# The Binding Between ROCK1 and KIF2A Signals for the Centrosome Amplification Triggered by High Glucose, Insulin and Palmitic Acid

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# Abstract

Diabetes increases the risk for various cancers without established mechanisms. Centrosome amplification can initiate tumorigenesis in genetically modified cells. However, the findings from genetically modified experimental models may be far away from reality. We have reported that diabetes promotes the occurrence of centrosome amplification in different types of cells, implicating that centrosome amplification is a candidate mechanism underlying the diabetes-promoted tumorigenesis. In the present study, we investigated the molecular mechanisms of the centrosome amplification triggered by high glucose, insulin and palmitic acid using HCT116 colon cancer cells as an experimental model. We found that KIF2A was localized in the centrosomes. The experimental treatment induced the binding between ROCK1 and KIF2A, although did not increased the protein level of KIF2A. The molecular docking modeling analysis also showed that the two proteins had the binding/interaction potential. We used siRNA of each protein to knockdown their expression level, as a tool to disrupt the ROCK1-KIF2A complex, which attenuated the treatment-induced centrosome amplification. In conclusion, our results suggest that the binding between ROCK1 and KIF2A signals for the diabetes-associated centrosome amplification.


# Introduction

Type 2 diabetes accounts for the majority of diabetes cases, which increases the risk for cancers and promotes cancer metastasis, including colon cancer (1, 2) without established mechanisms. Centrosome amplification (CA) refers to a cell with more than two centrosomes. Genetically modified cells forced to have CA can form tumors in recipient animals, and, in genetically modified animal model of CA, spontaneous tumorigenesis can occur (3, 4). Thus, CA is sufficient to cause tumorigenesis. CA is also found to increase the invasion potential of cancer cells *in vitro* (5), implicating that CA can promote cancer cell metastasis. The question is whether the observations from genetically modified models reflect the carcinogenesis in the nature. Type e diabetes presents clinical features of increased blood glucose and free fatty acids. At some stage of the disease development, increased blood insulin level occurs. Palmitic acid is the most common saturate free fatty acid, which is often used to represent free fatty acids in the studies of the functional roles of free fatty acids, in particular the adverse functional effects. We have reported that type 2 diabetes increases the level of CA in peripheral mononuclear cells *in vivo* (6). Pathophysiological factors of type 2 diabetes, i.e., high glucose, insulin and acid (representing free fatty acids) can induce CA *in vitro* in non-cancerous and cancer cell models via upregulation and centrosomal translocation of the Rho-associated coiled-coil containing protein kinase 1 (*ROCK1)* (6). Thus, it is possible that diabetes promotes tumorigenesis and metastasis via CA. Understanding the signaling pathways of the CA is helpful for the examination of the roles of the CA in the development of cancer in type 2 diabetes. CA occurs due to over-replication of centrosomes in many cases, in which protein-protein interaction, binding and recruitment are common events, alteration of which can cause CA (7). To initiate centrosome duplication, PLK4 is firstly recruited to one side of mother centriole by CEP152 and CEP63 complex, which phosphorylates and recruits STIL as well as SAS6 to form a cartwheel structure which is a platform to recruit proteins for centrioles extension, such as CEP135 and CPAP. Then

CEP135 recruits CEP120 and CPAP recruits CP110. During the maturation phase, a series of proteins are recruited to the PCM, such as CEP192, PLK1 and γ-tubulin, which are the key proteins for PCM assembling. At the end of G2 phase, the duplicated centrosomes are separated to form two poles. During this process, NEK2 is recruits to the centrosomes, which works with other proteins for centrosome separation.

We recently attempted to identify the ROCK1 binding partners using coimmunoprecipitation technology in combination with proteomic analysis, and found that antibody against ROCK1 pulled down several proteins which included KIF2A (8). In the study, we examined whether ROCK1 bound to KIF2A, and whether the binding contributed to the CA by high glucose, insulin and palmitic acid. Understanding the signals for the CA is helpful for the investigations of the CA in tumorigenesis and metastasis.

## Materials And Methods

### *Chemicals, reagents, antibodies and cells.*

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) and Beyotine Biotechnology (Shanghai, China) respectively. Antibody against the Rho-associated coiled-coil containing protein kinase 1 (ROCK1; #4035, rabbit antibody) and the Kinesin family member 2A (KIF2A; #D125969, rabbit antibody) antibodies were purchased from the Cell signaling technology (Boston, MA, USA) and (Sangon biotech (Shanghai China), respectively. Antibody against γ-tubulin was provided by Abcam (Cambridge, UK). Immortalized non-cancerous human colon epithelial NCM460 cells and human colon cancer HCT116 cells were purchased from the Procell Life Science & Technology (Wuhan, China) and the cell culture medium and supplements were from Gibco (Beijing, China).

### *Cell culture and experimental treatment.*

Cells were maintained in low-glucose (5 mM) DMED medium in an incubator under 5% $CO_2$ at 37 $^{o}$C. For experiments, cells seeded on cover slips which were placed in 6-well plates were treated with high glucose (15 mM), insulin (5 nM) and palmitic acid (150 μM) for 48 hours for assessment of CA level. Palmitic acid was used to represent free fatty acids. It was conjugated to BSA by incubating palmitic acid and BSA for 1 hour at 37 $^{o}$C just before use. The molar ratio of palmitic acid and BSA was 3:1.

### *Immunofluorecent staining and microscopy.*

Cells on cover slips were fixed in pre-cooled methanol and acetone (1:1 v:v; 6 min; -20°C), washed for 3 times using PBS. Cells were incubated with various agents in the order of 0.1% Triton X-100 (15 min), and 3% BSA (60 min) and primary antibody (overnight; 4°C). Cells were washed twice with PBS and incubated with secondary antibody in 3% BSA (60 min; room temperature). Finally, cells were mounted with mounting medium containing DAPI. Confocal microscopic examination was performed using Zeiss

LSM880 microscope (Oberkochen, Germany). Captured images were processed using Zen software (Oberkochen, Germany). At least 100 cells were counted to calculate the percentage of cells with CA.

## *Co-immunoprecipitation.*

Cells were lysed in lysis buffer followed by centrifugation and supernatants were collected then incubated with 20 ul of Protein G Plus/Protein A agarose (IP05, Miliproe) with gentle shaking for 2 hours. Agarose was removed after centrifugation, which was to minimize non-specific bindings. Supernatants were collected for incubation overnight under gentle shaking with antibody against specific protein. Afterwards, 30ul of agarose were added and incubated for 4 hours under gentle shaking to pull target protein and its binding partners. At the end, agarose was collected by centrifugation, re-suspended in loading buffer, heated to 100 $^{O}$C for 30 seco0nds and then centrifuged (14000g, 1 min). The supernatants were used for detection of protein that was pulled down with target protein, the binding partner of target protein.

## *Western blot.*

Cells were lysed in RIPA buffer. Cellular proteins were separated by polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membrane, blocked in TBST buffer containing 0.05% v/v Tween-20 and 5% w/v non-fat milk) for 1 hour, incubated with primary antibody (overnight; 4°C), washed with TBST containing 0.05% Tween-20, and incubated with secondary antibody (room temperature; 1 hour. Protein bands were visualized using ECL reagents (#32106, Thermo Biosciences), which were captured on X-ray films.

## *Knockdown of protein level using siRNA.*

Pre-designed siRNA oligonucleotides were obtained from Songon Technology (Shanghai, China): (1) ROCK1, 5’- UGAUCUUGUAGCUCCCGCAUGUGUC -3’ (sense) and 5’ GACACAUGCGGGAGCUACAAGAUCA 3’ (antisense); (2) KIF2A, 5’ GGCAAAGAGAUUGACCUGGTT 3’ (sense) and 5’ CCAGGUCAAUCUUUGCCTT 3’ (antisense). Cells cultured in a 6-well plate were transfected with 200 nM siRNA oligonucleotides using Lipofectamine 2000 transfection reagent (11668-019, Invitrogen), according to the manufacturer’s instructions. Transfection efficiency was evaluated by Western blot analysis 24 hours after transfection.

## *Molecular docking modeling.*

The protein crystal structures of ROCK1 and KIF2A were downloaded from PDB database with PDB IDs of 5WNH and 6BBN, respectively. Proteins were hydrogenated, prepared and endowed with force field. Discovery Studio version 3.5 was used to place two proteins in a CELL lattice at the same time, docking in the DOCK PROTEINS module, setting the Euler angle to 6 degrees to sample the binding configuration.

Through further protein conformation analysis, top 100 optimized proteins conformations were accessed, and the optimal results were analyzed.

## *Statistics.*

Student t-test was performed to compare the difference between means using the software package SPSS21. A $p<0.05$ was considered to be statistically significant.

# Results

## *High glucose, insulin and palmitic acid triggers centrosome amplification.*

Cell viability was not compromised by the treatment using high glucose, insulin and palmitic acid (Fig. 1A). Figure 1B was a representative image of CA. The experimental treatment increased the level of CA by approximately 6 times in the non-cancerous (NCM460) and cancerous (HCT116) colon cells (Fig. 1C).

## *The experimental treatment increases the binding between ROCK1 and KIF2A.*

Upon the experimental treatment, the protein level of ROCK1 was increased at 12 and 24 hours, whereas, the protein level of KIF2A started to decrease at 24 hours (Fig. 2A). KIF2A was pulled down together with ROCK1 from the treated cells, but not from the control samples (Fig. 2B). ROCK1 was pulled down together with KIF2A in both control and treated sample. However, the level of KIF2A pulled down with ROCK1 from the treated cells was much higher than that from the control samples (Fig. 2C).

## *KIF2A is localized in the centrosome.*

We reported that the experimental treatment increased the centrosomal translocation of ROCK1 (6). Here, we examined whether KIF2A was localized in centrosome. Indeed, as shown in Figure 3, KIF2A was present in only one centrosome at early stage of cell division, and then appeared in two centrosomes along the cell cycle progress. It was also clearly distributed in the spindles. When multipolar division occurred due to CA, KIF2A was present multipolar spindles.

## *Disruption of ROCK1-KIF2A complex attenuates the CA.*

We used siRNA technology to knockdown the protein level either RCOK1 or KIF2A, attempting to inhibit the formation or disrupt the protein complex, and examined whether it inhibited the treatment-caused CA.

Indeed, knockdown of ROCK1 (Fig. 4A) or KIF2A (Fig. 4B) did inhibit the CA (Figs 4C and 4D).

## *Molecular docking modeling for the interaction between ROCK1 and KIF2A.*

Figure 5A showed the model of protein-protein interaction between ROCK1 and KIF2A, the score of which reached as high as 20.3. In ROCK1, 50 amino acids were likely to be involved in the interacting and maintaining the complex stability, which were VAL38, TYR39, ASP42, PHE43, PRO44., ALA45, LYS60, ASN64, LYS65, ARG67, ASP68, LYS72, ALA73, GLU74, TYR76, GLU77, VAL78, VAL79, LYS80, VAL81, ARG84, GLY85, ALA86, GLU89, GLU91, VAL 93, LYS96, MET104, LEU106, GLU111, LYS114, ASP145, ASP146, ARG147, TYR148, TYR150, SER166, ASP370, LEU371, GLU372, GLU373, ASP374, GLU377, GLU378, GLU379, THR380, PHR381, PRO382, ILE383, and LYS385. The key sequence pieces for the interacting were VAL38-ALA45, ASN64-LYS96 and ASP370-LYS385. There were 52 amino acids in KIF2A, which involved in the interacting, which were VAL188, ARG229, LYS230, ARG231, PRO232, LEU233, ASN234, LYS235, LYS236, GLU237, ASP281, ASP282, SER283, ALA284, PRO285, ASN286, GLU287, GLY316, SER317, GLY318, LYS319, THR320, HIS321, THR322, GLY234, GLY325, ASP326, SER335, LYS336, ARG344, LEU377, LEU378, ARG380, GLU408, ASP409, LEU411, LYS412, ASP415, ILE416, GLY417, ASN418, SER419, LYS420, ARG421, THR422, SER423, HIS431, SER432, SER433, ARG434, ASP457, and THR529. The key sequence pieces were ARG229-GLU237, GLY316-LYS336 and GLU408-ARG434. The surface structures of ROCK1 and KIF2A presented a highly complementary feature (Fig. 5B), which could form a dimer with strong stability. Moreover, we alignment of the sequences of ROCK1 and KIF2A revealed that the amino acids sequence similarity was only 16% (Fig. 5C).

## Discussion

Our results suggest that the binding between ROCK1 and KIF2A signals for the CA, implicating that interfering the binding is a useful method to inhibit or block the CA. The binding may occur in or translocate to the centrosomes, as the experimental treatment increases the centrosomal distribution of both ROCK1 (6) and KIF2A (Figure 3). Understanding the signaling pathways of the CA is meaningful for investigating the roles of CA in diabetes-promoted tumorigenesis and metastasis, in which it may require to show that inhibition of the CA can prevent the development of cancer. It should also be mentioned that CA disturbs cilia signaling (9). Dysregulation of ciliogenesis might disturb the functions of kidney, vascular system and retina, as these tissues have cilia (10-12). In the light that diabetes has renal, vascular and retinal complications, the diabetes-associated CA may have functional consequences beyond cancer, such as in the development of the diabetic complications.

As reviewed by Julian and Olson (13), ROCK1 contains 33 exons and are located on chromosome 18 (18q11.1), and is ubiquitously expressed throughout embryogenesis and in adult tissues. It has been shown to regulate cell proliferation, actin organization and cytokinesis, and apoptosis. Interestingly, there is an association between ROCK1 and cancer (13). The polymorphisms in ROCK1 gene are associated

with cancer development; in particular, the microsatellite instability in ROCK1 gene is associated with colon cancer. ROCK1 and ROCK2 are both present in centrosomes, which regulates centrosome homeostasis, dysregulation of which results in CA (14), which leads to genomic instability that is thought to cause cell malignant transformation. There is also evidence that ROCK1 favors cell survival by promoting proliferation and inhibiting cell death. As reviewed by Niwa as well as Hirokawa and Takemura (15, 16), a total of 45 KIFs have been identified in mouse and human genomes so far. They are classified into three major types on the basis of the position of the motor domain: N- terminal, middle, and C-terminal motor domain types. The KIF2 family belongs to the middle motor domain type. KIFs, together with dyneins, serve as motors that move along microtubules carrying cargoes such as membranous organelles, protein complexes, and mRNAs. Moreover, KIFs have been found to regulate microtubule dynamics via depolymerizing or stabilizing microtubule. KIF2A has microtubule-depolymerizing activity, which is thought to be ATP dependent. It is not essential for cell division, as KIF2A-knockout mice develop into neonates. However, the knockout mice die after birth because of neuronal defects due to abnormal microtubule elongation. In relevance to cancer, increased expression of KIF2A is associated with worse prognosis and unfavorable clinicopathological features, suggesting that KIF2A favors cancer progression (17). Indeed, inhibition of KIF2A attenuates cancer cell invasion potential (18, 19). We have evidence that ROCK1 also binds to 14-3-3σ and DCTN2, which signals for the CA (7, 8). It would be interesting to investigate whether ROCK1, KIF2A, DCTN2 and 14-3-3σ form a giant complex that promotes the CA. It would also be interesting that the complex acts on PLK4, as KIF2A promotes PLK4 expression (18). We bear in mind that more evidence is required to support the binding between ROCK1 and KIF2A. For example, *in vitro* direct binding assays can be performed to support that the two proteins can bind to each other. Mutation(s) of binding sites can be created to test whether the binding potential is lost.

In conclusion, our results suggest that the binding between ROCK1 and KIF2A mediates the centrosome amplification triggered by high glucose, insulin and palmitic acid.

## Declarations

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# Conflicts of interests:

Non

# Ethical statement:

This article does not contain any studies with human precipitants or animals performed by any of the authors

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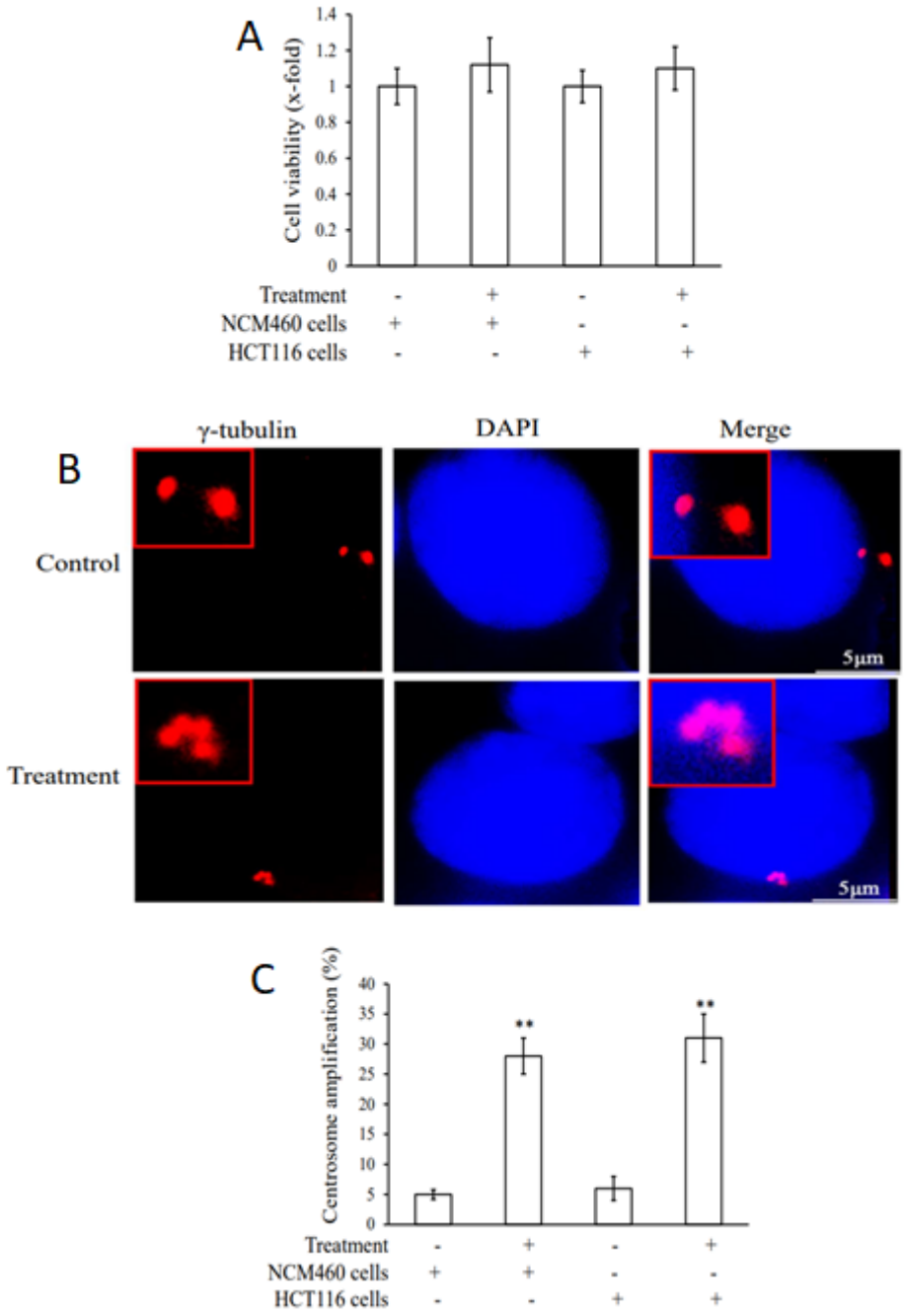
# Figures

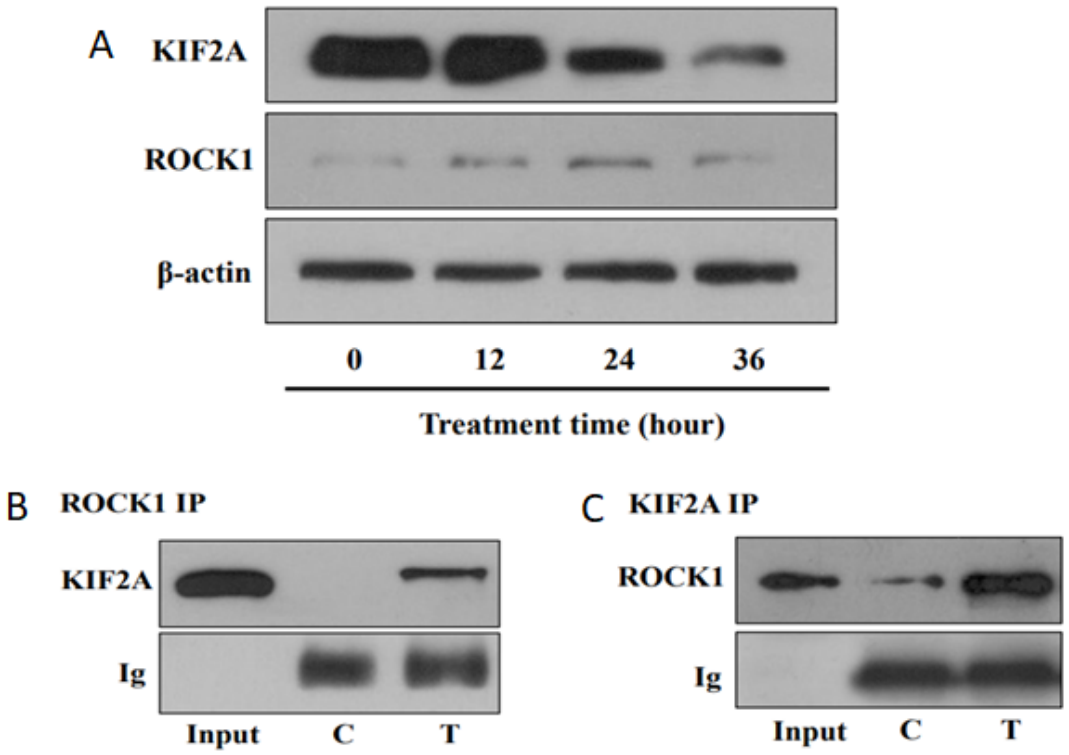
Figure 1




## Figure 1

High glucose, insulin and palmitic acid induce the centrosome amplification in non-cancerous (NCM460) and cancerous (HCT116) colon cells. (A) The experimental treatment was not toxic to the cells. (B) The representative image of the centrosome amplification. (C) The experimental treatment increased the level of centrosome amplification by approximately 6 folds. **: $p<0.01$, compared to the controls.

Figure 2




## Figure 2

The experimental treatment induces the binding between ROCK1 and KIF2A. (A) The experimental treatment increased the protein level of ROCK1 but decreased the protein level of KIF2A. (B) The antibody against ROCK1 pulled down KIF2A from the treated but not the control samples. (C) The antibody against KIF2A pulled sown more ROCK1 from the treated sample than that from the control sample. C: control samples; T: samples treated by high glucose, insulin and palmitic acid.

Figure 3

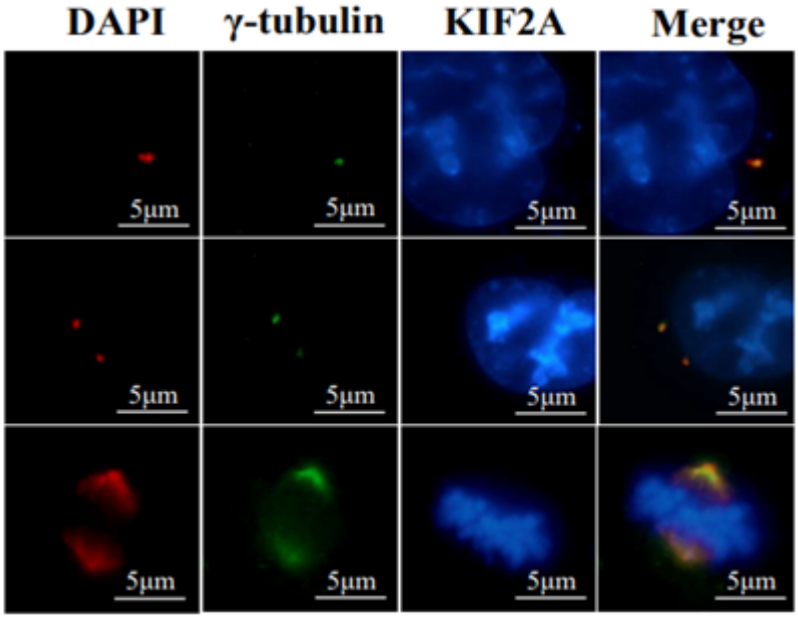
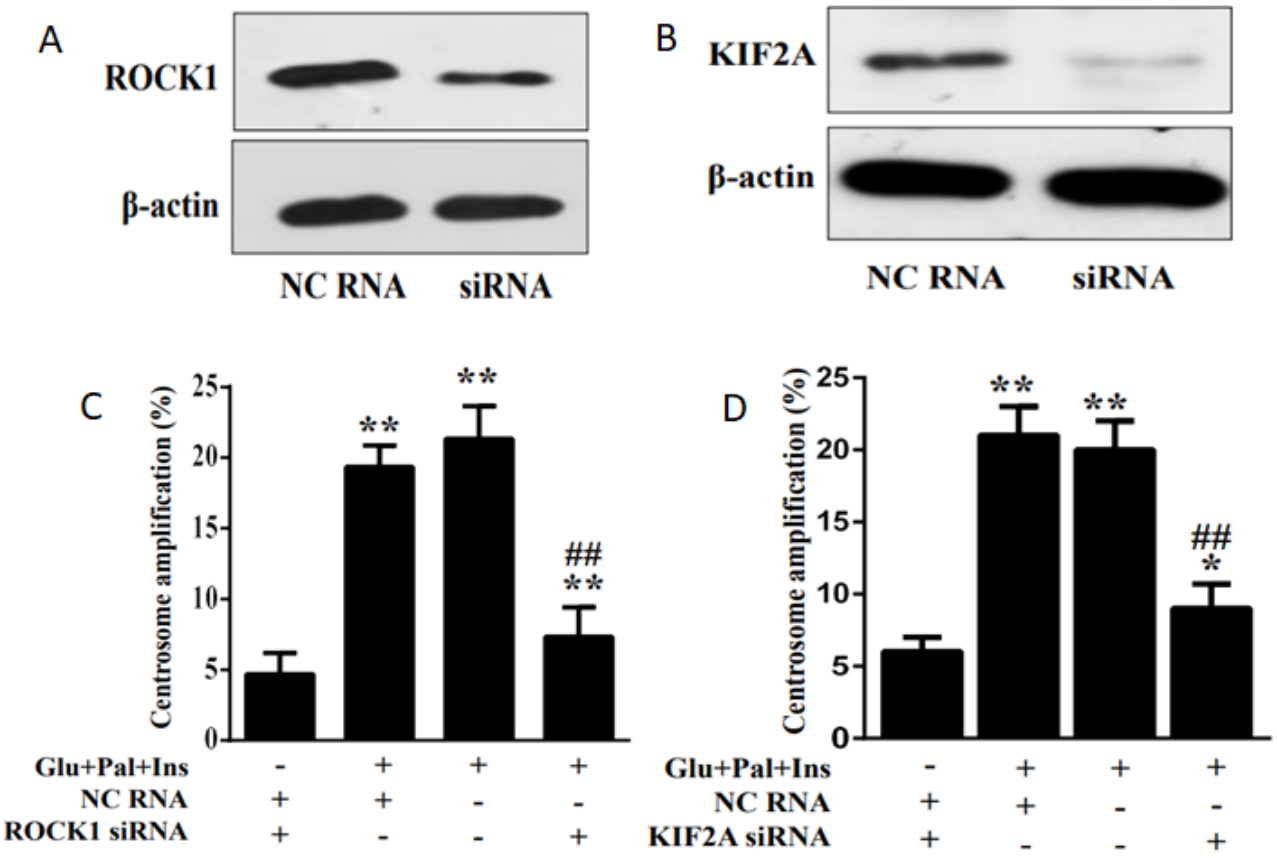



**Figure 3**

KIF2A is present in the centrosome and spindles. It was present in only one centrosome at early stage of cell division, and then appeared in two centrosomes along the cell cycle progress. It was also clearly distributed in the spindles.

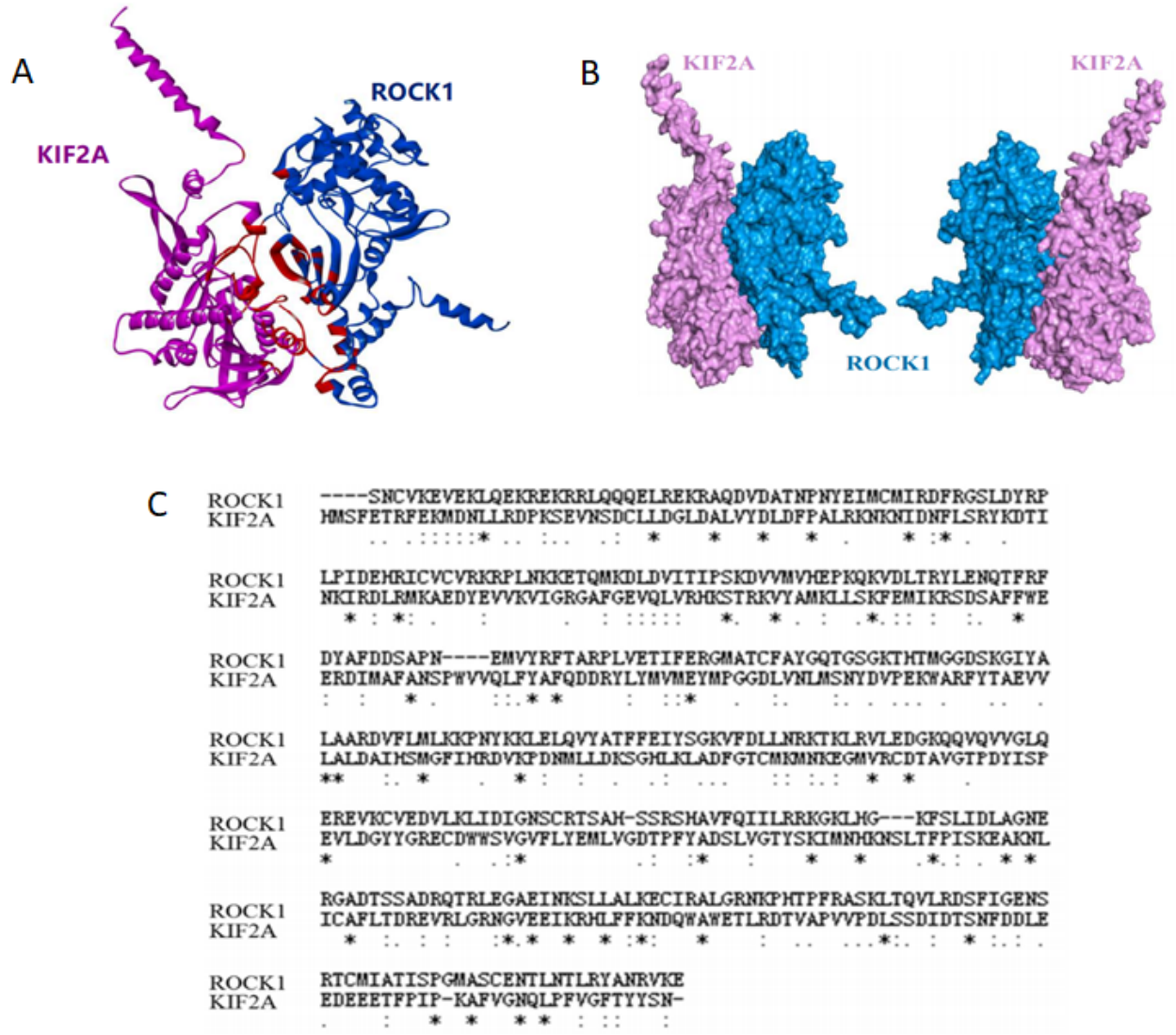
Figure 4




## Figure 4

Disruption of the ROCK1-KIF2A complex attenuates the centrosome amplification. (A) Knockdown of ROCK1 protein level using siRNA. (B) Knockdown of KIF2A protein level using siRNA. (C) Knockdown of wither ROCK1 or KIF2Ainhibited the treatment-induced centrosome amplification. **: p<0.01, compared with the control; ##: p<0.01, compared with the treatment group.

Figure 5




## Figure 5

Molecular modeling suggests that ROCK1 and KIF2A have the potential for binding. Protein-protein interaction model. (B) The surface structures of ROCK1 and KIF2A presented a highly complementary feature. (C) The amino acids sequence similarity between ROCK1 and KIF2A was only 16%.