Priority Report

Cancer Research

Unconventional Role of the Inwardly Rectifying Potassium Channel Kir2.2 as a Constitutive Activator of RelA in Cancer

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Abstract

The constitutive activation of NF-κB is a major event leading to the initiation, development, and progression of cancer. Recently, we showed that the size of preestablished tumors was reduced after the depletion of Kir2.2, an inwardly rectifying potassium channel. To determine the precise mechanism of action of Kir2.2 in the control of tumor growth, we searched for interacting proteins. Notably, NF-κB p65/RelA was identified as a binding partner of Kir2.2 in a yeast two-hybrid analysis. Further analyses revealed that Kir2.2 directly interacted with RelA *in vitro* and coimmunoprecipitated with RelA from cell lysates. Kir2.2 increased RelA phosphorylation at S536 and facilitated its translocation from the cytoplasm to the nucleus, thereby activating the transcription factor and increasing the expression level of NF-κB targets, including cyclin D1, matrix metalloproteinase (MMP)9, and VEGF. Kir2.2 was overexpressed in human cancer and the expression level was correlated with increased colony formation and tumor growth in mouse tumor models. On the basis of these findings, we propose an unconventional role for Kir2.2 as a constitutive RelA-activating protein, which is likely to contribute to tumor progression *in vivo*. *Cancer Res; 73(3); 1056–62. ©2012 AACR.*

Introduction

Kir2.2, localized on chromosome 17p11.1 (1), encodes a 48-kDa protein with sequence homology to the inwardly rectifying potassium channel 2 family. Pharmacologic evidence has shown that K^+ channels play important regulatory roles in cell-cycle progression (2, 3), and the overexpression of ion channels has been reported in multiple cancers (4–7). The role of K^+ channels in these events has been explained by the indirect effect of K^+ channels on the intracellular calcium concentration (8) or cell volume control (9). K^+ channels may serve as parts of larger multiprotein complexes that include components of the cytoskeleton, transport proteins, kinases, phosphatases, and extracellular matrix proteins (10). Therefore, K^+ channels interact with other intracellular proteins, and these protein interactions can lead to the translocation of the proteins to specific subcellular compartments and the recruitment of signaling molecules.

Recently, we showed that the depletion of Kir2.2 could trigger cell-cycle arrest in multiple cancer cell types and reduce the size of preestablished tumors *in vivo* (3). Although the role of Kir2.2 as a channel protein has been intensively studied in normal cells, the role of Kir2.2 in malignant cancer cells remains unknown.

This study was designed to determine the mechanism through which Kir2.2 regulates malignant cell proliferation. Previously, we showed that regardless of the K^+ transport activity, inhibition of the channel function did not affect the growth of malignant cells (3), suggesting that the growth modulatory function of Kir2.2 may rely on a protein–protein interaction. Molecular characterizations of the interaction of Kir2.2 suggest that it controls the growth of cancer cells by regulating RelA-activated NF-κB signaling. RelA is constitutively active in many cancers, in which it plays a critical role in the transcriptional activation of oncogenic genes. In this study, we show for the first time that Kir2.2 controls the growth of cancer cells by increasing cyclin D1 via the constitutive activation of RelA.

Materials and Methods

Cell culture and reagents

PC-3, DU145, HEK293, immortalized (RWPE-1), and transformed (RWPE-2) prostate epithelial cells (PrEC) were purchased from American Type Culture Collection. Normal human PrEC were obtained from Lonza. Kir2.2 siRNAs were purchased from Dharmacon (siGENOME, M-006242), unless otherwise indicated.

Yeast two-hybrid analysis

Yeast strain PBN204 (Panbionet) was cotransformed with two-hybrid plasmids, a bait plasmid pBCT-Kir2.2 that encodes a GAL4 DNA BD-fused Kir2.2 cDNA and pACT2 plasmid that encodes the human HeLa cell cDNA fused to GAL4 AD. Three

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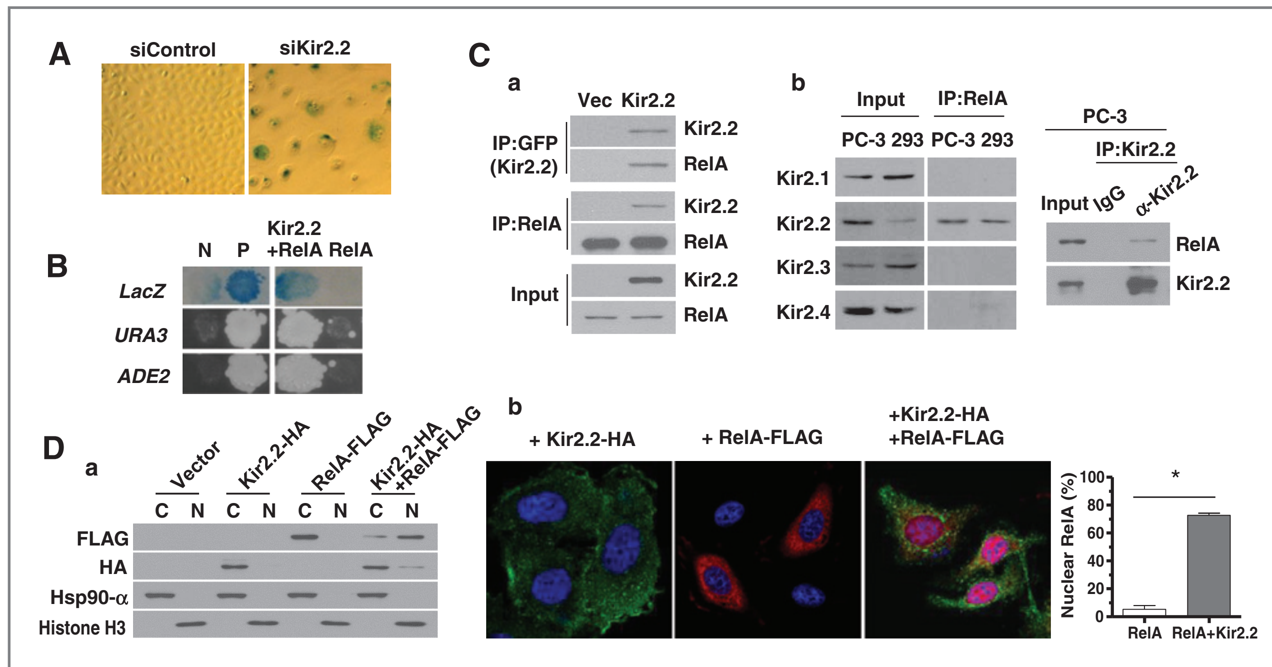

Figure 1. Kir2.2 physically interacts with RelA. A, Kir2.2 knockdown attenuated the growth of PC-3 cells. PC-3 cells transfected with siC or siKir2.2 (20 nmol/L) were stained for SA-β-Gal activity 5 days after transfection. B, RelA was identified as a Kir2.2-interacting molecule using yeast two-hybrid screening. Yeast was cotransformed with GAL4-BD–fused pBCT-Kir2.2 and GAL4-AD–fused pACT2-RelA. Upon interactions of the 2 proteins, cotransformants expressed lacZ, URA3, and Ade2. N, negative control; P, positive control. C, coimmunoprecipitation of RelA with Kir2.2. a, total cell lysates from Kir2.2-transfected HEK293 cells were immunoprecipitated (IP) with anti-GFP or RelA antibody, and the interactions between RelA and Kir2.2-GFP were assessed with immunoblot (IB) analysis. b, endogenous interaction of Kir2.2 with RelA. Total cell lysates from HEK293 and PC-3 cells were immunoprecipitated with an anti-RelA antibody or an anti-Kir2.2 antibody, and the levels of various proteins were determined by immunoblotting using the indicated antibodies. D, Kir2.2 enhances nuclear localization of RelA. PC-3 cells were transfected with indicated plasmids. a, cytoplasmic (C) and nuclear (N) fractions were prepared, and equal amounts of proteins from each fraction subjected to Western blot analysis using the indicated antibodies. b, cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI; blue) and proteins localization examined with fluorescence microscopy. Merged image of RelA (red) and Kir2.2 (green) clearly depicts increased nuclear location of RelA in the presence of Kir2.2. The percentages of cells containing nuclear RelA are shown on the right. IgG, immunoglobulin G.

different reporter genes, *URA3*, *ADE2*, and *lacZ*, each of which was under the control of different GAL4-binding sites, were used to minimize false positives. The transformants were spread on selective media lacking leucine, tryptophan, and uracil and containing 2% glucose (SD-LWU), where the transformants can grow when BD-Kir2.2 interacts with AD-prey proteins. Colonies showed a blue color in X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which was confirmed by filter assay to allow for the detection of β-galactosidase expression. pBCT-polypyrimidine tract-binding protein (PTB) and pACT2-PTB served as the positive control for the protein–protein interaction. pBCT (Panbionet) and pACT2 (Clontech) were used as the negative control.

Expression plasmids, transfection, antibodies, and reagents

cDNAs from Open Biosystems and Origene were further cloned into pCMVTag4C plasmid (Clonetech) or pCMVTag2B expression vectors. Kir2.2 and RelA expression constructs were verified by sequencing. Cells were transfected with siRNA or plasmids using Effectene (Qiagen). To establish stable cell lines overexpressing Kir2.2 (C2, C13, and C18), PC-3 cells were transfected with a vector containing hemagglutinin (HA)-tagged Kir2.2. Successfully transfected cells were selected with G418 (500 μg/mL), and overexpression of Kir2.2 was confirmed by Western blotting using an anti-HA antibody. The expression level of Kir2.2 was in the order C18 > C2 > C13.

Antibodies to RelA, RelB, c-Rel, NF-κBp50, NF-κBp52, IκBα, cyclin D1, GFP, HA, Myc, matrix metalloproteinase (MMP)9, VEGF, proliferating cell nuclear antigen (PCNA), Hsp90, and Lamin B were from Santa Cruz. Antibodies to β-actin, Flag, and Kir2.2 (Sigma), and to the phosphorylated forms of RelA (Cell Signaling) were also used. Horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz. Reactive proteins were visualized using Amersham ECL kit.

Cell growth assessment and colony formation assay

To assess cell numbers, an equal volume of 0.4% (w/v) Trypan blue (Sigma) was added to each cell suspension, and viability was determined on the basis of the ability of live cells to exclude the vital dye. Viable cells were counted using a hemocytometer. To measure colony formation, exponentially growing cells were seeded in triplicate in 60-mm diameter dishes at 2×10^2 cells per dish. After incubation at 37°C for 2 weeks, cells were stained with 0.005% (w/v) crystal violet.

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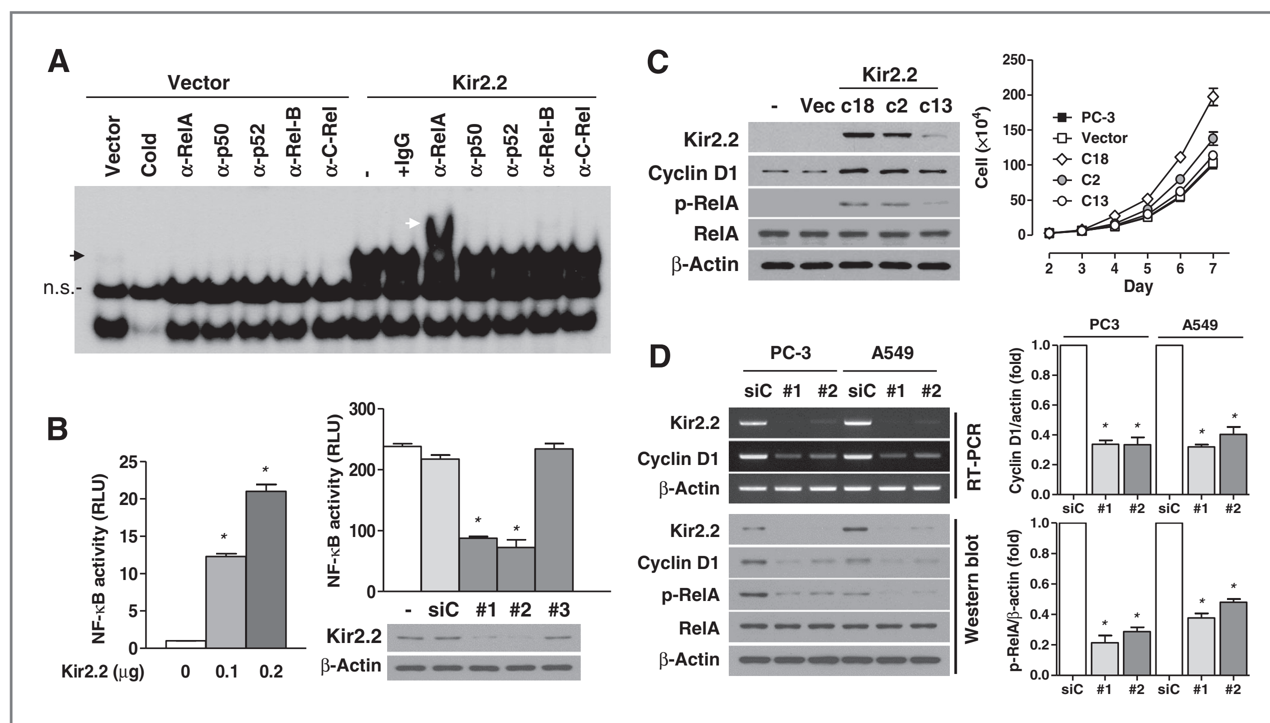

Figure 2. Kir2.2 enhances NF-κB activity and cell proliferation. A, DNA binding of NF-κB was determined with EMSA using nuclear extracts prepared from PC-3 cancer cells transfected with empty vector or Kir2.2-containing vector. Supershifting with the indicated antibody was conducted to verify the identity of the RelA complex band. n.s., nonspecific. B, Kir2.2 modulates NF-κB activity. NF-κB luciferase assay using PC-3 cells transiently cotransfected with pGL3-NF-κB-Luc, together with empty vector or the indicated amounts of Kir2.2 or siRNA. The siRNAs used were #1, 5′-AAG AAU GGC CAG UGC AAC AUU-3′; #2, 5′-UAC UCG CAC UUC CAC AAG AUU-3′; and #3, 5′-GUG CGA AGG AUC UGG UAG AUU-3′. RLU, relative luciferase unit (*, $P < 0.05$, vs. control). C, effects of Kir2.2 expression on RelA phosphorylation, cyclin D1 expression (left), and cell growth (right). D, Kir2.2 is required for the maintenance of endogenous cyclin D1 mRNA and protein expression. PC-3 and A549 cells were transfected with a nonspecific control or Kir2.2-specific siRNAs. Right, bands on the Western blot analyses were quantified by densitometry and the information is presented in histogram format. Data represent mean ± SEM from 3 independent experiments (P values, t test; bars, SEMs). *, $P < 0.05$, versus siC. RT-PCR, reverse transcription PCR.

Colonies, defined as cell groups containing a minimum of 50 cells, were counted under a phase contrast microscope.

Immunofluorescence, immunohistochemistry, and immunoprecipitation

HEK293 cells (5×10^5), transfected with pHA-Kir2.2, were incubated at room temperature for 1 hour with rabbit anti-HA (1:200) and mouse anti-RelA (1:500). Secondary antibodies were anti-rabbit Alexa 488 (1:800) and anti-mouse Alexa 594 (1:800). A streptavidin peroxidase procedure was used for immunohistochemical detection of GFP, RelA, cyclin D1, VEGF, MMP9, and PCNA. Briefly, paraffin-embedded tissue sections were deparaffinized and dehydrated in a graded series of alcohol. Antigenic epitopes were unmasked by autoclaving for 15 minutes in target retrieval solution (DAKO). For immunoprecipitation, HEK293 cells, transfected with pGFP-Kir2.2, were washed with cold PBS, and lysed in a buffer [20 mmol/L HEPES, pH 7.0, 150 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 1× protease inhibitor cocktail]. Anti-HA antibody (Cell Signaling) was incubated with cell lysates overnight at 4°C. The immune complexes were pulled down by addition of protein A agarose. After wash with lysis buffer, immunoprecipitates were analyzed by SDS–PAGE and Western blotting.

Luciferase assay

To examine the influence of Kir2.2 on cyclin D1 and NF-κB activity, pGL3-cyclin D1-Luc and pGL3-NF-κB-Luc (Clontech), which contained luciferase gene under the control of a cyclin D1- and a NF-κB-responsive element, were used. For NF-κB reporter assays, cells were transfected with either empty vector or pHA-Kir2.2 plasmid together with luciferase vector. After 24 hours, the cells were lysed and luciferase activity was determined according to the supplier's protocol (Promega). Luciferase activity is given as mean ± SD of triplicated experiments.

Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear protein extracts were obtained from the PC-3 and HEK293 cells and electrophoretic mobility shift assays (EMSA) were carried out with an EMSA kit (Promega). Double-stranded oligonucleotide probes (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were used. Hundred-fold unlabeled probe was added for cold competition. For antibody supershift, antibody (1 μg) was preincubated for 20 minutes before the addition of radiolabeled probe.

***In vivo* tumorigenesis**

To examine the effect of Kir2.2 expression in tumor formation, 1.5×10^6 PC-3 cells were implanted subcutaneously into

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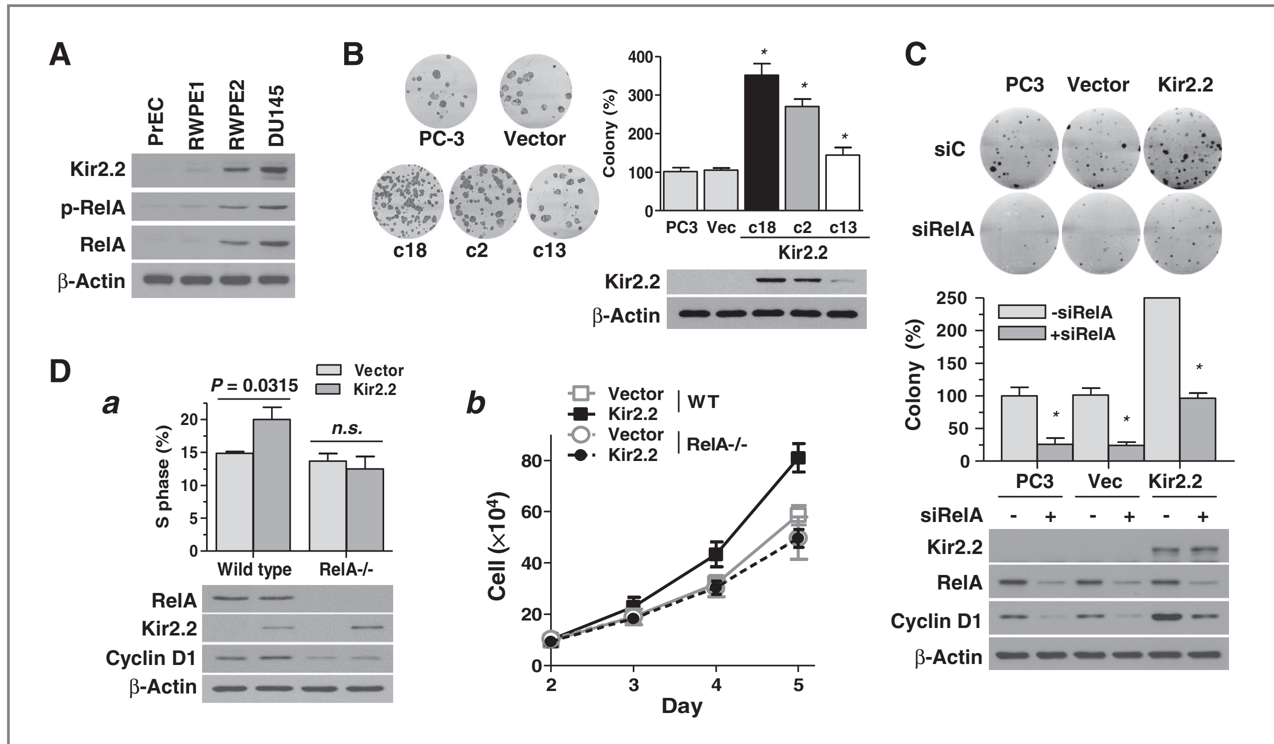

Figure 3. Kir2.2 is required for maintenance of the transformed phenotype. A, expression of Kir2.2 and RelA phosphorylation in normal (PrEC), immortalized (RWPE1), and transformed (RWPE2) human prostate cell lines as well as prostate cancer–derived cell line (DU145). B, Kir2.2 expression-dependent increase in colony formation of stable PC-3 cell clones. (*, $P < 0.05$, vs. vector). C, abrogation of Kir2.2-induced colony formation and cyclin D1 expression upon depletion of RelA. Stable Kir2.2-expressing PC-3 cell clones were transfected with a nonspecific control or RelA-specific siRNA. (*, $P < 0.05$, vs. control siRNA). D, RelA mediates the effect of Kir2.2 on cell cycle and cell proliferation. Wild-type (WT) and RelA-null MEFs were transfected with empty vector or Kir2.2, followed by cell-cycle analysis using flow cytometry (a) and MTT assays at the indicated time points (b). RelA, Kir2.2, and cyclin D1 expression was assessed with immunoblotting.

the 4-week-old female BALB/c nude mice and the tumor growth was monitored using calipers every 3 to 4 days. All mice were obtained from Charles River Laboratories, maintained and sacrificed according to institutional guidelines, and the procedures were approved by the Institutional Committee on the Use and Care of Animals and Recombinant DNA research.

Statistical analysis

Data shown in graphs are mean ± SD of values obtained in at least 3 independent experiments. Student *t* test was used to determine the significance of differences in mean values. Intergroup comparisons were conducted using a paired two-sample *t* test. A difference was considered significant if the *P* value was less than 0.05.

Results and Discussion

Previously, we showed that the loss of Kir2.2 triggered reactive oxygen species (ROS)-induced cell-cycle arrest in multiple cancer cell lines (3). Figure 1A shows that the growth of PC-3 cells was specifically inhibited by the depletion of Kir2.2. To identify Kir2.2-interacting proteins, we used a yeast two-hybrid system. When an analysis was conducted using a cytoplasmic portion (a.a. 301-430) of Kir2.2 cDNA as bait, the NF-κB p65 protein (encoded by RelA) was identified as a direct interacting partner of Kir2.2 (Fig. 1B). This result was not surprising, because NF-κB activation is essential for the proliferation of cancer cells (11, 12) and increasing ROS scavengers (13). Coimmunoprecipitation experiments using HEK293 cell lysates showed that GFP-tagged Kir2.2 interacted with endogenous RelA (Fig. 1C, a). Endogenous RelA coprecipitated with endogenous Kir2.2, but not with other members of the Kir2.x family, in HEK293 and PC-3 cells (Fig. 1C, b). Likewise, other NF-κB family members (p50, p52, and RelB) did not interact with Kir2.2 (Supplementary Fig. S1). Next, we examined the cellular localization of RelA. Immunoblotting of PC-3 cells with the empty vector (control) and cells overexpressing Kir2.2 showed that Kir2.2 enhanced the nuclear level of RelA (Fig. 1D, a). Wild-type Kir2.2 was present exclusively in the cytoplasm in the absence of RelA. However, upon coexpression of the latter protein, Kir2.2 colocalized with RelA in the nucleus (Fig. 1D, a and b). Enhanced immunofluorescence staining of nuclear RelA in the presence of Kir2.2 provided further evidence for the existence of this translocation (Fig. 1D, b). However, Kir2.2-NES, a fusion protein with a nuclear exclusion signal, failed to trigger nuclear translocation of RelA in mouse embryonic fibroblast (MEF) cells (Supplementary Fig. S2). These results indicate that Kir2.2 may interact with RelA in the cytoplasm and subsequently

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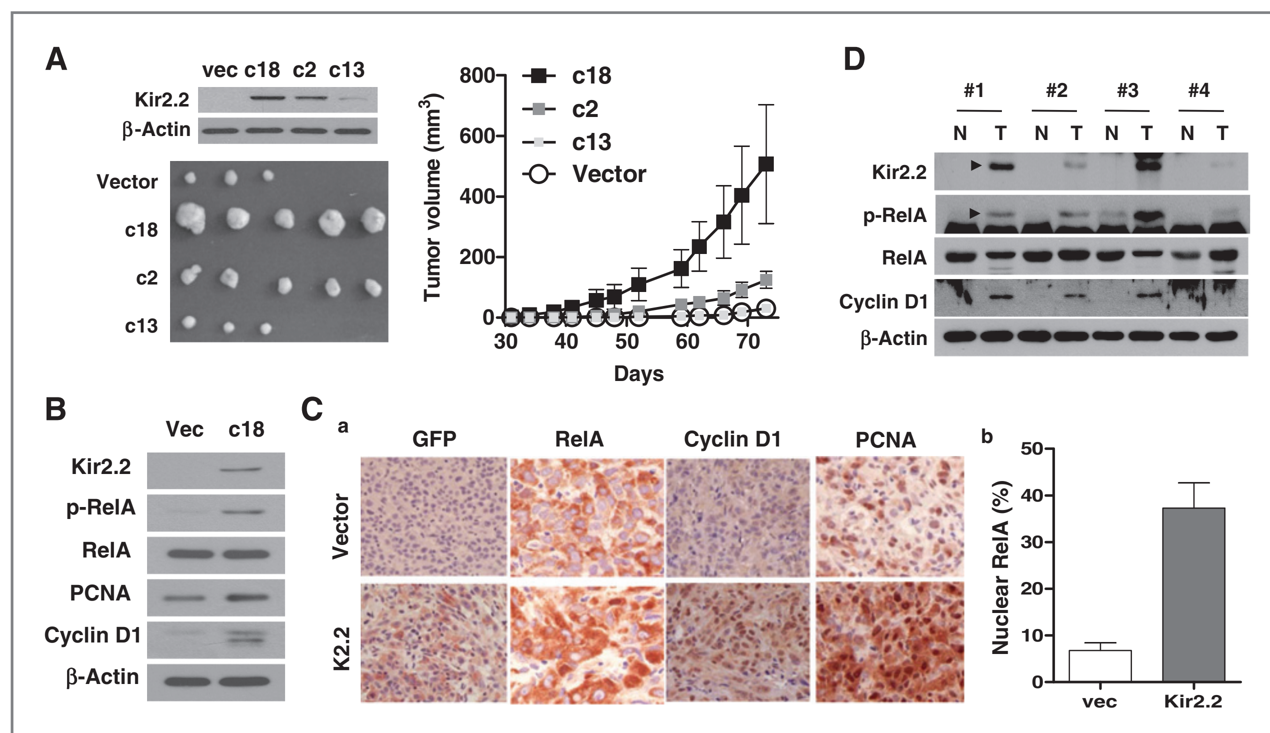

Figure 4. Kir2.2 increases RelA phosphorylation, nuclear translocation, cyclin D1 levels, and *in vivo* tumorigenesis. A–C, stable PC-3 cell clones were xenotransplanted subcutaneously into the athymic nu/nu mice. A, effects of Kir2.2 expression level on *in vivo* tumor growth (mean ± SEM; $n = 5$). B, Western blot analysis shows Kir2.2-dependent increases in RelA phosphorylation and cyclin D1 expression in the representative tumor samples. C, Kir2.2 expression increased nuclear RelA, cyclin D1, and PCNA from implanted tumors. a, immunohistochemical staining of the indicated proteins from formalin-fixed and paraffin-embedded tumor samples. b, percentage of cells containing nuclear RelA. Quantification of results shown in C, a. D, Western blot analysis of Kir2.2, RelA, phospho-RelA, and cyclin D1 in human lung tissues. Freshly frozen specimens of lung tumors (T) exhibit increased Kir2.2 expression, cyclin D1, and RelA phosphorylation compared with corresponding normal tissues (N).

translocate with RelA to the nucleus. Consistent with this finding, the interaction of RelA with IκB decreased in the presence of Kir2.2 (Supplementary Fig. S3).

Although nuclear localization is necessary for RelA function as a transcription factor, it is not sufficient for the transactivation of target gene expression (14). In Kir2.2-expressing cancer cells, RelA was constitutively bound to its consensus DNA sequence, as determined by EMSA (Fig. 2A). An anti-RelA antibody caused a supershift of the band but antibodies against RelB, C-Rel, p50, or p52 did not. In addition, an NF-κB-responsive luciferase reporter assay showed that Kir2.2 overexpression increased NF-κB activity more than 10-fold, whereas Kir2.2 knockdown decreased NF-κB activity in PC-3 (Fig. 2B) and other cancer cell lines (Supplementary Fig. S4). It has been shown that the nuclear retention of RelA can be prolonged by RelA phosphorylation, which is required for its acetylation (14). Therefore, we tested whether Kir2.2 affected RelA phosphorylation. Kir2.2 overexpression increased RelA phosphorylation on Ser536 (Fig. 2C), whereas knockdown of Kir2.2 suppressed this phosphorylation (Fig. 2D). Together, these observations indicated that Kir2.2 is required for constitutive RelA activity in cancer. To determine whether Kir2.2 was also required for TNF-activated NF-κB induction, we stimulated control siRNA- or siKir2.2-transfected PC-3 cells with TNF-α and next measured the NF-κB activity level. TNF-α–induced NF-κB activation was significantly inhibited by Kir2.2 depletion of PC-3 cells (Supplementary Fig. S5). Thus, the Kir2.2 expression level may affect the extent of TNF-α–induced NF-κB signaling.

The best explored link between NF-κB activation and cell-cycle progression involves cyclin D1, a cyclin that is crucial to commitment to DNA synthesis (15). Supporting the earlier findings, Kir2.2 overexpression upregulated cyclin D1 expression and increased cell growth (Fig. 2C), whereas Kir2.2 knockdown downregulated cyclin D1 expression (Fig. 2D) and the luciferase activity driven by cyclin D1 (Supplementary Fig. S6A). Furthermore, RelA knockdown and the RelA inhibitor SN50 efficiently blocked both Kir2.2-induced cyclin D luciferase reporter activity (Supplementary Fig. S6A and S6B) and cell growth (Supplementary Fig. S6C). Importantly, Kir2.2 knockdown decreased cancer cell growth in a siRNA concentration-dependent manner, whereas knockdown had no effect on the proliferation of normal epithelial cells (Supplementary Fig. S7).

Induced NF-κB activity in normal cells can be quickly attenuated following termination of the signal; however, during chronic stimulation, the signal is counteracted to precisely control NF-κB activity (16). This indicates that the Kir2.2-induced chronic stimulation of RelA can be detrimental for the growth control of normal cells. Consistent with

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these data, we detected the increased expression of Kir2.2 in multiple cancer cell lines (Supplementary Fig. S8). When levels of Kir2.2 and RelA were quantified in normal, immortalized, or transformed human prostate cell lines or prostate cancer–derived cell lines, cellular Kir2.2 levels were increased in association with malignant progression (Fig. 3A). To examine whether the ectopic expression of Kir2.2 affected long-term colony formation, we established PC-3 and HEK293 clones that stably overexpressed Kir2.2. Our results revealed that colony formation was dose-dependent and was higher in Kir2.2-overexpressing PC-3 (Fig. 3B) and HEK293 (Supplementary Fig. S9) cells than in controls. In contrast, knockdown of RelA abrogated Kir2.2-induced colony formation and cyclin D1 expression (Fig. 3C). Therefore, Kir2.2-induced colony formation and cyclin D1 expression seem to be mediated by RelA. To further confirm that RelA is involved in the effects of Kir2.2, we overexpressed Kir2.2 in RelA knockout MEFs. Kir2.2 overexpression increased the levels of the NF-κB target cyclin D1 and the percentage of cells in S-phase of the cell cycle in wild-type MEFs but not RelA-null MEFs (Fig. 3D, a). Consistently, the MTT assay showed that increased Kir2.2 led to enhanced cell proliferation in wild-type MEFs but not RelA-null MEFs (Fig. 3D, b). Taken together, these data indicate that the effect of Kir2.2 on cancer cell growth and long-term colony formation requires intact RelA activity.

Next, we compared the tumor formation capability of stable Kir2.2-transfected cells and control cells following their subcutaneous implantation into athymic nude mice. Kir2.2 overexpressing cells exhibited a dose-dependent increase in tumorigenesis ($P = 0.0138$; *t* test comparing vector with Kir2.2-c13; Fig. 4A). Immunoblot analysis of resected tumors showed enhanced expression of phosphorylated RelA, cyclin D1, and PCNA in Kir2.2-transfected tumors (c18), but not in control vector-transfected tumors (Fig. 4B). An immunohistochemical analysis of paraffin-embedded resected tumors showed strong staining for nuclear RelA and cyclin D1 in Kir2.2-transfected tumors (Fig. 4C, a and b). RelA-inducible VEGF and MMP9, typical proteins associated with angiogenesis and metastasis, also showed strong staining (Supplementary Fig. S10). In addition, strong staining for PCNA indicated that cell proliferation was increased after Kir2.2 expression (Fig. 4C, a). Next, we examined levels of RelA phosphorylation in human lung cancer, in which NF-κB is frequently found to be activated (17). RelA phosphorylation and cyclin D1 expression level correlated with Kir2.2 expression in tumor samples, as evidenced by Western blot analysis (Fig. 4D).

In summary, these results uncover a functional link between the potassium channel Kir2.2 and NF-κB signaling in cancer. The inhibitor of IκB kinase (IKK)-mediated phosphorylation of RelA has been shown to lead to RelA acetylation and extend the half-life of nuclear RelA (18), thereby increasing RelA activity. Because Kir2.2 is a membrane-localized protein that interacts with a variety of other proteins (10), it would not be surprising if Kir2.2 acts as a scaffold to recruit RelA to IKK. Recently, "conditionally essential malignancy" genes have arisen as attractive molecular targets (19, 20). Notably, Kir2.2 is required for the survival of cancer cells, showing that this protein could be a conditionally oncogenic protein. Together with our previous report that the knockdown of Kir2.2 can slow the progression of preestablished tumors (3), the present results show that targeting the Kir2.2–NF-κB pathway could be a novel approach for therapeutic interventions in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W.K. Kang, C. Park
Development of methodology: I. Lee, W.K. Kang, C. Park
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Lee, S.-J. Lee, T.M. Kang, W.K. Kang, C. Park
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Lee, T.M. Kang, W.K. Kang, C. Park
Writing, review, and/or revision of the manuscript: W.K. Kang, C. Park
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W.K. Kang, C. Park
Study supervision: W.K. Kang, C. Park

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