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Raf-1 Kinase Inhibitor Protein Regulates Cell Migration and Proliferation of Mouse Embryonic Fibroblasts

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Raf-1 Kinase Inhibitor Protein Regulates Cell Migration and Proliferation of Mouse Embryonic Fibroblasts

Donghui Song

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2012
Raf-1 Kinase Inhibitor Protein Regulates Cell Migration and Proliferation of Mouse Embryonic Fibroblasts

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2012
Raf-1 Kinase Inhibitor Protein Regulates Cell Migration and Proliferation of Mouse Embryonic Fibroblasts

Donghui Song, B.S.

University of Connecticut, 2012

Raf-1 kinase inhibitor protein (RKIP) is a multifunctional protein that plays a regulatory role in a variety of biological activities, including cellular signaling pathways, cell proliferation, cell migration, cell cycle, apoptosis and cancer metastasis. In our previous study, we have identified that RKIP has a positive effect on the cell migration in Madin-Darby canine kidney (MDCK) cell line. Moreover, we also discovered that a small stretch of amino acids (146-RGKFKVASRFKK-157) as the nuclear localization signal (NLS) targets RKIP for translocation to the nucleus. In this study, we showed strong evidence that RKIP expression has no effect on cell migration in both wild type mouse embryonic fibroblasts (MEF), and MEF cells lacking the RKIP gene (MEF RKIP−/−) with RKIP reintroduced. Furthermore, we found that RKIP expression inhibits the cell proliferation of the MEF cell line. Furthermore, we found that the nuclear localization of RKIP has a regulatory role in cell migration, cell proliferation and cell cycle distributions. Interestingly, we have also determined the effects that RKIP has on cell migration, cell proliferation and cell cycle distribution are independent of MAPK/ERK activation suggesting the involvement of a different pathway.
ACKNOWLEDGEMENTS

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# I. INTRODUCTION TO RAF-1 KINASE INHIBITOR PROTEIN AND ITS BIOLOGICAL FUNCTIONS

1.1 An overview for Raf-1 kinase inhibitor protein ........................................ 1
1.2 Biological functions of Raf-1 kinase inhibitor protein ................................. 3

## 1.2.1 The role of RKIP in the regulation of signaling pathway

1.2.1.1 Regulation of Raf-1 signaling pathway .............................................. 4
1.2.1.2 Regulation of NF-κB signaling pathway ........................................... 9
1.2.1.3 Regulation of GPCR signaling pathway .......................................... 11

## 1.2.2 Role of RKIP in cell cycle

1.2.3 Role of RKIP in apoptosis ................................................................. 15
1.2.4 Role of RKIP in cancer metastasis ..................................................... 17
1.2.5 Role of RKIP in cell migration .......................................................... 18

# II. RAF-1 KINASE INHIBITOR PROTEIN NUCLEAR LOCALIZATION HAS A REGULATORY EFFECT ON CELL MIGRATION AND PROLIFERATION INDEPENDENT OF THE MAPK/ERK PATHWAY

2.1 Introduction .......................................................................................... 20
2.2 Material and methods ........................................................................... 24
2.3 Results .................................................................................................. 29
2.4 Discussion ............................................................................................. 37

APPENDIX ............................................................................................... 42
REFERENCES ......................................................................................... 49
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCK-8</td>
<td>Cell Counting Kit-8</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GEF</td>
<td>GTP exchange factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein coupled receptor kinase</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IKKα</td>
<td>Inhibitor of κB kinase α</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitor of κB kinase β</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Inflammatory cytokines interleukin 1 beta</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
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<tr>
<td>MEK</td>
<td>MAPK/extracellular signal regulated kinase kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Transcriptional activator nuclear factor kappa B</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NIK</td>
<td>Nuclear factor κB (NF-κB)-inducing kinase</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Process of programmed cell death</td>
</tr>
<tr>
<td>PEBP</td>
<td>Phosphatidylethanolamine binding protein</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RKIP</td>
<td>Raf-1 kinase inhibitor protein</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SOS</td>
<td>Son of sevenless</td>
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<tr>
<td>TAK</td>
<td>TGF-β activated kinase</td>
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<tr>
<td>TAP</td>
<td>Transcription factor activation protein</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl phorbol-13-acetate</td>
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</table>
LIST OF ABBREVIATIONS (Continued)

TRAIL	TNF-related apoptosis-inducing ligand

TRE	TPA response element
CHAPTER I

INTRODUCTION TO RAF-1 KINASE INHIBITOR PROTEIN AND ITS BIOLOGICAL FUNCTIONS

1.1 An overview of Raf-1 kinase inhibitor protein

Raf-1 kinase inhibitor protein (RKIP), a 21 kDa protein with 187 amino acids, has been discovered as a member of the phosphatidylethanolamine binding protein (PEBP) family [1, 2]. Initially, the name of this protein was phosphatidylethanolamine binding protein 1 (PEBP1) resulting from its ability to bind weakly with phosphatidylethanolamine [3]. With the further study on it, this protein was renamed as Raf-1 kinase inhibitor protein (RKIP), because it was found that RKIP can bind to Raf kinase and inhibit the interaction between Raf-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MAPK/ERK) [4]. RKIP is commonly seen in the brain, testis, liver and kidney of mammalian animals [5, 6]. As well as the other PEBP family proteins, it has been determined from the 3D X-ray crystal structure of RKIP that there are two main domains, ligand binding pocket and globular structure [7, 8] (Figure 1). It has been reported that the ligand binding pocket provides the binding site not only for phosphatidylethanolamine, but also for the newly discovered small molecule inhibitor locostatin [9]. However, the mechanism of binding between RKIP and its ligands, as well as the specific amino acid binding sites has not as of yet been identified. Furthermore, the globular structure domain also plays a key role in a multitude of biological functions, due to its globular surface being able to provide
enough surface area for association with various cellular function modulator proteins. For example, previous studies have shown that RKIP is able to interact with several proteins that function as cellular signaling pathway regulators, such as Raf-1, B-Raf, MAPK/extracellular signal regulated kinase kinase (MEK), G protein coupled receptor kinase 2 (GRK2), and nuclear factor κB (NF-κB)-inducing kinase (NIK), transforming growth factor (TGF)-β activated kinase 1 (TAK1), inhibitor of κB kinase α (IKKα) and β (IKKβ) [4, 10-15]. Although the binding sites of RKIP and most of the proteins described above have not been revealed, a critical residue (Ser153) on RKIP was identified to have the relationship with the interaction between RKIP and Raf-1 [10]. The evidence has shown that RKIP is phosphorylated at Ser153. Further studies have shown that the phosphorylation at Ser153 has a crucial role in the regulation of the interaction between RKIP and Raf-1. Protein kinase C (PKC) phosphorylates the Ser153 residue leading to the dissociation of the RKIP-Raf-1 complex. It has been assumed that the steric hindrance contributes more to the dissociation than electrostatic repulsion [10]. Nevertheless, this is not the end of this story of the relationship between the structure of RKIP and its biological function. There are still many questions, such as the number and location of binding sites corresponding to different binding partners that has yet to be determined, so that a better understanding of this regulatory protein may be obtained.
1.2 Biological functions of Raf-1 Kinase Inhibitor Protein

1.2.1 Role of RKIP in the regulation of signaling pathway

1.2.1.1 Regulation of Raf-1 signaling pathway

Numerous experimental findings have revealed the fact that numerous biological activities, including cell proliferation, cell survival and cell differentiation, are mediated
by the Raf-1/MEK/ERK pathway in which RKIP plays a critical role in modulating the
aforementioned cell functions [16-22].

Presented in this thesis is how the Raf-1/MEK/ERK pathway functions at the molecular
level (Figure 2). Initially, the stimulus from the environment is sensed at the growth
factor cytokine. The signal is propagated by various growth factors, such as epidermal
growth factor (EGF), platelet-derived growth factor (PDGF) and nerve growth factor
(NGF). Afterwards, the receptor tyrosine kinase (RTK) is activated via phosphorylation
on its tyrosine residue. RTK acts as an initiator for many signaling cascades including
Raf-1/MEK/ERK and NF-κB signaling cascades [16, 17]. Then RTK activates Ras [23],
a small protein which belongs to G protein family, through a number of growth factor
receptor proteins. The growth factor receptor-bound protein 2 (Grb-2) and its domain, son
of sevenless (SOS), are also involved in this Ras activation process. SOS on Grb-2
interacts with Ras through its GTP exchange factor (GEF) domain, where GDP is
phosphorylated to GTP [23-25]. After Ras is activated, it binds with Raf-1 kinase and
activates it [26]. Subsequently, activated Raf-1 phosphorylates and activates
MAPK/extracellular signal regulated kinase kinase (MEK) directly. In the same way,
MEK then activates extracellular signal-regulated kinase (ERK) by phosphorylation [27].
To our knowledge, the Raf-1/MEK/ERK signaling pathway has a crucial function in the
modulation of the gene expression. In the nucleus of the cell, phosphorylated ERK
converts Fos proteins to Fos phosphoproteins [28]. When phosphorylated Fos proteins
bind with the transcription factor activation protein 1 (AP-1), AP-1 is then activated and
associates with the 12-O-tetradecanoyl phorbol-13-acetate (TPA) response element (TRE)
[29-32]. As a result, the stimuli from the environment affect gene expression leading to
the modulation of biological activities. For example, when the signal impacts Bcl-2 gene expression through Raf-1/MEK/ERK signaling pathway, the cell apoptosis is modulated accordingly [33-35]. In other words, Bcl-2 is able to regulate cell. Moreover, besides Bcl-2, many more genes including cyclin-dependent kinases (CDKs), Bcl-xL and Mcl-1 are also involved in the signaling pathway to regulate some certain biological process, such as cell proliferation, apoptosis, differentiation and migration [36-38]. Thus, disruption of any part of the Raf-1/MEK/ERK signaling pathway will lead to a deleterious effect in the behavior of the cell resulting in many human diseases such as cancer.
Figure 2 The Raf-1/ MEK/ERK signaling pathway. This signaling pathway plays a key role in transferring signal from cell membrane to the nucleus, leading the AP-1 transcription. The main direction of the signal transduction can be described like this: 

\[ \text{RTK} \rightarrow \text{Grb-2} \rightarrow \text{Ras} \rightarrow \text{Raf-1} \rightarrow \text{MEK} \rightarrow \text{ERK} \rightarrow \text{AP-1} \rightarrow \text{gene expression} \].


After the discovery of the Raf-1/MEK/ERK signaling cascade, the regulation process has remained complex and difficult to understand until Kam Yeung *et al.* identified RKIP as an inhibitor of this signaling pathway [4]. As we know, Raf-1 associates with MEK and activates it by phosphorylation. Experimental results have shown that overexpression of RKIP decreases the efficiency of the signaling pathway by disrupting the association between Raf-1 and MEK. Subsequently, the phosphorylation of MEK is inhibited, so that ERK cannot be phosphorylated and activated by MEK. As such, the signal stops at the Raf-1-MEK complex and thus cannot be transferred to MEK. As a consequence, RKIP is discovered as having an inhibitory function on Raf-1/MEK/ERK cascade. A model has been made to explain the mechanism of the RKIP inhibitory function. RKIP is considered as a competitive inhibitor of the association between Raf-1 and MEK (Figure 3). The result from binding site mappings among RKIP, Raf-1 and MEK definitely proves this conclusion [14]. It is well known that RKIP is able to bind physically with both Raf-1 and MEK. It leads to the disruption of their association and prevents phosphorylation of MEK. Additional data was also found to provide more evidence for this mechanism. *In vitro*, it was identified that mitogens can down-regulate the association of RKIP and Raf-1. Interestingly, the signaling pathway is recovered through the negative regulation with mitogens. Thus, we believe that the molecular mechanism model shown in Figure 3 best
elucidates the role that RKIP plays in the inhibition of Raf-1/MEK/ERK signaling pathway.

Figure 3 A proposed mechanism of inhibition of the Raf-1/MEK/ERK signaling pathway by RKIP. The signal propagates only when the Raf-1 binds to MEK and activates it by phosphorylation. Overexpressed RKIP disrupts the association between Raf-1 and MEK. Then no signal comes out with the suppression function by RKIP. However, the signal is dramatically enhanced when the mitogens is used to dissociate the Raf-1-RKIP complex. Therefore, both of the experimental result described above shows that RKIP plays a role in reducing the signal in the pathway. The figure is referred from Mol. Cell. Bio. 2008 20(9): 3079-3085.

In addition to the inhibitory function by RKIP, recent studies show that Raf-1/MEK/ERK signaling pathway can be positively regulated by protein kinase C (PKC) [10, 39] (Figure 4). The PKC is a form of serine/threonine kinase that mediate the Raf-1 cascade by phosphorylating RKIP. It is already known that G protein coupled receptors (GPCRs) induce the PKC phosphorylation of the Serine 153 residue on RKIP when the cell is
stimulated. The phosphorylation on Ser153 leads to the dissociation of the Raf-1-RKIP complex possibly due to steric hindrance. As a consequence, the PKC prevents the suppression of Raf-1 cascade by RKIP. We also know that the PKC functions not only as an activator for Raf-1 [40-42], but also as an activator for MEK for the reason that they can phosphorylate MEK as well [43]. Thus, the PKC can be considered as an up-regulator for the Raf-1/MEK/ERK signaling pathway. However, the mechanism that the down-regulation by RKIP and up-regulation by PKC work coordinately to mediate the physiological process in the cell is still unclear. In that, we still do not know when the positive mediation is dominant, or under what conditions the negative modulation displays more apparently.

Figure 4 Raf-1 signaling pathway is regulated by both RKIP and PKC. By binding to Raf-1 and MEK, RKIP prevents the Raf-1 binding with and phosphorylating MEK. Here RKIP is considered as a downstream suppressor. By phosphorylating the Serine 153 residue on RKIP, PKC induces the dissociation between Raf-1 and MEK, which in turn

Although this signaling cascade was mapped years ago and a great progress has been made in the study on it, there are still many unsolved problems on the modulation of Raf-1/MEK/ERK signaling pathway including what other specific proteins besides RKIP participate in regulation, the mechanism of modulation, what are the binding sites between the regulator proteins and their binding partners involved in the pathway and the timing of phosphorylation and de-phosphorylation of the kinases in the cascades.

1.2.1.2 Regulation of NF-κB signaling pathway

Similar to the Raf-1 signaling pathway, the NF-κB pathway is also characterized as a key modulator for many physiological functions, including cell growth, cell apoptosis, cell adhesion and immunity [44-50]. Errors in the NF-κB pathway are found to be responsible for cancer, inflammation, neurological disease, and improper immune development [51-55]. RKIP participates in not only the regulation of Raf-1 cascade we introduced above, but also that of NF-κB signaling pathway. Moreover, RKIP is reported to have an inhibitory function on NF-κB pathway [56].

The transcriptional activator nuclear factor kappa B (NF-κB) is a form of REL family dimeric protein [57] which is essential for regulating gene expression which is responsible for cell proliferation, survival, adhesion and immunity. Recent studies have revealed that gene expression related to NF-κB is activated by several kinds of stimuli, such as stress and inflammation [58]. Based on the fact that proinflammatory cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) are activators for the
NF-κB signaling pathway [59, 60], they stimulate the transforming growth factor β-activated kinase 1 (TAK-1) and NF-κB-inducing kinase (NIK) upon binding with tumor necrosis factor receptor 1 (TNF-R1) [24]. Afterwards, as the signal continues to be propagated when the TAK-1 and NIK activate by phosphorylating the IκB (inhibitor of NF-κB) kinase (IKK) which contains two subunits IKKα and IKKβ [61-70]. Subsequently, IKK activates and phosphorylates IκB, afterwards phosphorylated IκB is degraded by 26S proteasome. As a consequence, the phosphorylation and degradation of IκB causes the NF-κB to depart from the IκB-NF-κB complex. Finally, upon dissociation with IκB, NF-κB translocate to the nucleus and regulate the gene expression that responds to the NF-κB signaling pathway [71] (Figure 5).
Figure 5 The NF-κB signaling pathway. The NF-κB cascade is initiated by TNF-α which acts as a signal activator. The signal is propagated as follows: TAK-1/NIK→IKKα/β→IκB/NF-κB→NF-κB→gene expression in nucleus. The figure is referred from *Adv Cancer Res* (2004) 91: 169-200.

Kam Yeung *et al.* demonstrated that RKIP can modulate the NF-κB signaling pathway, as well as the Raf-1 signaling pathway [56]. It is significant that RKIP is able to regulate two different pathways which are independent of each other. In their study, RKIP is discovered as an inhibitor of NF-κB release so that it down-regulates the NF-κB signaling pathway. Although the detailed mechanism of the inhibitory function by RKIP still remains unknown, what we can recognize is that RKIP can prevent the phosphorylation of IKKa and IKKβ by associating with TAK-1 and NIK. It is very similar with mechanism that RKIP negatively regulates the Raf-1 cascade by interacting with Raf-1 and MEK, which blocks the phosphorylation and activation of MEK. Furthermore, another model was built to show that RKIP can also interact with IKKα and IKKβ [72]. This interaction leads to the blocking of the phosphorylation on IκB, so that IκB cannot be degraded and NF-κB cannot be released to regulate the gene expression. Taken together, RKIP is found to interact with four important kinases involved in the NF-κB signaling pathway, including TAK-1, NIK, IKKα and IKKβ, which cause the deactivation of NF-κB. Therefore, RKIP here acts as a down-regulator for the NF-κB cascade.

### 1.2.1.3 Regulation of GPCR signaling pathway
Previous studies have revealed that GPCR is yet another important signaling pathway which serves as a modulator for a variety of biological processes, including inflammation, hormone and blood pressure regulation [73]. As it is shown in the Raf-1 pathway, PKC phosphorylates RKIP on its Serine 153 site and leads to the dissociation of Raf-1-RKIP complex. Then, the phosphorylated RKIP departs from Raf-1 and binds to G protein coupled receptor kinase 2 (GRK-2), which is a suppressor for the stimulation of G protein coupled receptors (GPCRs) through their phosphorylating [10, 11, 74], leading to the loss of inhibitory activity of GRK-2. As a result, the GPCR signaling pathway is promoted because the deactivation of GRK-2 by the PKC-mediated phosphorylation of RKIP (Figure 6). Therefore, RKIP we report here works as a positive modulator for this pathway.
Figure 6 An overview scheme for the role of RKIP in Raf-1, G protein coupled receptor (GPCR) and NF-κB signaling pathways. The figure is referred from *Expert Opin. Ther. Targets* (2008) 12(10): 1275-1287.

### 1.2.2 Role of RKIP in cell cycle

Cell cycle, which is also called cell-division cycle, is a series of events involved in the cell division process. In the eukaryotes cells, a cell cycle starts at a resting phase (G0 phase) during which the cell has left the previous cycle and is ready for the next cycle. Then the next step of a cell cycle is interphase including G1, S and G2 phase, during which the cell keeps growing, replicates its DNA and gets everything ready for its final step – mitosis phase. In the mitosis phase (M phase), the cell stops growing and it is eventually orderly divided to two daughter cells [75, 76].

Many recent studies on the relationship between RKIP and the cell cycle have demonstrated that RKIP does play a key role in cell cycle regulation. Like the other physiological processes, cell cycle is also modulated by multiple signaling pathways. Raf-1/MEK/ERK as discussed above is reported to be one of the signaling cascades that function as a modulator of cell cycle [77]. The cell cycle is ended at mitosis phase during which a spindle checkpoint is formed in the middle of the mitosis [78, 79]. The spindle checkpoint is considered to be a monitor for making sure the kinetochore is attached to the correct position of the chromosomes, so that all replicated chromosomes can be divided equally and orderly to the two daughter cells. As a consequence, it is obvious that genomic stability is determined by the formation of this spindle checkpoint. Recent reports have revealed that RKIP is able to modulate the formation of the checkpoint by
inhibiting Aurora B kinase [80] (Figure 7). As it was mentioned previously, RKIP inhibits the Raf-1 signaling pathway by interrupting the interaction between Raf-1 and MEK. So it can be clearly seen that the depletion of RKIP leads to the hyper-activation of Raf-1 cascade, which is found to be a reason for down-regulating the Aurora B kinase [81, 82]. The inhibition of Aurora B kinase by RKIP results in abnormal separation of the chromosomes which may bring about cell apoptosis or even ontogenesis [83].

Furthermore, Al-Mulla et al. recently reported that depletion of RKIP in HEK 293 cells enhances the rate of the cell proliferation [84]. The proposed mechanism is related to the glycogen synthase kinase-3β (GSK3β) pathway. It has been shown that the GSK3β inhibits the activation of cyclin D1, which plays an important role in cell cycle. RKIP is known to act as a deactivator of GSK3β [85]. Thus, RKIP here works for stabilizing the cyclin D1. The acceleration of cell proliferation can be well explained by this significant finding. As with cyclin D1, there may be other gene regulatory proteins involved in cell cycle that are modulated by RKIP [86-88]. In the case of RKIP silencing, it is apparent that the expression and functions of these gene regulatory proteins are impacted. A proposed explanation for the higher rate of cell proliferation is that the depletion of RKIP induces some genes involved in interphase and mitosis phase abnormally expressed which leads to the reduction of time in G1/S and G2/M phase transition.
Figure 7 Regulation of spindle checkpoint by RKIP. The formation of the checkpoint in the cell cycle process is directly modulated by Aurora B kinase, which is effected by the Raf-1/MEK/ERK signaling pathway. In the case when RKIP is depleted, the hyper-activation of the Raf-1 cascade inhibits Aurora B kinase, thereby leads to the abnormal cell cycle. The figure is referred from Expert Opin. Ther. Targets (2008) 12(10): 1275-1287.

1.2.3 Role of RKIP in apoptosis

Apoptosis is known as a biological process of programmed cell death (PCD) which usually takes place in multicellular organisms [89-91]. Compared with necrosis, apoptosis is a normal biological activity which is considered as an advantageous phenomenon in the life cycle [92]. For example, there are 50 – 70 billion cells die each day due to apoptosis in a human adult [93]. As we know, apoptosis plays a critical role in human health. Deregulation of cell apoptosis pathway may cause drug resistance. The
drug resistant cells may become cancer cells when they lose the apoptotic function [94-96]. Nowadays, chemotherapeutic drugs or irradiation induced apoptosis has been widely used as a therapeutic treatment of cancer.

As it was described in the role of RKIP in signaling pathway, there are two major pathways that are able to regulate cell apoptosis by mediating the gene expression. They are Raf-1 and NF-κB cascades. Since it is already known that RKIP inhibits both of these two signaling pathways, it is very likely RKIP also has an effect on the regulation of cell apoptosis (Figure 8). Recently, many studies have provided enough evidence for the role of RKIP in cell apoptosis. In non-Hodgkin’s lymphoma B cells, RKIP expression is induced by the chemotherapy drug which is called Rituximab [97]. Consequently, induced expression of RKIP then inhibits the Raf-1 pathway and leads the cell death. Moreover, in RKIP overexpressed prostate and melanoma cells, the cell apoptosis is found to be regulated through NF-κB pathway [98]. Here, the apoptosis of the cells is stimulated by death receptor ligands, such as tumor necrosis factor α (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL) [99]. Therefore, these results clearly illustrate that RKIP functions as a modulator for cell apoptosis by inhibiting either Raf-1 or NF-κB pathway.
Figure 8 Model of RKIP up-regulating chemotherapy drug-induced cell apoptosis. Chemotherapy drug-induced expression of RKIP promotes the cell apoptosis by inhibiting Raf-1, NF-κB pathways or both simultaneously. The figure is referred from *Expert Opin. Ther. Targets* (2008) 12(10): 1275-1287.

### 1.2.4 Role of RKIP in cancer metastasis

Cancer metastasis is defined as a cancer cells leaving the original tumor and colonizing other tissues, forming secondary tumors and aggravating the disease [100, 101]. Thus, research in cancer metastasis inhibition has becoming a pressing topic. Here we report the function of RKIP in the regulation of tumor metastasis. RKIP was initially found to have lower expression in C4-2B cells (a form of metastatic prostate cells) compared with its expression in LNCaP (a form of nonmetastatic prostate carcinoma cells, the parental cells of C4-2B cells) [102]. Moreover, reduced or nonexpression of RKIP was subsequently
discovered in other cancer or tumor cell lines, including breast cancer [103], colorectal cancer [104-106], anaplastic thyroid cancer [107], hepatocellular carcinoma [108, 109], insulinomas [110] and melanoma [12, 111]. In addition, there is no effect on the growth of the primary tumor cells when the RKIP is overexpressed. The overexpression of RKIP is only found to inhibit the tumor invasion. Therefore, these findings show that RKIP can be regarded as a potential suppressor of cancer metastasis.

However, there is little known about the mechanism of the regulation of cancer metastasis by RKIP. One proposed mechanism is that RKIP modulates the metastasis by controlling the apoptotic pathway. The abrogation of the cell apoptosis process is one aspect that leads to cancer. RKIP is discovered as an inhibitor of Raf-1 and NF-κB signaling cascades which are responsible for cell apoptosis. In the tumor cells, the lack of RKIP may cause the disruption of these two key signaling pathways. As a consequence, the apoptotic signal is disrupted, so that the tumor cells with low RKIP levels show resistance to apoptosis leading to their metastasis [112]. However, some recent reports demonstrate the opposite opinion to the mechanism above. Their results have shown the fact that knockdown of RKIP in Merkel cell does not affect the level of phosphorylation of ERK [113]. Thus, it seems that the mechanism of how RKIP regulates metastasis is still poorly understood. Much work is required to be done to get a definitive answer to this problem.

1.2.5 Role of RKIP in cell migration

Cell migration is a multi-step process that involves dynamic assembly and disassembly of cytoskeleton, attachment and detachment to extracellular proteins, and translocation of
the cell, which plays a key role in many biological processes including embryonic development, immune function, and wound healing [114]. Moreover, errors in cell migration cause some diseased processes such as tumor formation, cancer cell invasion and metastasis [115].

By now, the relationship between RKIP and cell migration is not clearly understood, due to the conclusions made by different investigators are controversial. In our previous study, Zhu et al. demonstrated that knockdown RKIP decreases the rate of cell migration of Madin-Darby canine kidney (MDCK) epithelial cells. Moreover, they discovered a small molecule inhibitor of RKIP, locostatin, inhibits the activity of RKIP by disrupting the association between RKIP and Raf-1 [116]. Based on the function of locostatin, we previously reported that the cell migration rate of RKIP overexpressed MDCK cell is dramatically higher than that of normal MDCK cells. In addition to it, we also found cell migration rate of RKIP overexpressed MDCK cell treated with locostatin is slower than that of the control. Taken together, our previous study reveals that RKIP plays a positive role in cell migration. However, opposite conclusion of the function of RKIP on cell migration was made by other groups. They demonstrated that the overexpression of RKIP leads to the decrease of the rate of cell migration in other cells, such as hepatoma and melanoma cell lines [84, 109, 117]. These contradictory findings show that the effect RKIP on cell migration is cell line dependent. The detailed mechanism for the role of RKIP in cell migration requires further study.
CHAPTER II

RAF-1 KINASE INHIBITOR PROTEIN NUCLEAR LOCALIZATION HAS A
REGULATORY EFFECT ON CELL MIGRATION AND PROLIFERATION
INDEPENDENT OF THE MAPK/ERK PATHWAY

2.1 Introduction

Raf-1 kinase inhibitor protein (RKIP) is a 21 kDa protein that was initially discovered as a member of phosphatidylethanolamine-binding protein (PEBP) family and named PEBP1, as it weakly binds to phophatidylethanolamine [118, 119]. PEBP1 was renamed as RKIP since it was found to inhibit Raf-1 kinase. From numerous experimental results, RKIP has been discovered as an essential protein in many signaling pathways. In the Raf-1/MEK/ERK signaling pathway, RKIP binds to Raf-1 kinase [120, 121] and disrupts the interaction between Raf-1 and mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MAPK/ERK), thus preventing phosphorylation and activation of MEK1 and results in the negative regulation of the Raf-1 signaling cascades [120, 121].

As we know, phosphorylation on the serine 153 residue of RKIP by Protein kinase C (PKC) leads to dissociation between RKIP and Raf-1. This results in a new association between RKIP and G protein-coupled receptor kinase 2 (GRK2) [122, 123]. In GPCR signaling pathway, GRK2 phosphorylates G protein-coupled receptors (GPCRs) leading to an internalization of the receptors and subsequent inhibition of their signaling activity [123]. The association between RKIP and GRK2 results in the inactivation of GRK2 which leads to a positive regulation of GPCR signal receptor activation [123]. Furthermore, RKIP expression has also been found to antagonize the activation of the
NF-κB pathway [124, 125]. In the NF-κB signaling pathway, RKIP was found to interact with the following kinases involved in the NF-κB cascade: transforming growth factor β-activated kinase 1 (TAK1) [124], nuclear factor κB (NF-κB)-inducing kinase (NIK) [124], inhibitor of κB kinase α (IKKα) and β (IKKβ) [124]. To our knowledge, the interaction between RKIP and the four kinases listed above is regarded as a supposed mechanism of the down-regulation of NF-κB signaling pathway by RKIP. Recently, it was discovered that RKIP enhances GSK3 signaling by stabilizing GSK3 [126]. Interestingly, the aforementioned RKIP modulated signaling pathways have all been shown to play a role in cell cycle progression [127-132]. However, the physiological role of RKIP is not limited to cell proliferation. RKIP has also been associated with apoptosis [133-136], cell adhesion [137], cell migration [128, 136-145], and as a tumor suppressor in a wide variety of cancer cell types [127, 128, 133, 134, 141, 146-159]. These studies suggest that the biological effects of RKIP are quite complex and cannot be elucidated by one pathway.

In order to formulate a conceivable mechanism by which RKIP regulates biological activities such as cell migration and proliferation, we have studied exhaustively on the relationship between localization of RKIP to the nucleus and the biological activities modulated by RKIP. To our knowledge, it has been reported that RKIP is able to translocate in the nucleus [129, 130]. However, the mechanism of RKIP translocation still remains unclear. So far, two mechanisms have been discovered as the pathways by which the small size proteins are transported into the nucleus. Proteins with a molecular mass is lower than 45 kDa are believed to translocate to the nucleus via passive diffusion. Thus, the translocation mechanism of RKIP was believed to be by passive diffusion due
to its limited size (For a review, see ref. [161]). For the proteins greater than 45kDa, the transportation of the proteins from the cytoplasm to the nucleus depends on the importin superfamily which is functions as transport proteins. In this translocation mechanism, nuclear localization signal (NLS), which is a short stretch of amino acids on the protein being transported. NLS plays a key role in the recognition between the target proteins and importin. Proteins with NCL are initially recognized by a heterodimer importin α/β, then translocated into the nucleus. In collaboration with Christian Argueta, we have previously reported that a small stretch of amino acids (146-RGKFKVASRFKK-157) is the NLS targets RKIP to the nucleus. Our experimental results demonstrated that the nuclear localization signal (NLS) on RKIP binds to the nuclear transport protein importin α and ultimately leads to nuclear translocation.

Based on the discovery of the nuclear localization signal on RKIP, we have done considerable work to determine if there is any connection between the nuclear localization of RKIP and cell migration and proliferation. Initially, we chose wild type RKIP expressing mouse embryonic fibroblasts (MEF RKIP+/+) cells to conduct the experiment. Interestingly, the mouse embryonic fibroblasts cells with green fluorescence protein (GFP) labeled RKIP expressed (MEF RKIP+/+ + GFP-RKIP) have a slightly higher rate of cell migration than wild type RKIP expressing mouse embryonic fibroblasts (MEF RKIP+/+) cells. In addition to this result, we discovered that MEF RKIP+/+ cells with NLS deficient mutants of RKIP expressed have a visibly slower rate of cell migration than the wild type MEF RKIP+/+ cells. Therefore, we can draw a conclusion that the nuclear localization of RKIP does have an obviously regulatory effect on the cell migration of MEF cell line. Furthermore, we also worked on exploring if NLS
has the same regulatory effect on cell proliferation. To our surprise, the cell proliferation result fails to prove this assumption. We found that MEF RKIP^{+/+} + GFP-RKIP cells have a slower rate of cell proliferation than MEF RKIP^{+/+} cells. However, some of MEF RKIP^{+/+} cells expressing NLS deficient RKIP mutants have a higher rate of cell proliferation than MEF RKIP^{+/+} cells, whereas some of them have the opposite effect.

Thus, we failed to see the nuclear localization of RKIP in MEF cell line plays a role in regulating the cell proliferation process.

Previous studies have been conducted on cells that are over-expressing RKIP or have knocked down the expression of RKIP. Recent studies have shown that RKIP depletion regulates the transcription of proteins involved in both cell migration and proliferation [128]. A few studies have gone so far as to completely remove RKIP from the mouse embryonic fibroblasts (MEF) [130, 139]. These studies have shed valuable light on the function of RKIP, and prompted us to see if we could determine whether RKIP had the same effect when it is reintroduced in to cells lacking the gene for RKIP. We believe the work on the cells lacking gene for RKIP is a complementary step to explain the cell migration and proliferation result we found before. With this new method, we can clearly see if RKIP is the main factor that plays an essential role in the regulation of cell motility and growth. Moreover, downregulation of RKIP has been previously shown to promote the activation of Raf-1 cascade in several cell lines. However, the modulation on Raf-1/MEK/ERK pathway is ambiguous and still poorly understood. A few evidences so far cannot prove that RKIP always has the regulatory function in inducing the activation of Raf-1 signaling pathway. It is already known that different cell systems present different results. Therefore, we can also use the mouse embryonic fibroblasts completely lacking
RKIP (MEF RKIP^{+/}) cell line to verify if RKIP has the same function on the activation process of Raf-1/MEK/ERK signaling pathway.

Here we reported that RKIP promoted the rate of migration when we reintroduced RKIP to MEF cells completely lacking RKIP. RKIP also inhibited proliferation in MEF cell line. The effects that RKIP has on cell migration and proliferation could be linked to its nuclear localization. We identified that GFP-RKIP expressing RKIP^{+/} cells had a significantly lower number of cells in G2/M phase when compared to RKIP^{-/-} cells. We also found that RKIP had no effect on Raf activation in serum-stimulated cells.

2.2 Materials and Methods

Cell culture

Mouse embryonic fibroblasts (MEF) expressing RKIP (wild type RKIP^{+/+}) and MEF deficient in RKIP (homozygous RKIP^{-/-}) were provided by Prof. Kam Yeung and cultured in Dulbecco’s modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C with 5% CO2. Early passages of cells cultured from frozen stock cultures were used in all experiments. Main cultures were grown in 75cm^{2} tissue culture flasks with medium changed every two days. When the cultures reached 90% confluence, the cells were gently washed twice with phosphate buffered saline (PBS), and treated with a solution of trypsin/ ethylenediaminetetraacetic acid (EDTA) in PBS to detach cells from the flasks. After cells were detached, an equal volume of fresh medium was added to inhibit the trypsin activity. The cells were resuspended in media and cell density was determined with a hemacytometer. Cells were
replanted in fresh medium in new tissue culture flasks for continued culture and multi-well tissue culture plate for experiments.

**Construction of stably transfected cell lines**

Human RKIP was cloned into the pEF6-myc-his B vector containing green fluorescent protein (GFP; a kind gift of Prof. David Knecht) in cloned into its Eco RI and Eco RV sites. The point mutants were constructed with the following primers:

- **R146N**: 5′-AACCGATCTGGAGACCACTGCCAATTCAAGGTGCC-3′ and 5′-GCCACCTTGAATTTCGCCATTGTGGTCTCCAGATCGGT-3′
- **K148N**: 5′-AGACCACCCTGGCAATTTCAAGGTGCCGGTC-3′ and 5′-GACGCCACCTTGAATTTCGCCACGGGTGGTCT-3′
- **K150N**: 5′-GACCACCCTGGCAATTTCAAGGTGCCGGTC-3′ and 5′-AAGGACGCCACATTGAATTTCGCCACGGGTGGTC-3′
- **R146N, K148N, K150N**: 5′-CCGATCTGGAGACCACTGCCAATTTCAGTGCCGGTCCTCCGTATA-3′ and 5′-GGCTAGACCTCCTGGTGTTACCGTTAAAGTTACCCGCAGGAAGGCAT-3′
- **R155N**: 5′-AATTCAAGGTGGCGTCCTTTCAATAAAAAAGTATGAGCTCAGGG-3′ and 5′-CCCTGAGCTCATACCTTTTATTGAAGGACGCCACCTTGAATT-3′
- **K156N**: 5′-GTGGCGTCCTTTCCGTAATAAGTATGAGCTCAGGG-3′ and 5′-CCCTGAGCTCATACCTTTTATTGAAGGACGCCACCTTGAATT-3′
- **K157N**: 5′-GTGGCGTCCTTTCCGTAATAAGTATGAGCTCAGGG-3′ and 5′-GCCCTGAGCTCATACCTTTTATTGAAGGACGCCACCTTGAATT-3′
- **R155N, K156N, K157N**: 5′-AAATTCAAGGTGGCGTCCTTTCCGTAATAAAATTATGAGCTCAGGGCCCGGT-3′
and 5’-
CACC GG GCCCTGAGCTCAT AATTATTATTGAAGGACGCCCACCTTGAAATT-3’
R155N, K148N, K150N, R155N, K156N, K157N:
5’AATTTCAATGTGGCCGT CCTTCAATAATAATTATGAGCTCAGGGCCACCATGGAATT-3’ and 5’-
CCCCCCGCCCTGAGCTCATAAATTATTATTGAAGGACGCCCACATTGAAATT-3’
All GST-tagged rat RKIP (a kind gift of Prof. Kam Yeung) deletion mutant constructs
were cloned in the pEF6-GFP-Myc-His B vector in between the Eco RV and Not I sites
using the following primers:
GST fusion: 5’-ATAGCGCGGCATATCATGTCCCCTATATTACCTAGG-3’ and 5’-
ATAGCGCCGCCGCTCAGCATGAATAAGCTT-3’
Once sequenced, the vectors were transfected using Lipofectamine 2000.
The GFP-NLS fusion construct was created by PCR amplification of the NLS with the
following primers:
NLS fusion: 5’-GCGCGCATAGAATTCTCGTG GCAAATTCAAG-3’ and 5’-
GCGCGCATAGATATCCTTTTACGGAAGGACG-3’
The amplification product was then digested with Eco RI and Eco RV and ligated into the
pEF6-GFP-Myc-His B vector.
Once sequenced, the GFP tagged Human RKIP and RKIP mutant proteins vectors
described above were transfected in to MEF+/+ and MEF−/− cells with Lipofectamine 2000
(Invitrogen) and selections was performed with 10 µg/mL Blasticidin S (InvivoGen) for
three weeks. After selection the cells were cultured in DMEM containing 10% FBS with
10 µg/mL Blasticidin S.
**Wound closure assay**

All of the MEF created were plated on to 24-well tissue culture plates at a density of 1x10^5 cells/mL and allowed to reach confluence. Once confluent the cells were treated with 10 µg/mL Mitomycin C and wounded manually with a small 0.5-10 µL pipette tip. After 3 hours the media was replaced with fresh DMEM and the experiment continued until the wounds were completely closed. Digital images were acquired as soon as the wounds were made and at 3, 6, 9, 12, and 24 hours post wounding. The open wound areas were obtained using NIH Image J software and were subsequently plotted against time using GraphPad Prism software [136].

**Cell Proliferation**

Cell proliferation was measured using the CCK-8 kit as described by Dojindo. MEF cells (124µL of a 4x10^4 cells/mL in DMEM growth medium) were plated in to two 96-well tissue culture plates and incubated at 37 °C for 24 hours. After 24 hours one plate was treated with WST-8 (CCK-8; Dojindo) in order to determine cell density at the start of the experiment. The parallel plate was treated with WST-8 48 hours later and compared to the numbers generated from the first plate. A ratio of 48hr/24hr was obtained for each MEF cell line generated and plotted using GraphPad Prism.

**Cell cycle analysis**

MEF were plated at a density of 2x10^6 cells/mL and allowed to adhere overnight. 24 hours later, the cells were washed three times with 1x Phosphate Buffered Solution (PBS) and trypsinized for 30 minutes. The cells were pelleted by centrifugation at 1000 rpm for 3 minutes and washed three times with PBS. The cells were then resuspended in mL PBS
and fixed by adding ice-cold ethanol drop-wise with vortexing to prevent aggregation. The cell suspension was then incubated overnight at 4°C. Following the fixation process, the cells were pelleted and washed 3 times and resuspended in PBS containing 0.1% Triton X-100, 250 μg/mL RNase and 50 μg/mL propidium iodide for 1 hour. Cell cycle analysis was performed by flow cytometry (BD Calibur, BD Biosciences) and analyzed using Cell Quest software.

**Raf activation assay**

MEFs were plated at 1x10^5 cells/mL in 6-well tissue culture plates and allowed to reach confluence. Once confluent, the cells were serum starved for 48 and activation was achieved by reintroducing FBS into their medium. After 30 minutes the cells were lysed in 100μL 2x SDS sample buffer and scraped into a centrifuge tube. The samples were boiled for 10 minutes and subsequently centrifuged at 15000 rpm for 10 minutes. After centrifugation, the samples were subjected to SDS-PAGE and transfected to polyvinylidifluoride membrane. ERK activation was determined using the rabbit anti phospho p-44/42 MAPK antibody (Cell Signaling Technology). The primary antibody was probed with a goat anti-rabbit HRP antibody (Santa Cruz Biotechnology) with visualization by enhanced chemiluminescence.

**Raf pull-down assay**

Raf pull down assays were performed by plating MEFs at 2x10^5 cells/mL in 100 mm dishes. The cells were allowed to adhere overnight and well lysed the following morning using ice cold 1xPBS supplemented with protease inhibitors (sigma) and 0.25% Triton X-100 for 30 minutes. The samples were then centrifuged at 15000 rpm to remove any
insoluble debris and the GFP fusion proteins were pulled down by incubating an anti-GFP (FL) antibody (Santa Cruz Biotechnology) for 3 hours followed by protein G magnetic beads for 1 hour. The beads were pelleted by at 1000 rpm for 3 minutes and washed 3 times with PBS. The pellets were resuspended in 100 μL 2x SDS sample buffer and subjected to SDS PAGE. The samples were transferred to PVDF and probed with a rabbit anti Raf-1 (C-12) antibody (SantaCruz Biotechnology). The Raf-1 antibody was probed with a goat anti rabbit HRP antibody and visualized by enhanced chemiluminescence.

2.3 Results

RKIP has no effect on cell migration

We have previously shown that RKIP expression de-regulates cell cell adhesion and that cells treated with a RKIP inhibitor migrated at a slower rate in Madin-Darby canine kidney cells [140]. To determine whether RKIP has any effect, positive or negative, on cell migration in MEF cells, we reintroduced RKIP into MEF RKIP+/+ and MEF RKIP−/− cells. The cells were treated with Mitomycin C (MMC) to eliminate the effect cell division and proliferation would have on the rate of wound closure. Following treatment the cells were scratch wounded with a sterile micropipette tip. The rate of migration was measured as function of wound area plotted against time until the wound was 100% closed (roughly 24 hours). Comparing the wound closure rate of MEF+/+ and that of MEF+/+ GFP RKIP, we can see there is no visible differences between rates of migration relevant to the amount of RKIP expression (Fig.1A). We can also draw a conclusion that GFP introduced in MEF+/+ does not have any effect on cell migration (Appendix Fig.2).
Therefore, we believe that RKIP is the only factor that controls the modulation of cell migration. We then reintroduce RKIP to MEF RKIP\(^{-/-}\) cells to see what role of RKIP can play in cell migration. To our surprise, MEF\(^{-/-}\) GFP RKIP has a dramatically higher rate of migration than MEF\(^{-/-}\) (Fig.1B). Although there is no obviously difference in the rates of migration between MEF\(^{+/+}\) and MEF\(^{-/-}\), we can still find that MEF\(^{+/+}\) migrates very slightly faster than MEF\(^{-/-}\). Therefore, these strong evidences reveal that RKIP has no effect on cell migration of MEF cell line.

Fig. 1A Wound closure in MEF RKIP\(^{+/+}\) and MEF RKIP\(^{+/+}\) GFP RKIP cells in the presence of mitomycin C. we cannot see there is any visible difference between rates of migration relevant to the amount of RKIP expression.
Fig. 1B Wound closure in MEF RKIP\(^{+/+}\), MEF RKIP\(^{-/-}\) and MEF RKIP\(^{-/-}\) GFP RKIP cells in the presence of mitomycin C. MEF RKIP\(^{-/-}\) GFP RKIP migrates dramatically faster than MEF RKIP\(^{-/-}\). There is no obviously difference in the rates of migration between MEF RKIP\(^{+/+}\) and MEF RKIP\(^{-/-}\), but we can still find that MEF RKIP\(^{+/+}\) migrates very slightly faster than MEF RKIP\(^{-/-}\).

**NLS of RKIP has a regulatory effect on cell migration**

We have demonstrated that RKIP is able to promote cell migration in MEF cell line. To determine whether or not NLS of RKIP expression has a regulatory effect on cell migration, we reintroduced NLS deficient point mutants of RKIP, which are described in construction of stably transfected cell lines of materials and methods part, into MEF RKIP\(^{+/+}\) and MEF RKIP\(^{-/-}\) cells. Although we have not collected the data for MEF RKIP\(^{-/-}\) cells yet, we can still find an interesting result from the MEF RKIP\(^{+/+}\) cell migration data. MEF RKIP\(^{+/+}\) GFP RKIP K148N has a positive effect on cell migration (Appendix
Fig. 6). However, MEF RKIP\textsuperscript{+/+} GFP RKIP R155N, MEF RKIP\textsuperscript{+/+} GFP RKIP K157N and MEF RKIP\textsuperscript{+/+} GFP GST RKIP (1-134) have a negative effect on cell migration (Appendix Fig. 8, 10 and 12). Moreover, we also found that MEF RKIP\textsuperscript{+/+} GFP RKIP R146N, MEF RKIP\textsuperscript{+/+} GFP RKIP K150N, MEF RKIP\textsuperscript{+/+} GFP RKIP K156N MEF RKIP\textsuperscript{+/+} GFP RKIP R146N K148N K150N, MEF RKIP\textsuperscript{+/+} GFP RKIP R155N K156N K157N and MEF RKIP\textsuperscript{+/+} GFP GST RKIP showed no effect on cell migration (Appendix Fig. 3, 5, 7, 9, 11 and 13).

**RKIP has a regulatory effect on cell proliferation**

To determine whether or not RKIP expression had a regulatory effect on cell proliferation, we plated MEF cells of the cells in 96-well tissue culture treated plates and measured their proliferation over 24 hours with WST-8. Wild type MEF cells exhibited a slower proliferation rate when compared to RKIP deficient RKIP\textsuperscript{-/-} cells (Fig. 2A). Similarly, RKIP\textsuperscript{-/-} cells exhibited a faster rate of proliferation when compared to RKIP\textsuperscript{-/-} cells re-expressing RKIP (Fig. 2B).
Fig. 2A MEF cell proliferation is regulated by RKIP expression. Wild type MEF RKIP$^{+/+}$ cells proliferate at a significantly slower rate when compared to MEF RKIP$^{+/−}$.

**NLS RKIP has a regulatory effect on cell proliferation**

We have demonstrated that RKIP is able to reduce the rate of cell proliferation in MEF cell line. To determine whether or not NLS of RKIP expression has a regulatory effect on cell migration, we reintroduced NLS deficient point mutants of RKIP, which are described in construction of stably transfed cell lines of materials and methods part, into MEF RKIP$^{+/−}$ cells. Surprisingly, MEF RKIP$^{+/−}$ cells expressing RKIP missing the NLS show the tendency to revert to the RKIP$^{−/−}$ proliferation rate (Fig. 2B). We can clearly see that, except for MEF RKIP$^{+/−}$ GFP RKIP K150N, the rest of the MEF RKIP$^{+/−}$ cells with NLS point mutants of RKIP expressed has the similar or higher rate of proliferation than MEF RKIP$^{+/−}$ GFP RKIP cells. Therefore, there seems to be a regulatory effect NLS of RKIP would have on the cell proliferation of MEF cell line.
Fig. 2B MEF cell proliferation is regulated by RKIP expression. MEF RKIP-/- cells with RKIP and various RKIP mutants proliferate at a slower rate much like wild type MEF RKIP+/+ cells. Mutations located in the NLS or completely removing the NLS in the C-terminus of RKIP negatively affects the proliferative effect observed in MEF RKIP+/+ cells. Asterisks indicate statistical significance (p<0.05 compared to controls) derived from three different experiments.
RKIP expression regulates cell cycle distributions

To prove that RKIP expression alone was enough to regulate the cell cycle cultured cells deficient in RKIP along with RKIP<sup>−/−</sup> with RKIP introduced. Following a 24-hour incubation the cells were fixed and the cell cycle distribution was analyzed using flow cytometry after staining with propidium iodide. Consistent with previous results, RKIP expression coincided with a larger amount of cells in the apoptotic and G2/M phases when compared to RKIP<sup>−/−</sup> cells expressing GFP (Fig. 3)

Fig. 3 Deletion of the C terminus of RKIP eliminates effect RKIP has on cell cycle distributions in MEF RKIP<sup>−/−</sup> cells. Cell cycle analysis of unsynchronized MEF RKIP<sup>−/−</sup> cells expressing RKIP showed increased apoptosis and a larger amount of cells in S phase with a decrease in the amounts of cells in G2/M. MEF RKIP<sup>−/−</sup> cells expressing RKIP (1-
134) reverted to MEF RKIP<sup>+/-</sup> profile. Asterisks indicate statistical significance (p<0.05 compared to controls) derived from three different experiments.

**RKIP expression has no effect on Raf-1 activation in cells lacking serum stimulation**

To determine whether a lack on nuclear accumulation resulted in decreased Raf-1 activity we serum starved and stimulated our engineered cell lines to observe the effect. No observable difference was detected after stimulation in all of engineered cell lines (Fig 4A). Interestingly, MEF<sup>+/+</sup> cells with RKIP NLS deficient mutants bound Raf but failed to mitigate its activity in the presence of serum stimulation.

![Western blotting of p-ERK in MEF whole cell lysates after serum starvation and subsequent stimulation with 10% serum. Experiment performed in triplicate. 1) MEF RKIP<sup>+/+</sup>, 2) MEF RKIP<sup>+</sup>, 3) MEF RKIP<sup>+</sup> GFP, 4) MEF RKIP<sup>+</sup> GFP-RKIP, 5) MEF RKIP<sup>+</sup> GFP-GST-RKIP, 6) MEF RKIP<sup>+</sup> GFP-GST-RKIP(1-134) and 7) MEF RKIP<sup>+</sup> GFP-GST-RKIP(60-187).](image-url)
Fig. 4B RKIP expression in MEF cells fails to suppress ERK activity in the presence of serum stimulation. Western blotting of Raf in MEF cells after co-immunoprecipitation of GFP. 1) MEF RKIP<sup>+/−</sup> GFP, 2) MEF RKIP<sup>+/−</sup> GFP-RKIP, 3) MEF RKIP<sup>+/−</sup> GFP-RKIP (146-NGNFNVASFNNN-157), 4) MEF RKIP<sup>+/−</sup> GFP-GST-RKIP, and 5) MEF RKIP<sup>+/−</sup> GFP-GST-RKIP(1-134).

2.4 Discussion

RKIP is a multifaceted protein that has been linked to tumor metastasis, cell migration, and cell proliferation. However, a consensus on the role RKIP plays in these processes has yet to be reached since different groups have seen different results. To study the effect RKIP has we decided to study wild type MEF cells along with MEF cells completely devoid of RKIP. We then reintroduced RKIP in to the cells lacking RKIP and observed some of the more variable results published in the RKIP field.

RKIP has been shown to inhibit [128, 139, 143], promote [137, 140, 144, 162], and have no effect [162] on cell migration. These seemingly contradictory results could depend on the migration analysis method in question. For example, some groups favor a larger wound and measure the time needed for the cells to fill in the gap created by the
wounding mechanism. Although this method is a valid way to measure migration, a larger wound takes longer to close allowing for cell division to take place during the healing process. The cell line used could also explain the seemingly variable results. Some cell lines migrate faster, making any role RKIP has in the process harder to measure. In addition, knockdowns do not completely eliminate the protein in question and some of the error could be due to remaining RKIP activity. To address these issues, we used MEF+/+, MEF+/−, MEF+/+ +GFP-RKIP and MEF+/− +GFP-RKIP cells to conduct the experiment. We also made small wounds that would lessen the effect cell division would have on the migration process. Considering there is still a remaining effect cell division in the wound healing process, we then treated the cells with MMC to eliminate this negative effect. We noticed that MMC did significantly slow the rate of cell migration (Appendix Fig.1). The data we have collected reveals that RKIP has no effect on the cell migration (Fig. 1). Furthermore, we found that MEF RKIP+/+ GFP RKIP with NLS deficient point mutants of RKIP expressed have a variety of regulatory effects on cell migration. The different results exhibited that they have positive, negative, and neutral effects on cell migration (Appendix Fig.5-13). The reason for these conflicting results is difficult to explain. But, in my opinion, NLS of RKIP still seems to have a regulatory function on cell migration. Perhaps some of the mutants do not affect RKIP binding to importin α and its translocation to nucleus in which RKIP plays a role in modulating the protein expression involved in cell migration process. The mutants that showed the opposite regulatory effect on cell migration can be accounted for the substitution of the amino acids on NLS disrupting the binding between RKIP and importin α preventing RKIP from being transported into nucleus. Without the regulation
of protein expression involved in cell migration process by RKIP, the cells exhibit a lower rate of cell migration.

Another biological process that seems to vary depending on the cell type is cell proliferation. A few groups have not seen any link between RKIP and cell proliferation [146, 147, 156], while the remainder of the studies have noticed a negative effect on proliferation [130, 143, 144, 163]. When comparing wild type MEF cells with RKIP deficient MEF cells we noticed that the cells expressing RKIP were proliferating at a higher rate (Fig. 2A). We then introduced GFP and GFP-RKIP into MEF RKIP\(^+\) cells and confirmed the role RKIP plays in reducing cell proliferation (Fig. 2B). Moreover, we transfected cells lacking RKIP with GFP-RKIP NLS point mutants and RKIP deletion mutants [121]. Interestingly, we found that RKIP 146-NGNFNVASFNNN-157 failed to reduce the cell proliferation as MEF RKIP\(^+\) GFP-RKIP did. However, MEF RKIP\(^-\) GFP-RKIP with single amino acid substitutions on NLS of RKIP still have the same regulatory effect on cell proliferation with MEF RKIP\(^+\) GFP-RKIP. Furthermore, GFP-GST-RKIP and GFP-GST-RKIP (60-187) exhibited regulation of proliferation while the GFP-GST-RKIP (1-134) reverted to the RKIP deficient proliferation profile. These results suggest that nuclear localization is necessary for RKIP to reduce cell proliferation. Although the mechanism that nuclear localization of RKIP regulates the cell proliferation remains ambiguous, in my opinion, it can be explained with the similar reason that I described above in the discussion part for cell migration. Cell proliferation of MEF cell line may be regulated only when RKIP goes into the nucleus and modulates the gene expression involved in cell proliferation process.
It has been reported that phosphorylation of RKIP causes dissociation of Raf [123] and association with centrosomes and kinetochores [130]. The authors found that RKIP depleted cells had lower Aurora B kinase activity [130], and they reasoned that RKIP depleted cells could override spindle checkpoints ultimately accelerating the cell cycle progression. To determine whether the effect on proliferation could be linked to cell cycle progression, we used the mutants that seemed to have the most detrimental effect and study the cell distribution by flow cytometry. Consistent with previous results [128, 133, 135, 164], RKIP seems to increase apoptosis and promote a shift of cell distribution in the S phase (Fig. 3). The authors reasoned that RKIP regulates a checkpoint in G2 that cells are required to go through in order to enter M phase. Absence of RKIP allows the cells to skip this checkpoint and proliferate at a higher rate.

RKIP was first initially found to disrupt the interaction between Raf and MEK subsequently preventing the activation of MAPK/ERK pathway [120]. The MAPK/ERK pathway has been previously implicated in cell cycle progression [165-170]. To determine whether the regulatory effect RKIP seems to have in MEF cells was directly related to Raf activation, we serum starved confluent cells for 48 hours and then stimulated the cells with the addition of 10% FBS. Both wild type and RKIP depleted MEF cells responded to the stimulus. The GFP-RKIP and GFP-RKIP NGNFSFNASFNNN expressing cells also exhibited normal Raf activation (Fig. 4A). Interestingly, and the GFP-GST-RKIP (1-134) mutant lacking the serine 153 phosphorylation site still exhibited Raf activation in the presence of serum. This result could imply that a different method of regulation is available or that the protein is inactive (Fig. 4B). To determine whether the C terminus deletion mutant was active we
performed pull down experiments for RKIP full length and C terminus deletion mutants. We found that RKIP and the RKIP deletion mutant associated with Raf, consistent with previous results [121]. Taken together, these results indicate that nuclear localization is required for the role regulatory role RKIP plays in both cell migration and cell proliferation. In addition, these regulatory activities are independent of Raf-1/MEK/ERK signaling pathway. Furthermore, these results also reveal that there may be a new signaling pathway in which RKIP has the regulatory function on cell migration and proliferation of MEF cell line. To discover what pathway it is, more work needs to be done in the future. However, what we know is that RKIP modulates a variety of processes in a large number of cell lines. Conflicting results can be attributed to both different cell lines and different methods used to manipulate RKIP expression.

In summary, we discovered that RKIP has no effect on the rate of migration of MEF cell line. RKIP also inhibited proliferation in MEF cell line. The effects that RKIP has on cell migration and proliferation could be linked to its nuclear localization. We did notice an increase in the amount of cells in apoptosis and S phase by flow cytometry in cells expressing RKIP, but not cells expressing RKIP without a NLS. We also confirmed that Raf activity was not the reason that RKIP reduced cell proliferation.
APPENDIX

Fig. 1 Mitomycin C can eliminate effect cell division and proliferation have on the rate of cell migration. We can obviously see MEF RKIP\(^{+/+}\) with mitomycin C treated migrates significantly slower than MEF RKIP\(^{+/+}\) without mitomycin C treated.

Fig. 2 GFP introduced into MEF RKIP\(^{+/+}\) does not have any effect on cell migration. MEF RKIP\(^{+/+}\) GFP has almost the same rate of cell migration with MEF RKIP\(^{+/+}\).
Fig. 3: GFP GST introduced into MEF RKIP+/+ does not have any effect on cell migration. MEF RKIP+/+ GFP GST has almost the same rate of cell migration with MEF RKIP+/+.

Fig. 4: Wound closure in MEF RKIP+/+ and MEF RKIP+/+ GFP RKIP cells in the presence of mitomycin C.
Fig. 5 Wound closure in MEF RKIP$^{+/+}$ and MEF RKIP$^{+/+}$ GFP RKIP R146N cells in the presence of mitomycin C.

Fig. 6 Wound closure in MEF RKIP$^{+/+}$ and MEF RKIP$^{+/+}$ GFP RKIP K148N cells in the presence of mitomycin C.
Fig. 7 Wound closure in MEF RKIP\textsuperscript{+/+} and MEF RKIP\textsuperscript{+/+} GFP RKIP K150N cells in the presence of mitomycin C.

Fig. 8 Wound closure in MEF RKIP\textsuperscript{+/+} and MEF RKIP\textsuperscript{+/+} GFP RKIP R155N cells in the presence of mitomycin C.
Fig. 9 Wound closure in MEF RKIP^{+/+} and MEF RKIP^{+/+} GFP RKIP K156N cells in the presence of mitomycin C.

Fig. 10 Wound closure in MEF RKIP^{+/+} and MEF RKIP^{+/+} GFP RKIP K157N cells in the presence of mitomycin C.
Fig. 11 Wound closure in MEF RKIP+/+ and MEF RKIP+/+ GFP RKIP (R146N, K148N, K150N) cells in the presence of mitomycin C.

Fig. 12 Wound closure in MEF RKIP+/+ and MEF RKIP+/+ GFP GST RKIP (1-134) cells in the presence of mitomycin C.
Fig. 13 Wound closure in MEF RKIP+/+ and MEF RKIP+/+ GFP RKIP (R155N, K156N, K157N) cells in the presence of mitomycin C.
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