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Jab1 interacts with brain-specific kinase 2 (BRSK2) and promotes its degradation in the ubiquitin–proteasome pathway

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ABSTRACT

Brain-specific kinase 2 (BRSK2) was classified as an AMP-activated protein kinase (AMPK)-related kinase and one of the substrates of LKB1. Studies on homologs of BRSK2 in mice, SADA and SADB, implied that it might be involved in the regulation of cell polarity and cell cycle. However, physiological functions and molecular regulatory mechanisms of BRSK2 are incompletely understood. In this study, we isolated a novel BRSK2-interacting protein, c-Jun activation domain-binding protein-1 (Jab1), which was reported to mediate degradation of multiple proteins and positively regulate cell cycle progression. GST pull-down and immunoprecipitation assays revealed the direct interaction between BRSK2 and Jab1 *in vitro* and *in vivo*, respectively. The co-localization between Jab1 and BRSK2 in the perinuclear region was observed. Intriguingly, Jab1 promoted the ubiquitination and proteasome-dependent degradation of BRSK2. Silencing of endogenous Jab1 increased the cellular BRSK2 protein level. Consistent with this, BRSK2-mediated cell cycle arrest at the G2/M phase in mammalian cells was reversed by exogenous Jab1. Taken together, our findings provide a novel regulatory mechanism of BRSK2 through direct interaction with Jab1.

1. Introduction

Brain-specific kinase 2 (BRSK2), one member of AMP-activated protein kinase (AMPK) subfamily [1], is named based on its high expression in mammalian forebrain. Previously the closet homologs of BRSK2, *Caenorhabditis elegans* SAD1 and mice SADA/SADB, have been well identified. Studies on SADA and SADB suggested that they were essential to promoting neuronal polarity and regulating neurotransmitter release [2–4]. Recent evidence implies the crucial role of SADB in the regulation of cell cycle progression. SADB governed the centrosome duplication through phosphorylation of γ-tubulin on serine 131 [5]. Either increased or reduced SADB levels impaired S phase entry. Functional investigation in polarized neurons revealed that SADA and SADB phosphorylated Wee1 and initiated its downregulation [6]. Wee1 is a well-characterized cell cycle checkpoint kinase that regulates the entry into mitosis in dividing cells. In addition, human SAD1 (BRSK1) was found to phosphorylate several cell cycle-related proteins, including Wee1A, Cdc25C, and Cdc25B. Overexpression of BRSK1 induced G2/M arrest in HeLa S2 cells [7]. All these studies indicate the possible involvement of SAD kinases in the regulation of cell cycle. However, the physiological functions of BRSK2 in human have not been investigated. Meanwhile, it is worth noting that kinase activity of SADB was found to fluctuate during cell cycle, suggesting that the kinase activity of SADB is under precise temporal control by upstream regulators.

LKB1 functions as a master upstream kinase which phosphorylates and activates BRSK2 [8]. However, other regulatory mechanisms of BRSK2 are not well understood. Studies in human genome revealed that BRSK2 contains an ubiquitin-associated (UBA) domain. Abdallah et al. (2008) demonstrated that atypical Lys^{29}/Lys^{33}-linked polyubiquitin chains polyubiquitinated AMPKs including BRSK2 *in vivo* [9]. Recently, two polyubiquitinated AMPKs in cells, NUAK1 (AMPK-related kinase 5) and MARK4 (microtubule-affinity-regulating kinase 4), were reported to be deubiquitinated through interaction with the deubiquitinating enzyme USP9X (ubiquitin specific protease-9) [9]. However, the cellular mechanism underlying the regulation of BRSK2 polyubiquitination remains largely under-investigated.

In this study, we isolated c-Jun activation domain-binding protein-1 (Jab1) as a novel BRSK2-interacting protein by yeast two-hybrid screening. Jab1 as a transcriptional co-activator of c-Jun, is also defined as CSN5 based on its involvement in the constitutive photomorphogenic-9 (COP9) signalosome (CSN) complex as the fifth component [10,11]. Previous reports showed that Jab1 interacted with and subsequently degraded a large number of proteins such as the cyclin-dependent kinase inhibitor $p27^{kip1}$(p27), p53, the capsid of West Nile virus and Smad4, resulting in cell proliferation, apoptosis and tumorigenesis, respectively [12–15]. Among them, p27 plays a pivotal role in controlling cell cycle progression

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via inhibiting cyclin E-Cdk2. In view of these evidence, Jab1 functions as a positive regulator of cell cycle progression.

Here, we demonstrated that Jab1 directly interacted with BRSK2 and promoted its degradation in ubiquitin–proteasome pathway. BRSK2-induced cell cycle arrest at G2/M phase was reversed by exogenous Jab1 in mammalian cells.

2. Materials and methods

2.1. Cell culture and transfection

PANC-1, HEK293T, HeLa, HepG2 and U2OS cells were obtained from the American Type Culture Collection. Cells were maintained in DMEM with 10% FBS at 37 °C in an atmosphere of 5% CO_2. Cells were transiently transfected using Lipofectamine (Invitrogen, USA) according to manufacturer's instructions.

2.2. Plasmid construction

The full-length *BRSK2* cDNA was cloned into the SfiI and XhoI sites of pCMV-Myc and pCMV-HA vectors. Jab1 vector was generated in a similar manner except the EcoRI and NotI sites for cloning into pCMV-Myc and pGEX-4T-2 vector.

2.3. Western blot

Cell lysates and immunoprecipitates were subjected to SDS–PAGE and proteins were transferred to nitrocellulose membranes (GE Healthcare). The membrane was blocked in TBS containing 5% non-fat milk and 0.1% Tween-20, washed twice in TBS, and incubated with primary antibody at room temperature for 2 h, followed by incubation with secondary antibody at room temperature for 1 h. Afterward, the proteins of interest were visualized using ECL chemiluminescence system (Santa Cruz).

2.4. RNA interference

The RNAi oligos were purchased from GenePharma (Shanghai, China). The RNAi oligo sequences for Jab1 are: Jab1-siRNA1: 5′-GCUCAGAGUAUCGAUGAAATT-3′, Jab1-siRNA2: 5′-CAUGCAGGAAG CUCAGAGUTT-3′. The sequence of negative control is: control siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3′.

2.5. GST pull-down assay

HEK293T cells were lysed 36 h after transfection with cell lysis buffer for 30 min at 4 °C. GST fusion proteins were immobilized on Glutathione–Sepharose beads (Amersham Biosciences). After washing with pull-down buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 10% glycerol, 1 mM EDTA, 2.5 mM $MgCl_2$ and 1 μg/ml leupeptin), the beads were incubated with lysates of transfected 293T cells for 4 h. The beads were then washed five times with binding buffer and resuspended in sample buffer. The bound proteins were subjected to SDS–PAGE.

2.6. Immunoprecipitation assay

Cells were lysed with cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM EGTA, 1 mM Na_2EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin) and the lysate was centrifuged (12,000*g* for 20 min). The supernatant was precleared with protein A/G beads (Sigma, USA), followed by incubation with 2 μl antibody for 2 h and thereafter with protein A/G beads for 2 h, all at 4 °C. Pellets were washed five times with lysis buffer and resuspended in sample buffer and analyzed by SDS–PAGE.

2.7. Immunofluorescence assay

PANC-1 cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100 for 5 min at room temperature. The coverslips were blocked with 10% normal horse serum plus 1% BSA for 1 h. Cells were stained with rabbit anti-BRSK2 antibody (1:800 dilution) or mouse anti-Jab1 antibody (1:800 dilution) and then with Alexa 488 (green fluorescence)-conjugated goat anti-rabbit secondary antibody or Alexa 546 (red fluorescence)-conjugated goat anti-mouse secondary antibody for 1 h, respectively. The nuclei were stained with DAPI (1:1000 dilution) for 10 min. Confocal images were captured using a Zeiss LSM 710 microscope.

2.8. Cell cycle analysis

HepG2 or U2OS cells were transfected with Myc-BRSK2 and Myc-Jab1 either alone or in combination and pCMV-Myc vector was used as a control. At 48 h after transfection, the cells were harvested. Then the cells were stained with 0.1% Triton X-100 containing propidium iodide and RNase for 15 min at room temperature. Fluorescence from the propidium iodide–DNA complex was measured by flow cytometer, and the percentage of cells in the G2/M phase was determined using Modifit software.

2.9. Statistical analysis

The data in this study were expressed as mean ± SD from three independent experiments.

3. Results

3.1. BRSK2 interacts with Jab1 in vitro and in vivo

Previous research implied the existence of polyubiquitinated BRSK2 in human cells. To identify the key components responsible for BRSK2 polyubiquitination, we performed a yeast two-hybrid screening to isolate BRSK2-interacting proteins using the full-length human BRSK2 protein as bait. A human cDNA library from fetal brain was cloned into prey vector and used in this screening assay. Among the 11 isolated proteins, we identified Jab1, which has been reported to direct the degradation of multiple proteins in ubiquitin–proteasome pathway. We investigated the expression patterns of BRSK2 and Jab1 in five different cell lines by Western blot. Jab1 was detected in all of the cell lines, while BRSK2 was only expressed in PANC-1, HEK293T and HeLa cells (Fig. 1A). In order to confirm the direct interaction between BRSK2 and Jab1, we performed *in vitro* GST-pull down assay. The lysates from HEK293T cells expressing HA–BRSK2 and GST–Jab1 fusion protein were analyzed for the binding between BRSK2 and Jab1. The direct binding of Jab1 with BRSK2 was observed (Fig. 1B). Moreover, co-immunoprecipitation assay was employed to assess the association of BRSK2 with Jab1 *in vivo*. When immunoprecipitated with anti-Myc antibody, HA-BRSK2 was detected in the co-immunoprecipitated complex from cells cotransfected with HA-BRSK2 (Fig. 1C). In addition, we verified the interaction between endogenous BRSK2 and Jab1 in mammalian cells. As shown in Fig. 1D, BRSK2 co-immunoprecipitated with Jab1, but not control IgG, from HEK293T cells. Taken together, the results indicate that BRSK2 directly interacts with Jab1 *in vitro* and *in vivo*.

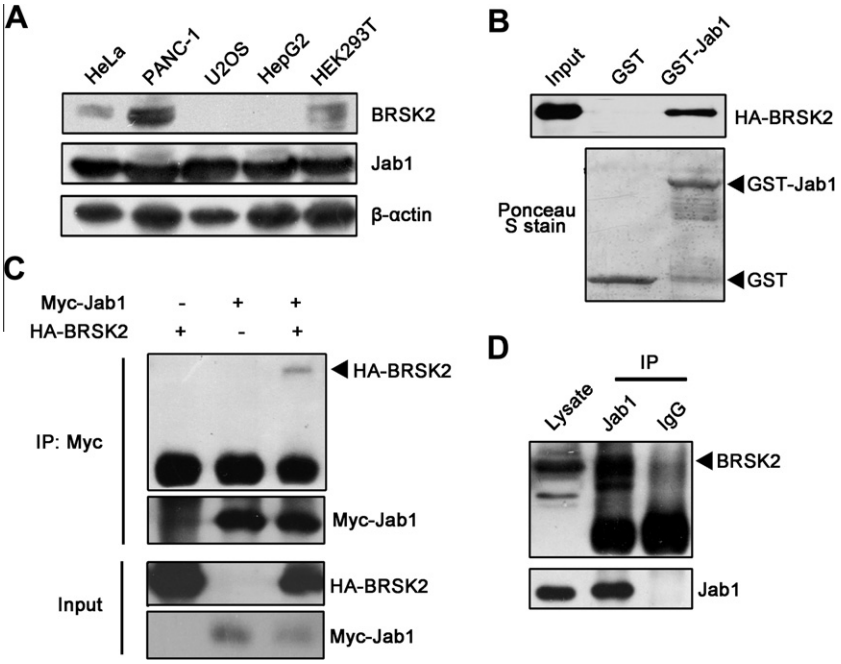

Fig. 1. BRSK2 interacts with Jab1 *in vitro* and *in vivo*. (A) Western blot analysis of BRSK2 and Jab1 expression in PANC-1, HeLa, U2OS, HepG2 and HEK293T whole cell lysates. (B) BRSK2 interacts with Jab1 *in vitro*. HEK293T cells were transfected with HA-BRSK2. After 36 h, cells were lysed and incubated with purified GST–Jab1 fusion protein conjugated with Glutathione–Sepharose beads for 2 h at 4 °C. GST alone was used as a negative control. Bottom panel: Ponceau S staining of the nitrocellulose membrane was used to visualize GST and GST–Jab1 fusion proteins. (C) BRSK2 interacts with Jab1 *in vivo*. HEK293T cells were co-transfected with HA-BRSK2 and Myc-Jab1 constructs. Cell lysates were prepared and subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were analyzed by Western blot. (D) The interaction between endogenous BRSK2 and Jab1. Endogenous BRSK2 was immunoprecipitated from HEK293T cells using anti-Jab1 antibody. An equal amount of non-specific mouse IgG was used as a control. The immunoprecipitates were resolved on SDS–PAGE followed by Western blot.

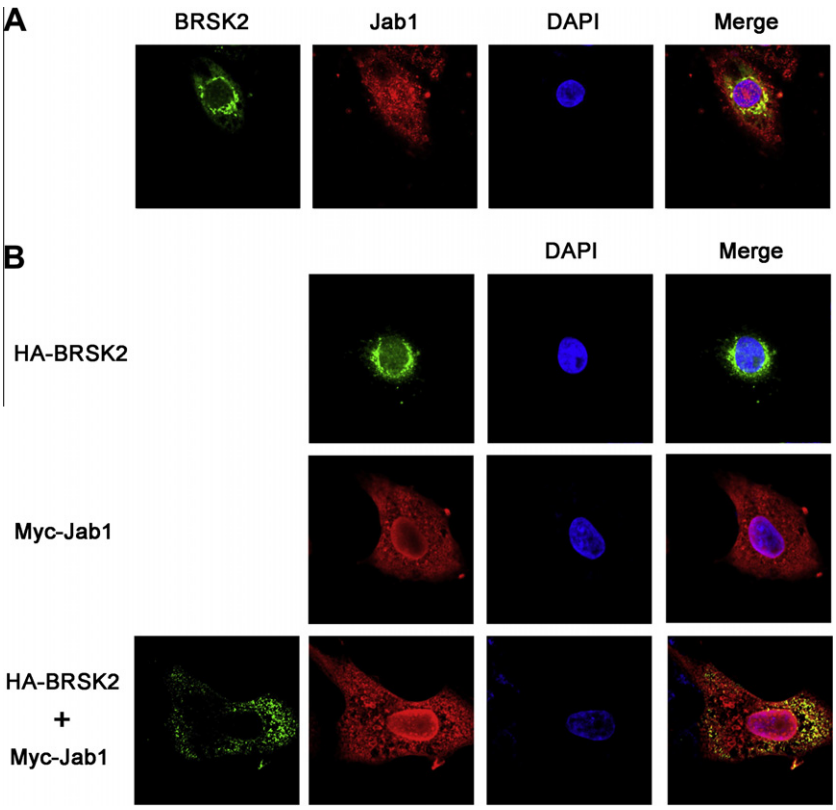

Fig. 2. BRSK2 is co-localized with Jab1. (A) The co-localization of endogenous BRSK2 and Jab1. PANC-1 cells were cultured on coverslips. After 36 h, cells were fixed as described in Section 2. (B) The co-localization of exogenous BRSK2 and Jab1. PANC-1 cells were transfected with HA-BRSK2 and Myc-Jab1 constructs either alone or in combination. After 36 h, BRSK2 or Jab1 were stained with rabbit-anti-HA or mouse-anti-Myc antibodies, respectively. The secondary antibodies were described in Section 2.

3.2. BRSK2 is co-localized with Jab1

The significant interaction between BRSK2 and Jab1 led us to consider their intracellular co-localization. Therefore, we performed immunofluorescence experiments of endogenous BRSK2 and Jab1 in PANC-1 cells. Jab1 was localized in both nucleus and cytosol, while BRSK2 was mainly found at the nuclear periphery. Their co-localization was observed in the perinuclear region (Fig. 2A). Furthermore, we expressed exogenous HA-BRSK2 and Myc-Jab1 in PANC-1 cells and monitored their co-localization. Immunofluorescence analysis showed that HA-BRSK2 or Myc-Jab1 transfected alone exhibited the same localization as endogenous protein. However, co-expression of Myc-Jab1 decreased HA-BRSK2 immunofluorescent signal and they co-localized in cytoplasm (Fig. 2B). These results provided further evidence to support the interaction between BRSK2 and Jab1 *in vivo*.

3.3. Jab1 promotes the degradation of BRSK2 via ubiquitin–proteasome pathway

In an attempt to test whether Jab1 mediates the degradation of BRSK2, we expressed the increasing doses of Jab1 in PANC-1 cells and examined endogenous BRSK2 protein level. As shown in Fig. 3A, BRSK2 protein level was significantly reduced in a dose-dependent manner when transfected with Myc-Jab1. In addition, knockdown of endogenous Jab1 by two specific siRNA resulted in higher BRSK2 protein level compared with the control in PANC-1 and HEK293T cells (Fig. 3B). Importantly, treatment with proteasome inhibitor MG132 rescued BRSK2 reduction mediated by Jab1 (Fig. 3C), suggesting that Jab1 induced BRSK2 degradation through proteosome pathway. To evaluate whether Jab1 regulated BRSK2 abundance at post-translational level, we determined the effects of Jab1 on BRSK2 protein turnover. The relative stability of the BRSK2 protein was examined at the indicated interval after treatment with cycloheximide (CHX) to block protein synthesis. Overexpression of Jab1 accelerated the protein degradation of BRSK2 significantly (Fig. 3D). Moreover, marked stabilization of endogenous BRSK2 protein was observed with siRNA against Jab1, compared with the control siRNA (Fig. 3E). A previous study showed that Jab1 regulated the degradation of ET_AR and ET_BR by promoting ubiquitination of these receptors [16]. Thus, we further investigated whether Jab1 promoted the ubiquitination of BRSK2. Ubiquitination assay revealed that overexpression of Jab1 enhanced the polyubiquitination of BRSK2 (Fig. 3F). The results above demonstrate that Jab1 promotes the degradation of BRSK2 *via* ubiquitin–proteasome pathway.

3.4. BRSK2-mediated cell cycle arrest at G2/M phase is reversed by Jab1 in mammalian cells

Previous findings implied the involvement of SAD kinases in regulating cell cycle progression. To explore the role of BRSK2 in the regulation of cell cycle, we introduced BRSK2 into HepG2 and U2OS cells that are lack of endogenous BRSK2. When transfected with DNA construct expressing BRSK2, cell cycle arrest was induced at the G2/M phase in both cell lines. The population rate of HepG2 cells transfected with mock vector was 18% at G2/M phase, and it increased to 30% when transfected with BRSK2. The co-transfection of Jab1 with BRSK2 reduced cell cycle arrest at G2/M phase from 30% to 19% (Fig. 4A and B). The similar results

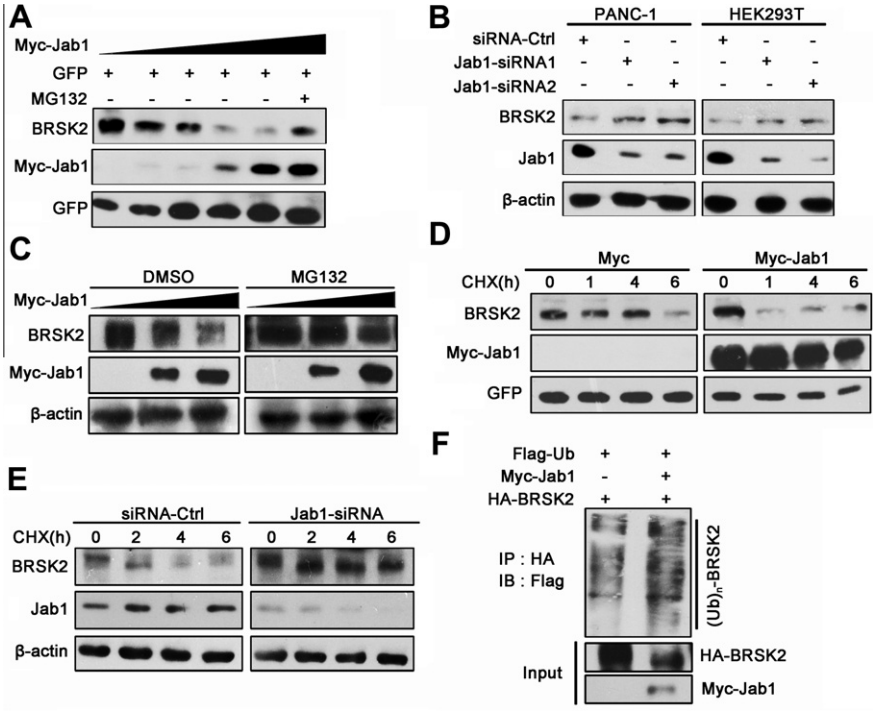

Fig. 3. Jab1 decreases the stability of BRSK2 *via* the proteosome pathway. (A) Transfection of Jab1 reduces endogenous BRSK2 protein level. PANC-1 cells were transfected with increasing amounts of Jab1. After 36 h, cells were lysed and protein levels were analysis by Western blot. (B) Knockdown of endogenous Jab1 results in higher BRSK2 protein level. PANC-1 cells were transfected with control or Jab1 siRNA oligos. After 36 h, the protein levels of endogenous Jab1 and BRSK2 were determined by Western blot. Similar knockdown experiment was performed in HEK293T cells. (C) Jab1-mediated BRSK2 degradation is proteasome-dependent. Increasing amounts of Jab1 were transfected into PANC-1 cells. Cells were treated with MG132 (20 μM) or DMSO for 6 h, and the cell lysates were determined by Western blot. (D) Jab1 accelerates BRSK2 protein turnover. PANC-1 cells transfected with Myc-Jab1 or pCMV-Myc vector were treated with 50 μM CHX and cells were lysed at the indicated times. Stability of endogenous BRSK2 was determined by Western blot. (E) Knockdown of endogenous Jab1 decelerates BRSK2 protein turnover. PANC-1 cells were transfected with control or Jab1 siRNA oligos. Cells were treated with 50 μM CHX for indicated times. The protein level of BRSK2 and Jab1 was measured by Western blot. (F) Jab1 promotes the ubiquitination of BRSK2. HA-BRSK2, FLAG-Ubiquitin and Myc-Jab1, or pCMV-Myc vectors were co-transfected into HEK293T cells. BRSK2 proteins were immunoprecipitated by HA antibody. The immunoprecipitates were resolved by SDS–PAGE. The polyubiquitinated forms of BRSK2 were detected by Western blot with anti-FLAG antibody.

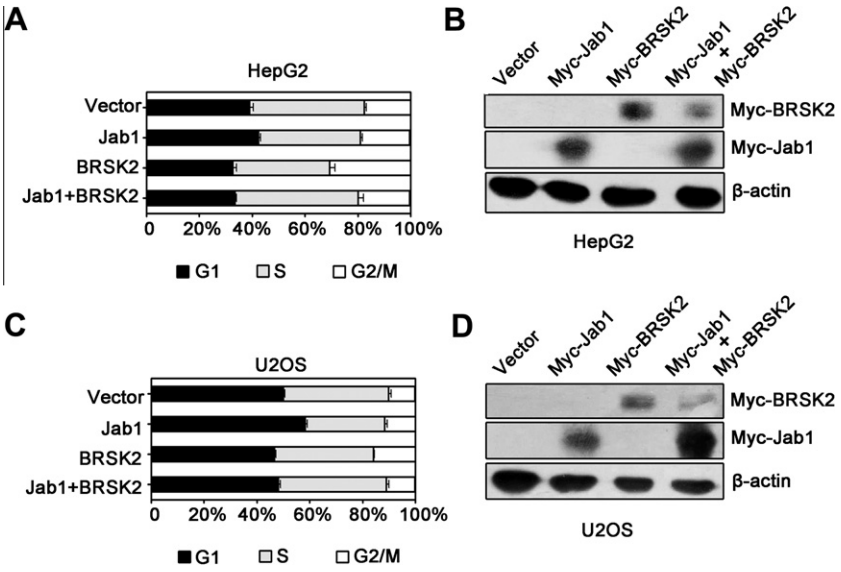

Fig. 4. BRSK2-induced cell cycle arrest at G2/M phase is reversed by Jab1. (A and C) BRSK2 induces cell cycle arrest at G2/M phase. HepG2 or U2OS cells were transfected with Myc-BRSK2 and Myc-Jab1 constructs either alone or in combination. After 48 h, the cells were harvested and percentages of cells in individual cell cycle phases were determined by flow cytometry (mean ± SD, $n = 3$). pCMV-Myc vector was used as a control. (B and D) Cells described in panel A and C are analyzed by Western blot.

were observed in U2OS cells (Fig. 4C and D). Transfection of Jab1 alone was used as a negative control which didn't exert significant influence on G2/M phase. The results suggest that Jab1 is able to reverse BRSK2-mediated G2/M phase arrest in mammalian cells.

4. Discussion

BRSK2 shares high sequence homology with two mice SAD kinases. They play a pivotal role in the regulation of neuronal polarization and cell cycle progression. Knockout of both SAD kinases in mice exhibited defects in neuronal polarity and the double mutant died within 2 h of birth [3]. In addition, SADB functions as a key regulator of centrosome duplication [5]. Changes in SADB activity were associated with centrosome duplication defects and reduction of S phase progression. These evidence imply that the protein levels and kinase activities of SAD kinases are tightly regulated in mammalian cells.

Up to now, the regulation of BRSK2 activity through LKB1 phosphorylation has been well demonstrated [8,17], but other regulatory mechanisms of BRSK2 are largely unknown. A recent study determined that BRSK2 and other AMPK family members were polyubiquitinated in cells. However, the molecular mechanisms underlying the polyubiquitination of BRSK2 are under investigation. In our study, Jab1 was identified as a novel binding partner for BRSK2 (Fig. 1). We provided the evidence that Jab1 promoted the ubiquitination and degradation of BRSK2. Silencing of Jab1 increased BRSK2 protein level in both HEK293T and PANC-1 cells (Fig. 3). These data support a novel regulatory mechanism for BRSK2 through its interaction with Jab1. AMPK has been reported to function as a crucial energy sensor and maintain cellular energy homeostasis [18]. Thus, investigation on whether the cellular energy metabolism is involved in the degradation process of BRSK2 will help us to understand the regulatory mechanisms of BRSK2 stability in cells.

BRSK1 has been demonstrated to phosphorylate Wee1A and CDC25B/C, resulting in G2 phase arrest in HeLa cells [7]. Our data showed that overexpression of BRSK2 induced cell cycle arrest at G2/M phase in HepG2 and U2OS cells. Moreover, exogenous Jab1 reversed BRSK2-mediated G2/M arrest (Fig. 4), indicating that BRSK2 is involved in the regulation of cell cycle in mammalian cells. Jab1 has been identified as a positive regulator of cell cycle progression through promoting p27 degradation [12]. Based on our results, Jab1 regulates cell cycle *via* a novel mechanism that involves the degradation of BRSK2.

Recent studies have indicated that both the protein stability and kinase activity of BRSK2 were reduced in the Lkb1-deficient pancreas, resulting in defective acinar cell polarity and subsequently leading to the development of serious cystadenoma [19]. Nevertheless, the role of BRSK2 in cancers occurrence and development has not been well understood. Jab1 has been found to mediate the degradation of multiple proteins which are important regulators of disease progression in diverse cancers, including p27, p53 and Smad4/7. High expression of Jab1 was detected in a number of human malignant cancers, such as pancreatic adenocarcinoma, breast carcinoma and hepatocellular carcinoma [20–22]. Therefore, it will be meaningful to find out whether Jab1-mediated BRSK2 degradation is associated with oncogenic processes.

In conclusion, we present novel findings showing that BRSK2 directly interacts with Jab1 *in vitro* and *in vivo*. Moreover, Jab1 promotes polyubiquitination and degradation of BRSK2. Consistent with this, BRSK2-mediated cell cycle arrest at the G2/M phase in mammalian cells is reversed by exogenous Jab1.

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