprivation conditions. Specifically, glucose deprivation activates p90 RSK, which phosphorylates CHK1 at Ser280 and triggers CHK1 polyubiquitination and degradation. Biologically, silencing of p90 RSK induced apoptosis upon glucose deprivation by blocking CHK1 degradation and p90 RSK activity was inversely correlated with CHK1 levels in solid tumors. Our study reveals the cross-talk among p90 RSK, CHK1, and the tumor microenvironment and shows that p90 RSK favors cell survival by CHK1 degradation upon glucose deprivation.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

HEK293 (ATCC-CRL-1573), SK-BR3 (ATCC-HTB-30), MDA-MB231 (ATCC-HTB-26), and H1299 (ATCC-CRL-5803) cells were obtained from ATCC. All the cell lines were authenticated by ATCC, and were expanded and preserved in liquid nitrogen upon receipt. Cells for experiments were passaged fewer than 25-30 times. Ampk WT and Ampk DKO MEFs have been described previously.[14] All the cell lines were maintained in DMEM (C11995500BT; Gibco) containing 10% FBS and 1% penicillin/streptomycin. Glucose deprivation treatment and transfection were carried out as described previously.[13]

2.2 | Antibodies and chemicals

Antibodies against the following proteins were obtained from commercial sources: CHK1 for immunoblotting (8408; Santa Cruz Biotechnology), CHK1 for immunohistochemistry (ab47574; Abcam), p-CHK1 Ser280 (2347), p-AMPK Thr172 (2535), AMPK (5831), p-BAD Ser112 (5284), BAD (9239), caspase-3 (9665), p-GSK3β Ser9 (9323), GSK3α/β (5676), PARP (9542), p-ERK Thr202/Tyr204 (9101), ERK (4696), p-H3 Ser10 (3377), p-p90 RSK Ser380 (9341), and p90 RSK (9355) (all Cell Signaling Technology), β-actin (A5441; Sigma), and FLAG (F1804; Sigma). Compounds BI-D1870 and CHX were obtained from Selleck and Sigma-Aldrich, respectively.

2.3 | Small interfering RNA silencing

Cells were transfected with the following siRNA oligonucleotides by using Lipofectamine 3000 (L3000-015; Invitrogen) according to the manufacturer's instructions: control (siCtrl), 5′-ATT GTA TGC GAT CGC AGA C-3′; siCHK1, 5′-TCG TGA GCG TTT GTT GAA C-3′; siRSK1, 5′-CCC AAC ATC ATC ACT CTG AAA-3′; and siRSK2: 5′-AGC GCT GAG AAT GGA CAG CAA-3′.

2.4 | Flow cytometry

Cells were treated with glucose deprivation for 36 hours and analyzed by flow cytometry. Apoptosis was determined using an annexin

V-FITC apoptosis detection kit (C1062; Beyotime) according to the manufacturer's instructions.

2.5 | Immunoblotting and in vivo ubiquitination assay

For immunoblotting, cells were harvested and washed with cold PBS. Cell pellets were lysed in lysis buffer with protease inhibitors and phosphatase inhibitors, followed by western blotting as previously described.[15] For the in vivo ubiquitination assay, cells were transfected with the indicated plasmids using PolyJet (SL100688; SignaGen Laboratories) for 48 hours and then treated with 20 μM MG132 or 10 μM BI-D1870 for 4 hours before being harvested. Thereafter, the cells were lysed in lysis buffer with protease inhibitors and incubated with bead-conjugated HA (A2095; Sigma) in a rotating incubator for 3 hours at 4°C. The immunoprecipitates were washed with lysis buffer and subjected to immunoblotting.[16]

2.6 | Immunohistochemistry

Mouse lung and lymphoma tissues for immunohistochemistry were isolated and immediately fixed in 4% paraformaldehyde, followed by dehydration and then embedding in paraffin wax. Following dewaxing, rehydration, and antigen retrieval in 0.01 M citrate buffer (pH 6.0) at 95°C for 15 minutes, 4-μm-thick serial sections were blocked for 60 minutes with blocking buffer (0.1% Triton X-100, 5% goat serum in 0.01 M PBS) and then immunostained with an anti-CHK1 (ab47574, Abcam; 1:100 dilution) or anti-phosphorylated GSK3β (Ser9) Ab (9323; Cell Signaling Technology; 1:100 dilution) overnight at 4°C, followed by incubation in an HRP-conjugated secondary Ab for 30 minutes at room temperature. After staining with 0.05% 3,3-diaminobenzidine tetrahydrochloride, the sections were counterstained with hematoxylin and scanned by using an Aperio Whole Slide Scanner.

2.7 | Mouse studies

For the animal study, all procedures were approved by the Zhejiang University Laboratory Animal Center. Animal care was provided in accordance with the principles and procedures outlined in the Chinese National Research Guide for the Care and Use of Laboratory Animals.

3 | RESULTS

3.1 | Glucose deprivation upregulates p90 RSK kinase activity

To explore cell signaling involved in cell survival upon glucose deprivation, we screened for the activity of cell signaling in response to glucose deprivation in MDA-MB231 and SK-BR3 breast cancer cells and H1299 lung cancer cells. Consistent with a previous study,[17] we found AMPK signaling was dynamically activated. Unexpectedly, we noticed remarkable elevation of p90 RSK activity at multiple time points in all three tested cells under glucose deprivation, compared with that under normal glucose conditions, as indicated by the increased phosphorylation of BAD at Ser112 and GSK3β at Ser9 (Figures 1 and S1A, lanes 6-9 vs 2-5), two well-known downstream phosphorylation targets of p90 RSK.[3-8] In addition, the phosphorylation of ERK1/2, which are upstream kinases for p90 RSK activation, was also increased in all three tested cell lines upon glucose deprivation (Figures 1 and S1A, lanes 6-9 vs 2-5). Consistently, the phosphorylation of p90 RSK was increased accordingly (Figures 1 and S1A, lanes 6-9 vs 2-5). Taken together, these results clearly indicate that p90 RSK signaling is activated upon glucose deprivation, and suggests that it might be involved in cell survival in response to glucose deprivation. Interestingly, following glucose deprivation for a shorter time course, p90 RSK was moderately activated in MDA-MB231 cells, but not in H1299 or SK-BR3 cells (Figure S1B), suggesting that the dynamic activation of p90 RSK in response to glucose deprivation might vary in a cell context-dependent manner.

3.2 | P90 RSK inactivation increases cell apoptosis upon glucose deprivation

To determine whether activated p90 RSK signaling has any effect on cell survival or cell death upon glucose deprivation, we blocked the activity of p90 RSK with BI-D1870 treatment, a p90 RSK inhibitor that inhibits the activities of p90 RSK1-4.[18] Apoptotic cells stained with annexin V^+ were moderately increased by BI-D1870 treatment in SK-BR3 cells (Figure S2), and further significantly increased along with glucose deprivation in both MDA-MB231 and SK-BR3 cells (Figures 2A and S2). Likewise, the levels of cleaved PARP and caspase-3, two hallmarks of apoptosis, were also obviously increased upon BI-D1870 treatment (Figure 2B, lane 4 vs 3) when the cells were treated with glucose deprivation. BI-D1870 is known to exert some off-target effects through the mTORC1-p70 S6K signaling pathway.[19] To further confirm that p90 RSK activation is responsible for apoptosis inhibition upon glucose deprivation, we silenced p90 RSK by transfecting siRNA oligos targeting p90 RSK1/2 and determined the apoptosis induction. Accordingly, we observed that p90 RSK silencing markedly increased the cleavage of both PARP and caspase-3 upon glucose deprivation (Figure 2C, lane 8 vs 4; Figure 5A, lane 4 vs 2), as well as the percentage of apoptotic cells stained with annexin V^+ (Figure 5B). These results suggest that p90 RSK activity is essential to cancer cell survival upon glucose deprivation.

3.3 | P90 RSK inactivation inhibits CHK1 degradation upon glucose deprivation

Our recent study showed that AMPK and $SCF^{\beta\text{-}TrCP}$-mediated CHK1 degradation facilitates cancer cell survival under glucose deprivation

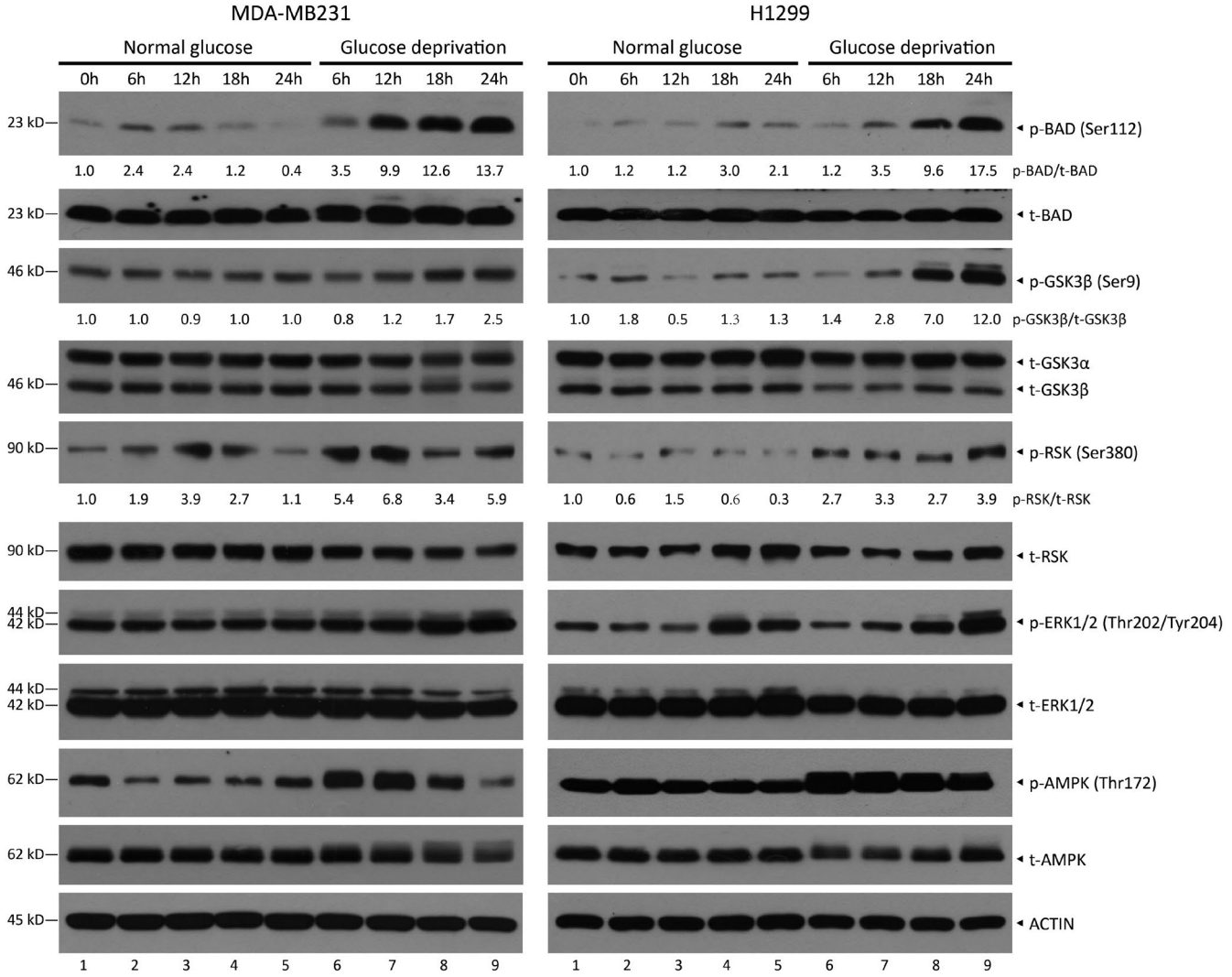


FIGURE 1 Glucose deprivation upregulates p90 ribosomal S6 kinase (RSK) activity. MDA-MB231 and H1299 cells were grown in normal glucose or glucose-free media for the indicated time periods. Cells were harvested and subjected to immunoblotting using the indicated Abs. Band density was quantified using ImageJ. Phosphorylated protein levels (p-) were normalized to total levels (t-) and expressed as fold change compared with the corresponding control by setting the control value as 1. AMPK, AMP-activated protein kinase; BAD, BCL2 associated agonist of cell death; GSK3β, glycogen synthase kinase 3β

conditions.[13] Using GPS2.0, a kinase prediction program,[20] we found that p90 RSK is one of the putative kinases involved in phosphorylating CHK1 on Ser280, which is located within the β-TrCP binding motif of CHK1. Following this lead, we next explored whether p90 RSK contributes to $SCF^{\beta\text{-}TrCP}$-mediated CHK1 degradation upon glucose deprivation. Consistent with our previous study,[13] glucose deprivation triggered CHK1 degradation in a time-dependent manner (Figures 3A and S3A). Interestingly, although AMPK inactivation by siRNA-based knockdown or genetic KO inhibited CHK1 degradation induced by glucose deprivation (Figure 3B, lane 5 vs 2), glucose deprivation still induced significant degradation of CHK1 in AMPK knocked-down H1299 cells or Ampk DKO MEFs (Figure 3B, lane 5 vs 4), which can be inhibited by MG132, a proteasome inhibitor (Figure 3B, lane 6 vs 5). This indicates that other kinase(s) are also involved in CHK1 degradation through proteasomes as well as AMPK. Therefore, we knocked down AMPK or p90 RSK alone or in combination in MDA-MB231 and H1299 cells. Indeed, knocking down either AMPK or p90 RSK alone inhibited CHK1 degradation, whereas combined knockdown further blocked CHK1 degradation induced by glucose deprivation (Figure 3C).

To further determine whether p90 RSK activity is important for regulating CHK1 protein levels under glucose deprivation, we treated cells with BI-D1870 and found that the reduction of CHK1 protein levels in response to glucose deprivation was significantly inhibited by BI-D1870 in all three tested cell lines, including MDA-MB231, SK-BR3, and H1299 cells (Figures 3D and S3B). Given that CHK1 protein abundance is controlled by both protein synthesis and degradation, we further determined whether BI-D1870 treatment had any effect on CHK1 protein stability. We treated cells with CHX, an inhibitor of new protein synthesis that blocks protein translation during elongation, in combination with glucose deprivation and then undertook immunoblotting

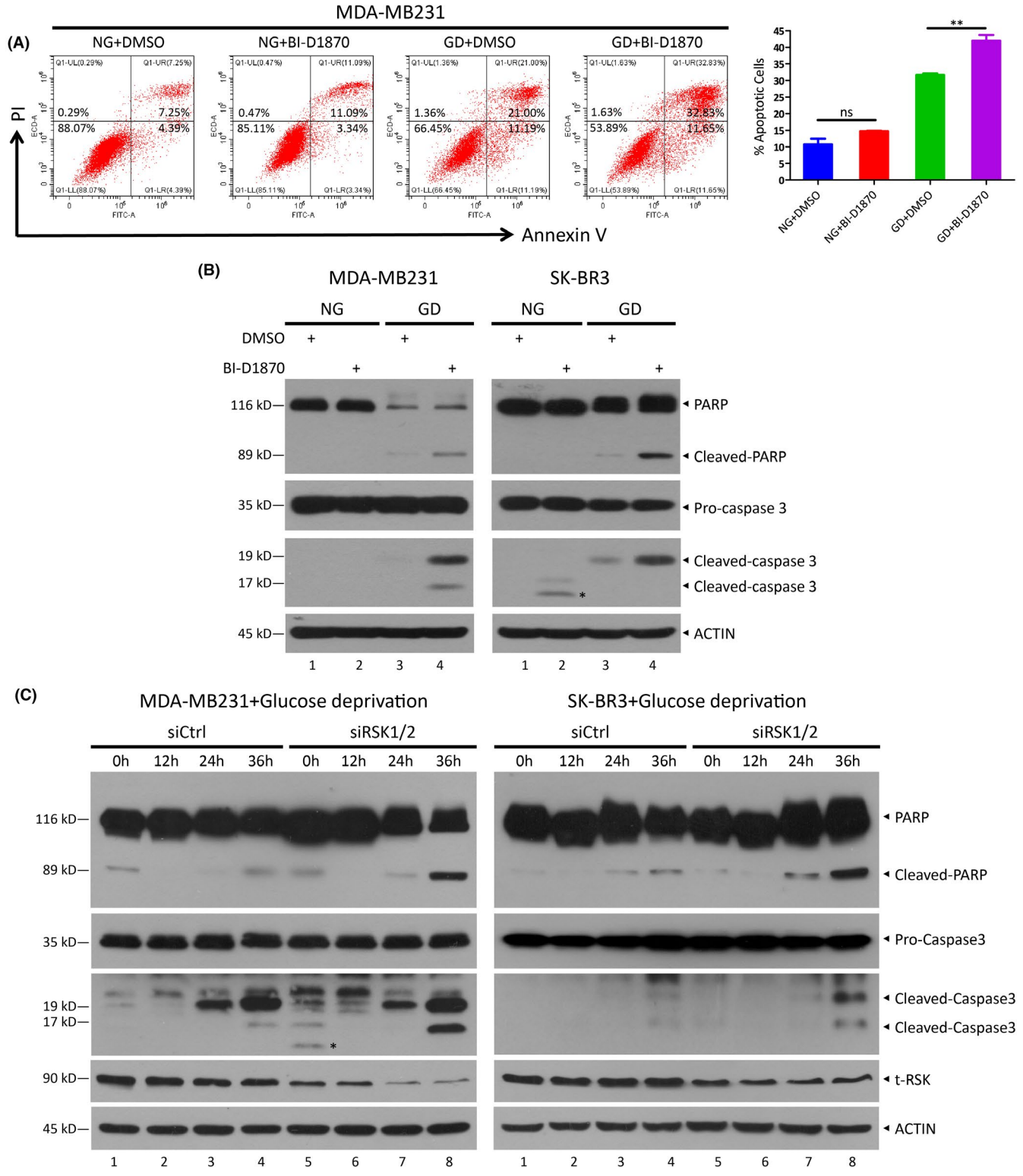


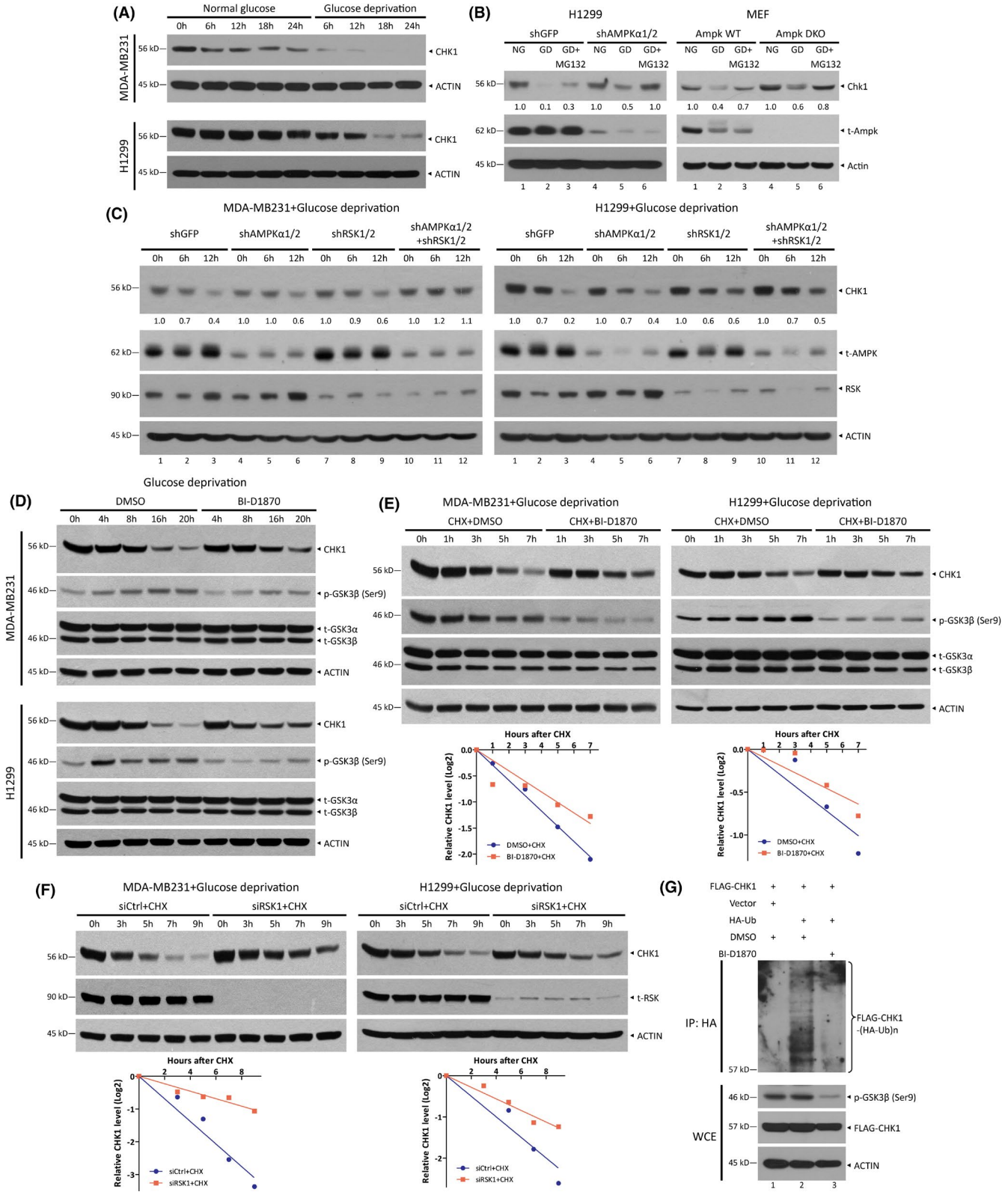
FIGURE 2 P90 ribosomal S6 kinase (RSK) inactivation increases cell apoptosis upon glucose deprivation (GD). A, B, MDA-MB231 cells were transferred to glucose-free media for 36 h with or without 10 μM BI-D1870, and subjected to FACS analysis to determine the apoptotic population (A, left, a representative FACS profile; A, right, the percentage of annexin V^+ cells, mean ± SEM; n = 3; ns, not significant; $^{**}P < .01$), or immunoblotting with the indicated Abs (B). C, MDA-MB231 and SK-BR3 cells were transfected with siRNA targeting p90 RSK1/2 (siRSK1+2) or scrambled control siRNA (siCtrl) for 48 h, and then transferred to glucose-free media for indicated time periods, followed by immunoblotting with the indicated Abs. NG, normal glucose; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; t-RSK, total RSK. *Nonspecific band

to determine the protein half-life of endogenous CHK1 with or without BI-D1870 treatment. We found that the half-life of CHK1 protein was significantly extended in the presence of BI-D1870, compared with that in the DMSO group, in all tested cell lines (Figures 3E and S3C). To further confirm that p90 RSK activation is responsible for CHK1 degradation upon glucose deprivation, we knocked down p90 RSK by transfecting with siRNA oligos targeting p90 RSK and determined CHK1 protein half-life. Accordingly, we observed that depletion of p90 RSK markedly extended the half-life of CHK1 upon glucose deprivation (Figure 3F and S3D). It is well established that covalent linkage of a poly-ubiquitin chain to a targeted protein is required as a signal for protein degradation by the 26S proteasome. Hence, we used an in vivo polyubiquitination assay to determine whether CHK1 ubiquitination is regulated by p90 RSK activity. Indeed, we found that BI-D1870 treatment significantly suppressed CHK1 polyubiquitination (Figure 3G, lane 3 vs 2). Taken together, these results suggest that p90 RSK controls CHK1 polyubiquitination and degradation upon glucose starvation.

3.4 | P90 RSK phosphorylates CHK1 at Ser280 to promote CHK1 degradation upon glucose deprivation

It is well established that the β-TrCP binding motif of substrates would be phosphorylated before $SCF^{\beta\text{-}TrCP}$ promotes their ubiquitination for degradation.[21] Next, we detected CHK1 phosphorylation at Ser280 in response to glucose deprivation with or without BI-D1870. First, we observed the accumulation of phospho-CHK1 at Ser280 (Figure 4A), but not at Ser317, Ser345, or Ser296 (Figure S4A), under glucose deprivation while cells were simultaneously treated with the proteasome inhibitor MG132 to block CHK1 degradation. Furthermore, the inhibition of p90 RSK activity by BI-D1870 obviously suppressed the phosphorylation of CHK1 at Ser280 induced by glucose deprivation (Figure 4A). Likewise, we confirmed the involvement of p90 RSK in CHK1 phosphorylation at Ser280 using an siRNA-mediated knockdown approach. As shown in Figure 4B, silencing of p90 RSK significantly decreased the phosphorylation levels of CHK1 at Ser280 triggered by glucose deprivation. Furthermore, we synchronized MDA-MB231 shGFP and shRSK1/2 cells at the end of G_1 phase by double thymidine treatment and then released them back into the cell cycle, along with glucose deprivation, to evaluate the role of p90 RSK-mediated CHK1 phosphorylation at Ser280 in cell cycle progression. Checkpoint kinase 1 was largely phosphorylated at Ser280 by glucose deprivation in M phase, marked by phosphorylation of histone H3 (p-H3), and CHK1 phosphorylation was significantly inhibited by p90 RSK knockdown, indicating that p90 RSK-mediated CHK1 phosphorylation at Ser280 occurs in a cell cycle-dependent manner (Figure 4C, lanes 11-12 vs 5-6). Interestingly, upon UV irradiation, p90 RSK phosphorylates CHK1 at Ser280 to rapidly activate CHK1 in the G_1 phase.[22] However, upon G_2 arrest induced by low dose of doxorubicin, p90 RSK phosphorylates CHK1 and inhibits its activity to facilitate checkpoint recovery.[23] These findings suggest that the phosphorylation of CHK1 at Ser280 by p90 RSK plays a specific role in a cell cycle-dependent manner upon various stresses. Finally, we mutated Ser280 or Ser284 to glutamic acid (S to E) to mimic phosphorylated CHK1, and found that the protein half-lives of FLAG-CHK1 (S280E) and FLAG-CHK1 (S284E) were significantly shortened, indicating that the phosphorylation of CHK1 at Ser280 and Ser284 is important for CHK1 degradation (Figure S5A). Moreover, the

FIGURE 3 P90 RSK regulates CHK1 protein stability upon glucose deprivation. A, Glucose deprivation decreases the protein levels of CHK1. MDA-MB231 and H1299 cells were grown in normal glucose or glucose-free media for the indicated time periods. Cells were harvested and then subjected to immunoblotting using the indicated antibodies. B, Glucose deprivation promotes the proteasomal degradation of CHK1 in cells with AMPK inactivation. H1299 cells infected with lentivirus expressing indicated shRNA were treated with glucose deprivation or/and MG132 for 6 h. Paired Ampk WT and DKO MEFs were treated with glucose deprivation or/and MG132 for 12 h. Cells were harvested and then subjected to immunoblotting using the indicated antibodies. The band density was quantified using ImageJ, and the alteration of CHK1 levels was expressed as fold change compared with the corresponding control (NG) by setting the control value as 1. GD, glucose deprivation; NG, normal glucose. C, AMPK and p90 RSK coordinatedly control CHK1 degradation in response to glucose deprivation. MDA-MB231 and H1299 cells transfected with indicated siRNA oligos were transferred to glucose-free media for the indicated time periods. Cells were harvested and then subjected to immunoblotting using the indicated antibodies. D, Blockage of p90 RSK activity inhibits the reduction in CHK1 protein levels in response to glucose deprivation. Cells were transferred to glucose-free media with or without 10 μM BI-D1870 for the indicated time periods. Cells were harvested and then subjected to immunoblotting using the indicated antibodies. E, Blockage of p90 RSK activity extends the CHK1 protein half-life under glucose deprivation. Cells were transferred to glucose-free media containing CHX with or without 10 μM BI-D1870 for the indicated time periods. Cells were then harvested and subjected to immunoblotting using the indicated antibodies. F, Silencing of p90 RSK extends the CHK1 protein half-life under glucose deprivation. Cells transfected with siRNA oligos targeting p90 RSK or scrambled control siRNA were transferred to glucose-free media with 100 μg/ml CHX and then harvested at the indicated time points. Cell lysates were subjected to immunoblotting using the indicated antibodies. Densitometry quantification was performed with ImageJ, and the relative intensity values were calculated by normalization to the intensity at the 0 h time point. Decay curves are shown (E-F, bottom). G, Blockage of p90 RSK activity inhibits CHK1 polyubiquitination upon glucose deprivation. HEK293 cells were transfected with FLAG-CHK1 either alone or in combination with HA-Ub, as indicated. Cells grown in glucose-free media were treated with MG132 for the last 6 h with or without BI-D1870 for 6 h. Cells were harvested and lysed, and then Ub-conjugated proteins were pulled down with HA beads. Pull-downs and whole cell extracts were separated by SDS-PAGE and immunoblotted using the indicated antibodies



protein half-life of FLAG-CHK1 (S280E) was extended upon β-TrCP knockdown (Figure S5B). Together, these results indicate that p90 RSK participates in CHK1 phosphorylation at Ser280, leading to CHK1 degradation mediated by $SCF^{\beta\text{-}TrCP}$ upon glucose deprivation.

3.5 | P90 RSK confers cancer cell survival by promoting CHK1 degradation upon glucose deprivation

As glucose deprivation activated p90 RSK, activated p90 RSK phosphorylated CHK1 to trigger its degradation, and CHK1

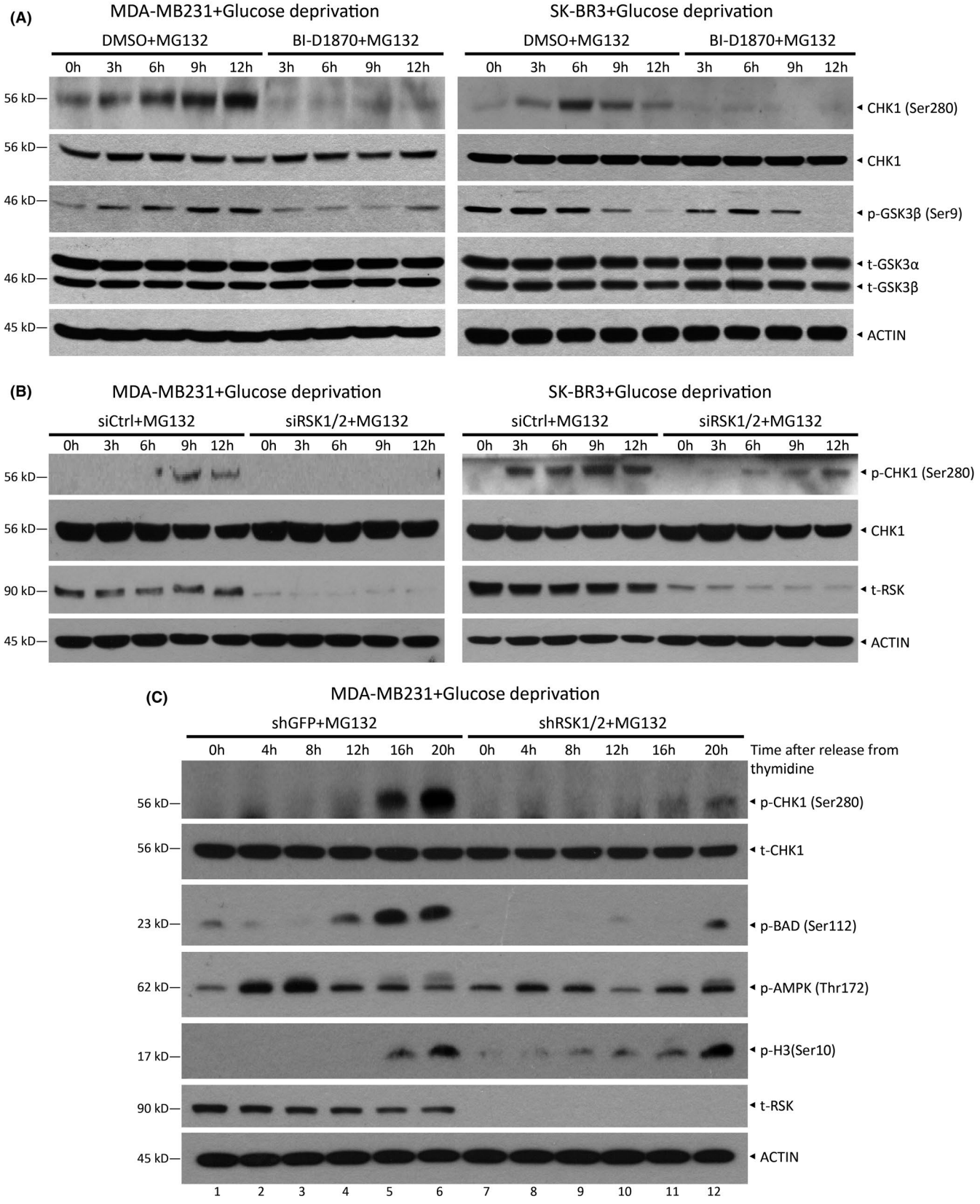

(A)
MDA-MB231+Glucose deprivation
SK-BR3+Glucose deprivation
DMSO+MG132
BI-D1870+MG132
0h 3h 6h 9h 12h 3h 6h 9h 12h
56 kD
46 kD
45 kD
CHK1 (Ser280)
CHK1
p-GSK3β (Ser9)
t-GSK3α
t-GSK3β
ACTIN
(B)
MDA-MB231+Glucose deprivation
SK-BR3+Glucose deprivation
siCtrl+MG132
siRSK1/2+MG132
0h 3h 6h 9h 12h 0h 3h 6h 9h 12h
56 kD
90 kD
45 kD
p-CHK1 (Ser280)
CHK1
t-RSK
ACTIN
(C)
MDA-MB231+Glucose deprivation
shGFP+MG132
shRSK1/2+MG132
0h 4h 8h 12h 16h 20h 0h 4h 8h 12h 16h 20h
Time after release from thymidine
56 kD
23 kD
62 kD
17 kD
90 kD
45 kD
p-CHK1 (Ser280)
t-CHK1
p-BAD (Ser112)
p-AMPK (Thr172)
p-H3(Ser10)
t-RSK
ACTIN
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 4 P90 RSK phosphorylates CHK1 at Ser280 upon glucose deprivation. A, Blocking p90 RSK activity inhibits CHK1 phosphorylation at Ser280 upon glucose deprivation. MDA-MB231 and SK-BR3 cells were transferred to glucose-free media containing MG132 with or without 10 μM BI-D1870 for the indicated time periods. Cells were then harvested and subjected to immunoblotting using the indicated antibodies. B, Silencing of p90 RSK suppresses CHK1 phosphorylation at Ser280 upon glucose deprivation. Cells transfected with siRNA oligos targeting p90 RSK or scrambled control siRNA were transferred to glucose-free media containing MG132. Cells were harvested at the indicated time points and subjected to immunoblotting using the indicated antibodies. C, P90 RSK phosphorylates CHK1 at Ser280 in a cell-cycle-dependent manner. MDA-MB231 cells infected with lentivirus expressing indicated shRNA were treated with 2 mM thymidine for 14 h and then released for 9 h. Next, cells were treated with thymidine for an additional 14 h and then incubated in fresh glucose-free media along with MG132 for various time periods. Cells were harvested and then subjected to immunoblotting using the indicated antibodies

degradation promoted cancer cell survival upon glucose deprivation,[13] we hypothesized that p90 RSK-mediated CHK1 degradation facilitated cancer cell survival in response to glucose deprivation. Indeed, p90 RSK knockdown stabilized CHK1 (Figure 5A, lane 4 vs 2), leading to more induction of apoptosis by glucose deprivation, as reflected by increased cleavage of both PARP and caspase-3 (Figure 5A, lane 4 vs 2). The simultaneous CHK1 knockdown partially abrogated the increased apoptosis caused by p90 RSK knockdown (Figure 5A, lanes 6 vs 4 and 2). Likewise, the percentage of annexin V^+ apoptotic cells were also significantly increased in p90 RSK knockdown cells upon glucose deprivation, which can be largely rescued by simultaneous CHK1 knockdown (Figure 5B). Interestingly, CHK1 knockdown alone had minimal, if any, effect on the induction of apoptosis by glucose deprivation (Figure 5A, lane 8 vs 2, and 5B). These results suggest that p90 RSK-mediated CHK1 degradation contributes to cancer cell survival under glucose deprivation conditions.

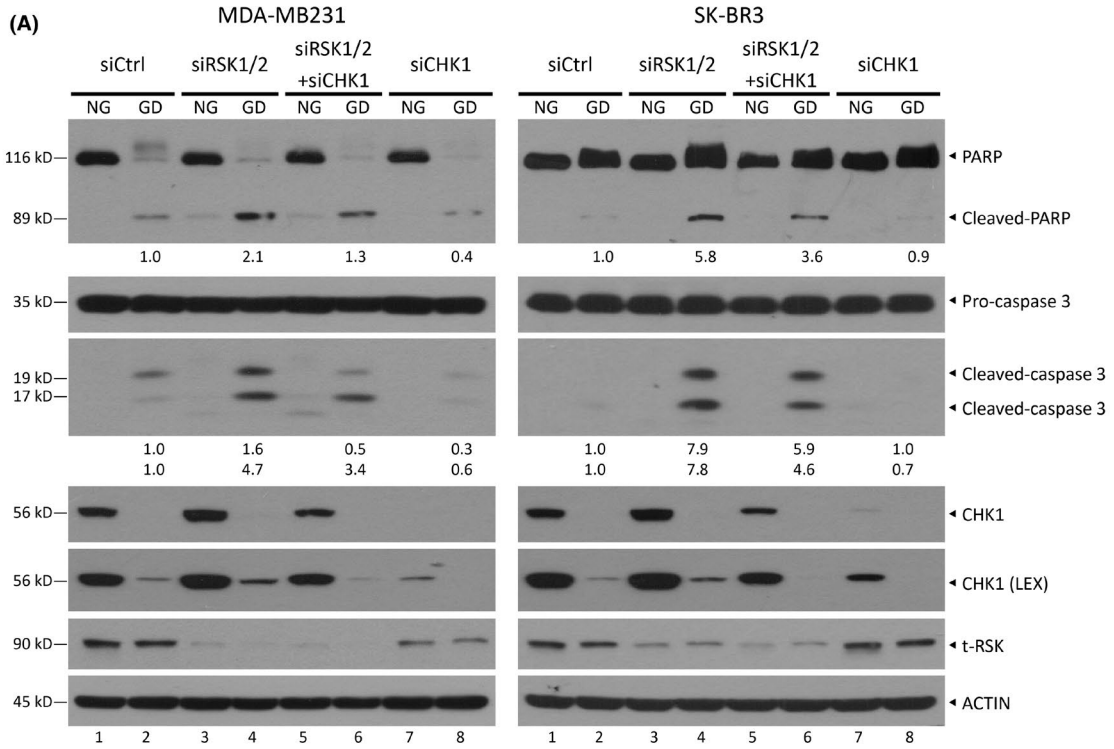
3.6 | Inverse correlation of CHK1 levels and p90 RSK activity within tumor mass

The blood flow in solid tumors is chaotic and heterogeneous, resulting in pronounced differences in glucose concentrations within the solid tumor mass.[24] The central portions of solid tumors are hypovascular, compared with the periphery, and therefore often suffer from hypoxia, glucose deprivation, and even necrosis.[24] To determine the correlation between p90 RSK activity and CHK1 protein levels in vivo, we assessed endogenous levels of CHK1 and phosphorylated GSK3β (Ser9) in spontaneous solid tumors. To obtain whole lung tumors, we used a well-established *LSL* (Loxp-STOP-Loxp)-$Kras^{G12D}$;$p53^{fl/fl}$ conditional mouse lung cancer model. The lung tumors were isolated and fixed after Cre-recombinase activates $Kras^{G12D}$ and deletes p53 for 9 weeks.[25] Additionally, blood vessels are rare and necrosis is a common phenomenon in the center of spontaneous lymphomas, another solid tumor, developed in $Pten^{+/-}$ mice at the age of 10 months, which is also suitable to assess the correlation of the levels of Chk1 and glucose in vivo. Immunohistochemical analysis showed that Chk1 levels were decreased in the central area of tumors, where the blood supply was insufficient and the glucose concentration was low, compared to the levels at the margin of tumors, which is consistent with glucose deprivation-triggered Chk1 degradation in cell culture settings. Conversely, we found higher phosphorylation levels of Gsk3β at Ser9 in the central area of tumors, indicating higher p90 Rsk activity, whereas low phosphorylation levels of Gsk3β at Ser9 were found at the margin of tumors (Figures 6A and S6A). The inverse correlation between Chk1 and p-Gsk3β (Ser9) in the center versus the margin of the solid tumor mass was confirmed by immunoblotting, as shown by lower Chk1 levels and higher p90 Rsk activity in the center of tumors, but higher Chk1 levels and lower p90 Rsk activity at the margin of tumors (Figure S6B). Taken together, these results indicate an inverse correlation of Chk1 levels and p90 Rsk activity within the tumor mass, which likely resulted from variance in glucose concentrations.

4 | DISCUSSION

The RSKs are a group of conserved Ser/Thr kinases that regulate a variety of cellular processes, such as cell growth, proliferation, and survival.[3] In this study, we showed that p90 RSK is essential for cancer cell survival by promoting CHK1 degradation under glucose deprivation conditions. Our conclusion is supported by the following lines of evidence: (a) P90 RSK is activated by glucose deprivation in a time-dependent manner; (b) P90 RSK inactivation increases cell apoptosis upon glucose deprivation; (c) activated p90 RSK promotes CHK1 degradation by phosphorylating CHK1 at Ser280; (d) simultaneous CHK1 knockdown abrogates the increased apoptosis by p90 RSK knockdown; and (e) CHK1 levels are inversely correlated with p90 RSK activity at the center/margin of lung tumors and lymphoma tissues. Thus, our results revealed a novel mechanism for cancer cell survival in response to low-glucose stress.

Activated p90 RSK has been shown to phosphorylate CHK1 to regulate its subcellular location by serum stimulation[22] or to regulate its activity by PMA stimulation.[23] To the best of our knowledge, this is the first study to observe that glucose deprivation increases p90 RSK activity in all of the tested cell lines (Figures 1 and S1; MDA-MB231, SK-BR3, and H1299 cells), suggesting a general phenomenon and not one that occurs in a context-dependent manner. P90 RSK plays a prosurvival role by phosphorylating proapoptotic proteins (eg, BAD and DAPK) and inhibiting their functions.[3,7,8,26] Accordingly, we showed that in response to glucose deprivation, p90 RSK silencing promoted cell apoptosis by inhibiting the reduction of CHK1 (Figure 5), suggesting that the activation of p90 RSK by glucose deprivation allows cancer cells to survive in conditions of low glucose, which are often observed in solid tumor microenvironments.


(A)
MDA-MB231
SK-BR3
siCtrl
siRSK1/2
siRSK1/2 +siCHK1
siCHK1
NG
GD
116 kD
89 kD
PARP
Cleaved-PARP
1.0 2.1 1.3 0.4
1.0 5.8 3.6 0.9
35 kD
Pro-caspase 3
19 kD
17 kD
Cleaved-caspase 3
Cleaved-caspase 3
1.0 1.6 0.5 0.3
1.0 4.7 3.4 0.6
1.0 7.9 5.9 1.0
1.0 7.8 4.6 0.7
56 kD
CHK1
56 kD
CHK1 (LEX)
90 kD
t-RSK
45 kD
ACTIN
1 2 3 4 5 6 7 8

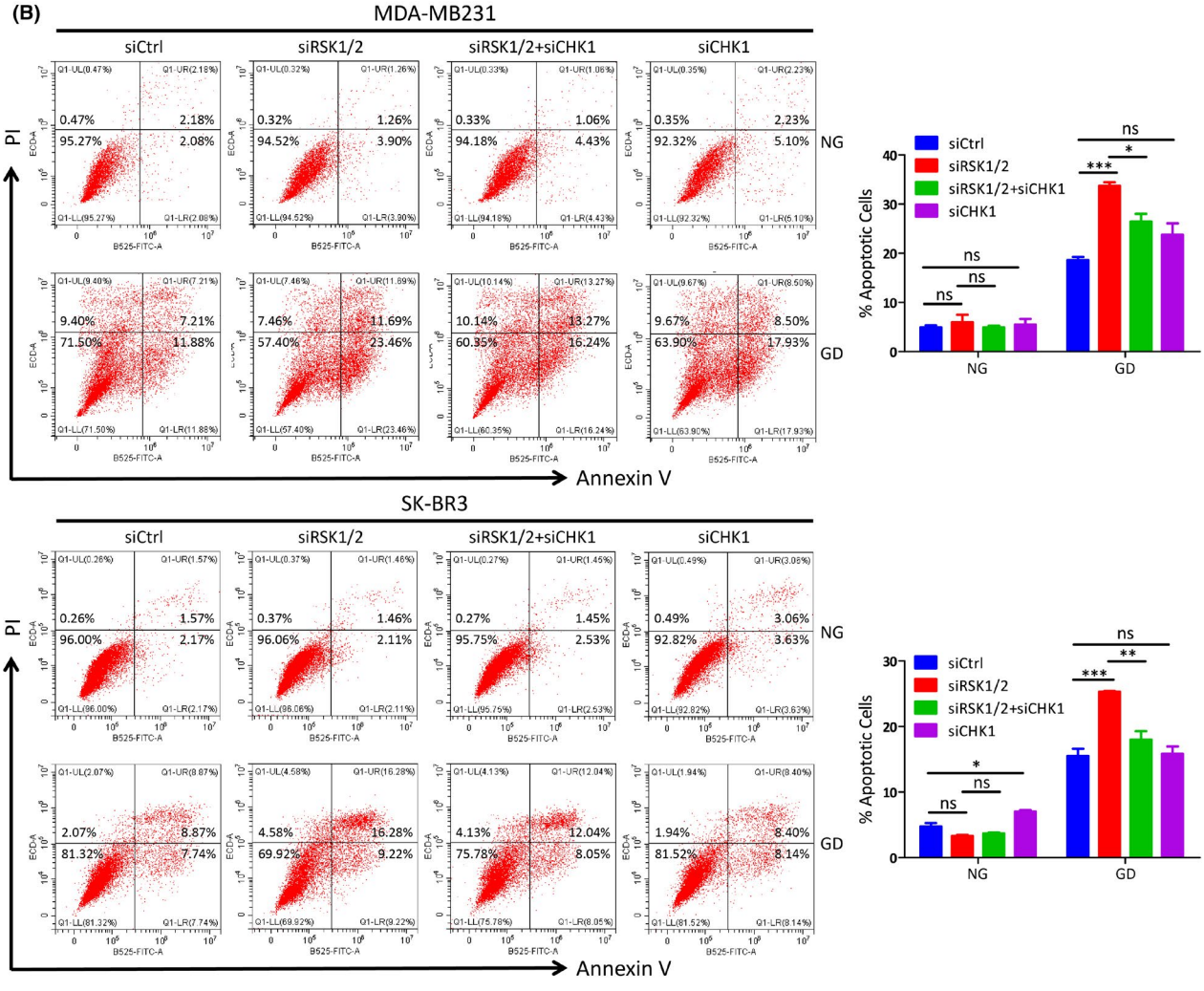
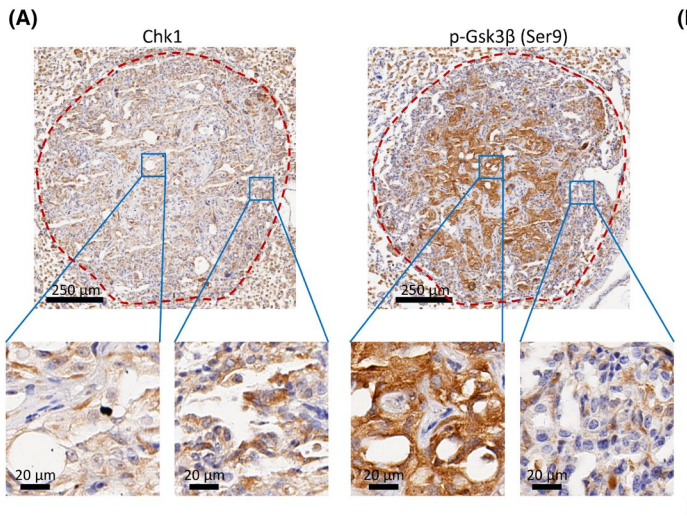

(B)
MDA-MB231
siCtrl
siRSK1/2
siRSK1/2+siCHK1
siCHK1
PI
Annexin V
NG
GD
0.47% 2.18% 95.27% 2.08%
0.32% 1.26% 94.52% 3.90%
0.33% 1.06% 94.18% 4.43%
0.35% 2.23% 92.32% 5.10%
9.40% 7.21% 71.50% 11.88%
7.46% 11.69% 57.40% 23.46%
10.14% 13.27% 60.35% 16.24%
9.67% 8.50% 63.90% 17.93%
% Apoptotic Cells
ns
*
***
SK-BR3
0.26% 1.57% 96.00% 2.17%
0.37% 1.46% 96.06% 2.11%
0.27% 1.45% 95.75% 2.53%
0.49% 3.06% 92.82% 3.63%
2.07% 8.87% 81.32% 7.74%
4.58% 16.28% 69.92% 9.22%
4.13% 12.04% 75.78% 8.05%
1.94% 8.40% 81.52% 8.14%
**

FIGURE 5 P90 RSK inhibits apoptosis by promoting CHK1 degradation upon glucose deprivation. A-B, MDA-MB231 and SK-BR3 cells transfected with indicated siRNA oligos were transferred to glucose-free media for 36 h. Cells were then harvested and subjected to immunoblotting using the indicated antibodies (A) or FACS analysis to determine the apoptotic population (B, left, a representative FACS profile, and right, the percentage of Annexin V^+ cells, mean ± SEM; n = 3; ns, not significant, $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$). The band density was quantified using ImageJ. The cleaved protein levels were normalized to total protein levels treated by normal glucose, and expressed as fold change compared with the corresponding control by setting the control value as 1. GD, glucose deprivation; NG, normal glucose



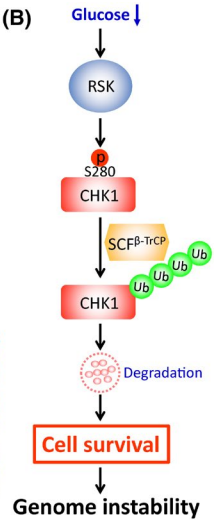


FIGURE 6 Inverse correlation of checkpoint kinase 1 (CHK1) levels and p90 ribosomal S6 kinase (RSK) activity within the tumor mass. A, Lung tumors that spontaneously developed from *LSL-Kras^{G12D};p53$^{fl/fl}$* mice after Cre-recombinase treatment for 9 weeks were isolated and fixed. Tumors indicated by dashed line were then immunohistochemically stained with an anti-Chk1 or anti-phosphorylated Gsk3β (Ser9) Abs. Representative staining images are shown . B, A model for p90 RSK-mediated CHK1 degradation upon glucose deprivation

Although the precise mechanisms underlying p90 RSK activation upon glucose deprivation still require substantial characterization, our finding that activated p90 RSK triggers CHK1 degradation could provide novel insight to explain how p90 RSK promotes the silencing of the G_2/M checkpoint in response to DNA damage, which was recently characterized through inhibition of CHK1 activity.[23]

We recently showed that AMPK phosphorylates CHK1 at Ser280/Ser284 and mediates its degradation upon glucose deprivation.[13] Indeed, glucose deprivation increased the levels of CHK1 phosphorylation at Ser280 in a time-dependent manner in shGFP cells, whereas the increased CHK1 phosphorylation was significantly delayed in MDA-MB231 shAMPK α1/α2 cells, or reduced in H1299 shAMPK α1/α2 cells, indicating that AMPK participates in CHK1 phosphorylation in response to glucose deprivation (Figure S4B). The relationship of p90 RSK and AMPK in regulating CHK1 degradation remains an open question. We first speculated that AMPK and p90 RSK might have opposite effects on CHK1 degradation, due to their opposite effects on cell proliferation: AMPK negatively regulates cell proliferation, whereas p90 RSK positively regulates cell proliferation.[3,27] Moreover, both AMPK and p90 RSK phosphorylate the cyclin-dependent kinase inhibitor p27 on Thr198,[28,29] but with different biological consequences. However, in this study, we found that inhibition of p90 RSK attenuated the reduction of CHK1, prolonged the CHK1 protein half-life, and suppressed CHK1 polyubiquitination (Figure 3), which are similar effects to those observed under AMPK-mediated CHK1 degradation.[13] More importantly, p90 RSK silencing blocked CHK1 degradation and promoted cell apoptosis upon glucose deprivation, which is consistent with the obvious induction of apoptosis induced by overexpression of CHK1 and glucose deprivation.[13] This finding implied that CHK1 degradation is essential for cell survival under glucose starvation. Thus, these findings led us to believe that p90 RSK works in concert with AMPK to ensure CHK1 degradation in response to glucose deprivation. This hypothesis is further supported by: (a) the observed increase in p90 RSK activity, which compensates for the deficit of AMPK activity after long-term glucose deprivation treatment in MDA-MB231 cells (Figure 1, lane 9 vs 5); and (b) further inhibition of CHK1 degradation following the simultaneous knockdown of AMPK and p90 RSK (Figure 3C, lanes 10-12 vs 4-9). Interestingly, a study showed that the ERK-p90 RSK axis negatively regulates AMPK activation by phosphorylating LKB1 to compromise the binding and activation of AMPK in melanoma cells,[30] adding a layer of complexity to the relationship between p90 RSK and AMPK in controlling CHK1 protein levels. Thus, further evidence of the following would help to elucidate the mechanism by which p90 RSK and AMPK cooperate or compete with each other in controlling CHK1 degradation to determine cell survival or death under low-glucose stress.

Another important question is why CHK1 must be degraded under glucose deprivation.[31] As a key transducer in checkpoint signaling pathways, CHK1 also regulates DNA repair and apoptosis, suggesting that the role of CHK1 extends beyond regulating the cell cycle.[32] Checkpoint kinase 1 has dual effects on apoptosis and cell survival: CHK1 not only monitors DNA replication and maintains genomic integrity during unperturbed cell cycle progression, but also determines whether cells survive or undergo apoptosis by regulating caspase-2/3 and p53 under stress conditions.[32,33] Under DNA damage stress, activated CHK1 triggers cell cycle checkpoints and induces cell cycle arrest. Thus, CHK1 degradation could promote checkpoint termination and the recovery of cell cycle progression after the completion of DNA repair.

However, under low-glucose stress, given that cell cycle progression continues, the degradation of CHK1 will give rise to genomic instability, which impairs the proper cell cycle checkpoint and the DNA damage response and repair in cells with naturally occurring replication errors, leading to the accumulation of mutations in the genome. Once abnormal cells achieve a selective growth advantage, they drive the onset and progression of tumors. The enhancement of mutagenesis by CHK1 degradation would also lead to tumor heterogeneity and the selection of cells that are resistant to the harsh tumor microenvironment. Tumor heterogeneity is also related to the complicated tumor microenvironment, which is much different from that in normal tissues and frequently includes regional metabolic stresses, such as poor oxygenation, energy deprivation, and high lactate levels.[34] Accordingly, we identified lower CHK1 levels and higher p90 RSK activity in the center of the solid tumor, where low glucose often occurs because of insufficient vascularization compared to that at the margin of the solid tumor (Figures 6A and S6). The correlation among the protein levels of CHK1, the tumor microenvironment, especially glucose concentrations, and the degree of accumulating mutations in the genome defined by single-cell sequencing in human solid tumors is a subject for future investigations, which will ultimately benefit the development of a therapeutic strategy that targets CHK1 for degradation in the center of solid tumors for cancer treatment.

In summary, our study supports the following working model. Glucose deprivation activates p90 RSK, and then activated p90 RSK phosphorylates CHK1 at Ser280 within the β-TrCP degron motif and triggers $SCF^{\beta\text{-}TrCP}$-dependent CHK1 polyubiquitination and degradation. Checkpoint kinase 1 degradation promotes cell survival and might give rise to genomic instability, leading to tumorigenesis (Figure 6B). Our study reveals a novel mechanism by which cancer cells strive to survive under a limited energy supply, as well as another important upstream mechanism of CHK1 degradation.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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