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Role of km23-1 in RhoA/actin-based cell migration

Qunyan Jin [a,1], Nageswara R. Pulipati [a,1], Weidong Zhou [b,1], Cory M. Staub [a], Lance A. Liotta [b], Kathleen M. Mulder [a,*]

[a] Department of Biochemistry and Molecular Biology, Penn State Hershey College of Medicine, PA 17033, United States
[b] Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA 20110, United States

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ABSTRACT

km23-1 was originally identified as a TGFß receptor-interacting protein that plays an important role in TGFß signaling. Moreover, km23-1 is actually part of an ancient superfamily of NTPase-regulatory proteins, widely represented in archaea and bacteria. To further elucidate the function of km23-1, we identified novel protein interacting partners for km23-1 by using tandem affinity purification (TAP) and tandem mass spectrometry (MS). Here we show that km23-1 interacted with a class of proteins involved in actin-based cell motility and modulation of the actin cytoskeleton. We further showed that km23-1 modulates the formation of a highly organized stress fiber network. More significantly, we demonstrated that knockdown (KD) of km23-1 decreased RhoA activation in Mv1Lu epithelial cells. Finally, our results demonstrated for the first time that depletion of km23-1 inhibited cell migration of human colon carcinoma cells (HCCCs) in wound-healing assays. Overall, our findings demonstrate that km23-1 regulates RhoA and motility-associated actin modulating proteins, suggesting that km23-1 may represent a novel target for anti-metastatic therapy.

1. Introduction

Cell migration is an important aspect of the tumor metastatic process that transforms tumor cells from local, noninvasive, confined cells to the migrating, metastatic cancer cells [1]. Cell migration is a highly integrated multistep process that is initiated by the protrusion of the cell membrane [2,3]. Protrusive structures formed by migrating and invading cells are termed filopodia, lamellipodia, and invadopodia/podosomes, dependent on their morphological, structural, and functional characteristics [3,4]. Formation of these protrusive structures is driven by spatially- and temporally-regulated actin polymerization at the leading edge [4].

To date, several important proteins that regulate reorganization of the actin cytoskeleton have been identified and found to be overexpressed in several types of cancers [3]. For example, the Wiskott–Aldrich syndrome protein (WASP) family/actin-related protein 2 and 3 (Arp2/3) complex, LIM-kinase (LIMK)/cofilin, and cortactin pathways have been studied extensively due to their recognized importance in cell migration and invasion [3]. In addition, the Rho family of GTPase proteins Rho, Rac, Cdc42 are well-established regulators of cell migration, and have been implicated in the process of tumor cell invasion and metastasis [5,6]. In particular, RhoA activity plays a key role in protrusion in addition to its previously reported role in cell retraction during cell motility [7,8]. For example, disruption of RhoA activity has been shown to lead to invasive properties in human pancreatic cancer [7]. As a result, there has been considerable interest in the possibility that specific proteins in the signal transduction pathways mediating these cytoskeletal events could be potential targets for cancer therapy [9].

km23-1 (also referred as km23 [10–12], Robl1 [13], DNLC2A [14], mLC7-1 [15], DYNLRB1 [16]) was originally identified as a TGFß receptor-interacting protein that is also a dynein light chain [15]. More recently, we have shown that Ras and km23-1 form a TGFß-regulated complex in vivo [17]. However, km23-1 is actually part of an ancient superfamily, widely represented in archaea and bacteria, which appears to be involved in regulating NTPase activity [18]. One of the members of this superfamily (MglB) displays considerable structural correspondence with km23-1. Further, in bacteria, MglB interacts with a Ras-like small G protein, MglA [19–21], and plays a critical role in the spatial control of directed motility [21]. By analogy to the bacterial model, km23-1 would be expected to regulate the activity and biological functions of Ras family members of the Rho type, in the control of actin-based cell motility.

In the current report, using TAP and MS methods, we have identified km23-1-binding proteins that are involved in actin-based cell motility and modulation of the actin cytoskeleton.

* Corresponding author. Address: Department of Biochemistry and Molecular Biology-MC H171, Penn State Hershey College of Medicine, 500 University Drive, Hershey, PA 17033, United States. Fax: +1 717 531 0939.
E-mail address: kmm15@psu.edu (K.M. Mulder).
[1] These authors contributed equally.

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Further, we have demonstrated a role for km23-1 in RhoA/actin-based cell migration.

2. Materials and methods

2.1. Reagents

The pGEX2T-RBD plasmid (15247) was from Addgene. The piLenti km23-1 siRNA-GFP (i006555) and piLenti NC siRNA-GFP vectors were from Applied Biological Materials Inc. (Canada). InterPlay TAP purification kit was from Agilent technologies (La Jolla, CA, USA). Anti-dynein intermediate chain (DIC) (MAB 1685) was from Chemicon (Temecula, CA). Anti-Flag (M2) mAb was form Sigma. Anti-SBP and -CBP epitope tag antibodies (Abs) (07-482) were from Upstate/Millipore. Anti-actin mAb was from Sigma. Anti-RhoA (ARH03-A) mAb was from Cytoskeleton. GST-RBD plasmid (15247) was from Addgene.

2.2. Construction of expression vectors

The full-length coding region of human km23-1 was digested from the pcDNA 3.1-km23-1 vector by BamH1 and Xho1 and was cloned into the pCTAP expression vector (Stratagene, La Jolla, CA, USA).

2.3. Cell culture

293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C, 5% CO_2. Madin Darby canine kidney (MDCK) cells (CCL-34) were grown in minimal essential medium-α, supplemented with 10% FBS. HCT116 cells were cultured in McCoy's 5A, supplemented with amino acids, pyruvate, and antibiotics (streptomycin, penicillin) with 10% FBS as described previously [22]. Cells were routinely screened for mycoplasma using Hoechst 33258 staining.

2.4. TAP and Western blot analyses

Protein complexes were purified by the InterPlay TAP purification kit (Agilent technologies La Jolla, CA, USA) according to the manufacturer's instructions. Proteins were separated on 5–20% SDS–PAGE and visualized by Sypro Ruby Red staining (Invitrogen) according to the manufacturer's instructions. For Westerns, the TAP purified samples were resolved by 4–15% Bis-Tris SDS–PAGE gels and transferred to a PVDF membrane.

2.5. In-gel digestion, MS, and MS data analysis

The Sypro Ruby stained bands were excised from the gel and in-gel digested with trypsin. The proteins in the gel bands were identified by liquid chromatography (LC) tandem MS [23].

Stable transfections were performed as described previously [15] except that MDCK cells were used. Expression of km23-1 was verified by Western blot analysis, and stably transfected pools of km23-1-FLAG or (empty vector) EV were used for actin immunostaining.

2.6. Immunofluorescence microscopy analyses

Stably transfected pools of km23-1-FLAG or EV cells were grown in 6-well plates on top of coverslips. Immunofluorescence staining was performed as described previously [24]. Subsequently, these cells were incubated with an anti-actin Ab for 1 h. The bound primary Abs were visualized with FITC-conjugated goat anti-mouse IgG (green). Images were collected by fluorescent microscopy (Phase contrast-2, Diaphot, Nikon, Japan).

2.7. RhoA GTPase activation assays

The amount of activated, GTP-bound RhoA protein was measured using a technique similar to the method described [25].

2.8. Preparation of viruses/shRNA production and cell infection

Lentiviruses were generated by transfecting appropriate piLenti km23-1 shRNA green fluorescent protein (GFP) constructs with packaging plasmids into 293T cells using lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Viral supernatants were harvested 48 h after transfection and were filtered through a 0.45-mm filter. The virus was subsequently aliquoted (100 μl) and supernatants were stored at −80 °C. HCT116 cells were infected with lentivirus in the presence of 8 μg/ml polybrene.

2.9. Wound-healing assays

HCT116 cells were plated in 12-well plates and were infected with piLenti NC siRNA-GFP and piLenti km23-1 siRNA-GFP according to the manufacturer's instructions. 24 h after infection, cells were grown to confluence: Wound-healing assays were then performed as described previously [26].

The statistical significance was determined using the Student's *t*-test. The results are expressed as the mean ± SE.

3. Results

To validate the TAP protocol employed using our system, we purified the km23-1 protein complexes from 293T cells overexpressing km23-1-TAP. DIC was used as a positive control for detection of known km23-1-binding partners, since it is known to bind km23-1 [15]. As shown in Fig. 1A, in the EV-transfected cells, no band was detected (lane 1, 1st and 2nd panels) as expected. However, in the km23-1-TAP-transfected cells, DIC was detected after both the SBP pulldown (lane 2, 1st panel) and the SBP/CBP tandem pulldown (lane 2, 2nd panel). The expression of km23-1-TAP was confirmed by Western blot analyses (lane 2, 3rd panel), and GAPDH was used as a loading control (bottom panel). Our results demonstrate that the two-step tandem purification procedure can efficiently co-purify km23-1-TAP and its interacting partner DIC from cultured 293T cells.

To identify the proteins that were co-purified with km23-1-TAP, protein mixtures obtained from two successive affinity purification steps were fractionated on a 5–20% SDS–PAGE gradient gel and visualized by Sypro Ruby Red staining (Fig. 1B). Visible bands were excised and subjected to tryptic digestion. The extracted tryptic peptides were analyzed by the highly sensitive nanospray LC–MS/MS using an LTQ-Orbitrap mass spectrometer. The results were searched against the human NCBI database. The criteria used for identifying a protein interacting with km23-1-TAP were the identification of two and more independent peptides from a protein with a probability score cut-off of 1.0×10^{-7}. This means that the best match is the one with the smallest score [27]. The dynein complex proteins that met these criteria, such as the dynein heavy chain (DHC), DIC, km23-1 itself, and km23-2, are shown in Table 1. Since km23-1 is a dynein light chain (DLC), subunits of the dynein complex would be expected in the MS results, as would km23-1 itself and km23-2, which are known to form homodimers or hetrodimers [10]. Other dynein complex proteins (such as dynein light intermediate chain, DLC1, and DLC LC8-type 2) were identified

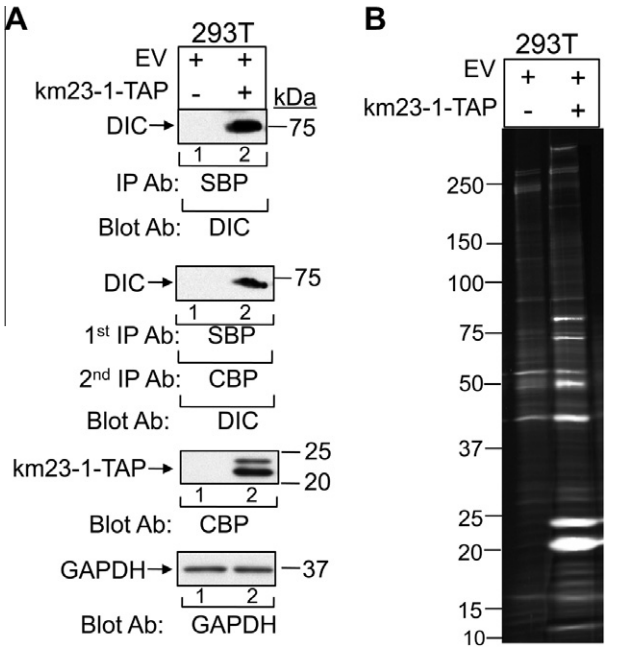

Fig. 1. TAP of km23-1 interacting protein complexes. (A) 293T cells were transiently transfected with either EV or km23-1-TAP, and protein complexes were purified by the TAP protocol described in Section 2. Aliquots were analyzed by Western blotting. All data are representative of three independent experiments, (B) Protein complexes obtained as in A were resolved by 5–20% SDS–PAGE. The gel was visualized with Sypro Ruby Red stain. The indicated bands were excised from the gel and identified by MS. The results are representative of two independent experiments.

Table 1
Subunits of the cytoplasmic dynein complex identified by LC–MS/MS from the km23-1-TAP purification.

NCBI Acc no.	Protein	*p*-Score	Peptides
7661822	km23-1/DYNLRB1	1.63E−11	71
18702323	km23-2/DYNLRB2	1.85E−08	6
33350932	Cytoplasmic dynein 1 heavy chain 1	1.61E−08	12
24307879	Cytoplasmic dynein 1 intermediate chain 2	5.55E−15	3
5453634	Dynein light intermediate polypeptide 2	5.60E−07	1
18087855	Dynein light chain, LC8-type 2	7.71E−06	1
83267866	Dynein light chain 1	1.11E−12	1

with one peptide (Table 1), possibly due to the low abundance of these proteins in our TAP sample. Overall, our TAP-MS results confirmed previous findings regarding the other subunits of the dynein motor complex [10], and validated the methodologies employed in our study.

In addition to the subunits of the dynein complex identified in Table 1, several other classes of km23-1-binding partners were identified, including proteins involved in vesicle transport/protein trafficking and transcriptional regulation. Of particular interest, various km23-1-binding partners that were related to actin-based motility met the criteria defined above and are shown in Table 2. Since these km23-1-interacting proteins have been shown to be involved in regulating reorganization of the actin cytoskeleton [3,28], we anticipated that forced expression of km23-1 would affect the actin cytoskeleton. Accordingly, we developed MDCK cell clones stably expressing EV or km23-1-FLAG, and confirmed km23-1 expression by Western blot analysis. As shown in Fig. 2, MDCK control cells displayed a polygonal shape, with actin staining more

Table 2
Motility-related km23-1-interacting proteins identified by LC–MS/MS from km23-1-TAP purification.

NCBI Acc no.	Protein	*p*-Score	Peptide
7656991	Coronin 1C	1.11E−10	3
21361358	Serine/threonine kinase 25 (STK25)	1.29E−10	2
4504789	1D-myo-inositol-trisphosphate 3-kinase (ITPKA)	1.94E−09	2
67906814	Rho GTPase activating protein (GAP)19	2.38E−08	2
33946278	Cofilin 2	3.91E−08	2
4507913	Wiskott–Aldrich syndrome protein family member (WASP) 1	1.42E−07	2

prominent at the cell cortex. In contrast, km23-1 MDCK pool cells exhibited an enlongated morphology, with actin staining demonstrating increased actin polymerization and a more highly organized stress fiber network. Thus, our results suggest that km23-1 may modulate the actin cytoskeleton, and in particular, stress fiber formation.

It is known that the RhoA signaling cascade plays an important role in regulating actin cytoskeletal organization and dynamics, inducing actin stress fiber and focal adhesion formation [29]. Since we have shown that overexpression of km23-1 significantly induces a highly organized actin stress fiber network, it was conceivable that KD of km23-1 might affect RhoA activation. Accordingly, we performed RhoA GTPase activation assays using Mv1Lu cells transient transfected with either NC siRNA or km23-1 siRNA (km23-1KD) in the absence or presence of TGFß. TGFß was used as the extracellular stimulus because it was previously shown to activate RhoA and induce actin stress fiber formation [30,31]. As expected, in the NC siRNA-transfected cells, TGFβ treatment demonstrated an accumulation of RhoA-GTP (Fig. 3., lane 1 vs. 2), consistent with a previous report [32]. In contrast, in km23-1KD cells, basal RhoA-GTP activity was significantly inhibited compared to NC siRNA-expressing cells (lane 3 vs. 1). While TGFß also activated RhoA in the km23-1KD cells (lanes 3, 4), TGFß induction of RhoA-GTP was reduced compared to the NC siRNA-expressing cells + TGFß (lane 2 vs. 4). Thus, our results demonstrate that km23-1 is required for RhoA activity in epithelial cells.

It was of interest that km23-1 depletion dramatically affected RhoA activity even in the absence of TGFß treatment. Since it is well-established that aggressive cancer cells with invasive properties are generally resistant to TGFß [33], it was conceivable that km23-1 depletion might reduce the migratory properties of in invasive, TGFß-resistant tumor cells. Toward this end, we examined whether km23-1KD would affect the cell migration of TGFβ-resistant HCT116 HCCCs using wound-healing assays. In the NC siRNA-infected cells, we observed an almost complete closure of the wound area within 24 h. In contrast, km23-1KD cells failed to close the wound area as effectively as control cells. As shown in Fig. 4, quantification for multiple wounds from multiple experiments revealed that km23-1KD cells exhibited a 43% decrease in migration compared to parental control cells. GFP expression was detectable in approximately 50% of both NC siRNA- and km23-1KD-infected cells, suggesting that the results plotted represent an underestimate of the actual effects of km23-1 depletion on tumor cell migration. Overall, however, our results demonstrate that km23-1 is required for the cell migration of highly invasive HCCCs.

4. Discussion

In the present study, we identified km23-1-interacting proteins that were involved in actin-based cell motility and modulation of the actin cytoskeleton, including cofilin, coronin, RhoGAP, serine/

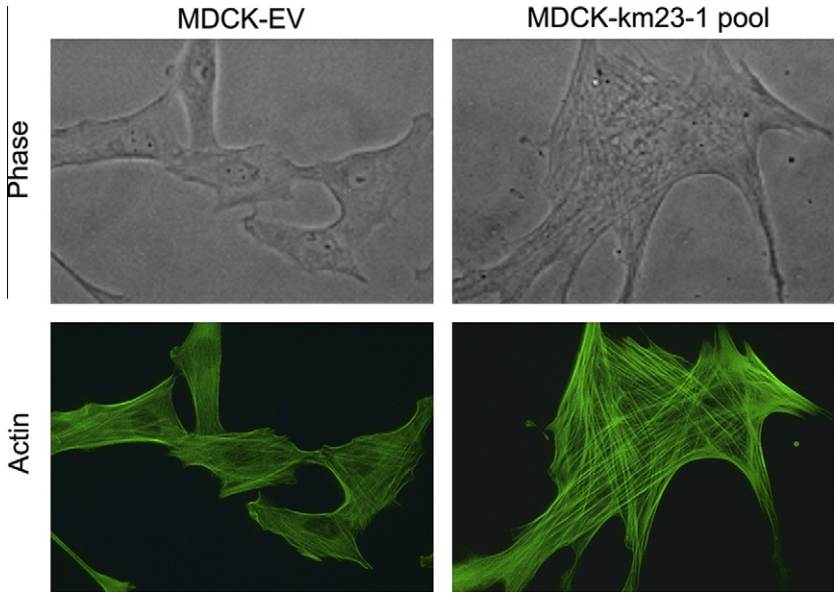

Fig. 2. Over-expression of km23-1 increases actin filaments in MDCK cells stably expressing km23-1. Actin immunostaining analyses were performed as described in Section 2, and representative photos are shown (400×).

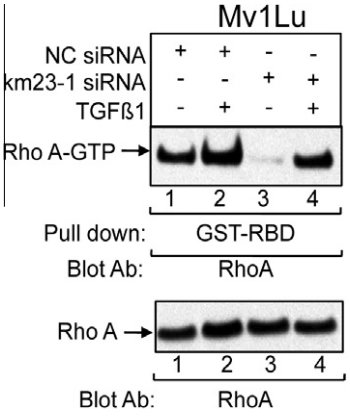

Fig. 3. KD of km23-1 decreases basal RhoA activation in Mv1Lu epithelial cells. Mv1Lu cells were transiently transfected with either NC siRNA or km23-1 siRNA. 24 h after transfection, RhoA activation assays were performed as described in Section 2.

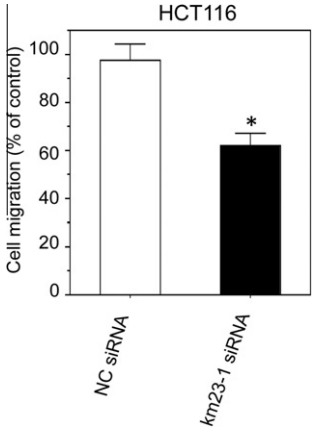

Fig. 4. Depletion of km23-1 inhibited cell migration of HCCCs. Wound-healing assays were performed as described in Section 2. Photographs were taken immediately after wounding and 24 h later. The results were quantified and normalized to parental control cells. Data plotted are the mean ± SE of triplicate wells from a representative experiment ($n = 4$). Asterisk (*) indicates $p < 0.01$ compared with control cells. All data are representative of two independent experiments.

threonine kinase 25 (STK25), 1D-myo-inositol-trisphosphate 3-kinase (ITPKA), and WASP-1. In addition, we showed herein that km23-1 is required for RhoA activation and actin stress fiber formation in epithelial cells. More importantly, depletion of km23-1 inhibited the cell migration of HCCCs. Overall, our results suggest that km23-1 is required for RhoA activity and cell migration via its association with key proteins involved in actin-based cell motility and modulation of the actin cytoskeleton.

Among the identified km23-1 interacting partners, coronins are WD-repeat proteins known to regulate cell motility by coordinating actin filament turnover in lamellipodia of migrating cells [34]. Recently, Samarin et al. [28] have shown that siRNA silencing of coronin 1C in intestinal epithelial cells enhanced cell migration and modulated lamellipodia dynamics by increasing the persistence of lamellipodial protrusions [28]. Since we have shown herein that km23-1 contributes to cell migration of HCCCs and that it is required for RhoA activation in epithelial cells, perhaps the km23-1-coronin 1C complex assists in modulating the activities of coronin 1C in this context.

Actin binding has been shown to be required for the synergistic effects between coronins and actin-depolymerizing factor (ADF)/cofilin in severing actin filaments and for the normal localization and functions of coronins in vivo [35]. However, cofilin not only severs or depolymerizes actin filaments, but also cooperates with other actin-binding proteins to assemble stress fibers and lamellipodia formed by filamentous actin [36]. For example, for stress fiber formation through Rho family GTPases, cofilin is inactivated through phosphorylation by LIMK1 [36]. In the currentreport, we have shown that ovexpression of km23-1 increases stress fiber formation. In addition, we have identified cofilin and RhoGAP as km23-1 interacting partners in our TAP-MS analyses. Of note, LIMK1 was also identified in the MS results with two peptides and a score of 1.22E-06, which did not meet the threshold criteria we chose for inclusion in Table 2.

However, it is still likely that LIMK may be in a km23-1-regulated complex that contains cofilin and other actin-modulating proteins. Accordingly, km23-1 might cooperate with actin-binding proteins such as cofilin and LIMK1, as well as regulatory factors for Rho family of GTPases, to mediate actin cytoskeletal reorganization.

As mentioned earlier, the Rho family of small GTPases plays critical roles in extracellular signal-regulated cytoskeletal changes and cell migration [29]. In particular, RhoA has been shown to play a unique role at the leading edge of specific types of cancer cells, where it is critical for cellular migration and invadopodia formation [29,37,38]. RhoGAPs enhance the intrinsic GTPase activity of RhoA to promote hydrolysis of GTP to GDP to inactivate RhoA [39]. Although recent studies suggest that RhoGAPs are regulated by various mechanisms, including protein–protein interactions, phospholipid interaction, phosphorylation, subcellular translocation, and proteolytic degradation [39], the precise mechanisms that control RhoGAP activity remain elusive in many cases. Our results have shown that km23-1 is required for RhoA activation and for cell migration of HCCCs. Consequently, km23-1 might negatively regulate RhoGAP activity, thereby inducing RhoA activation, leading to cell migration. In addition, blockade of the downstream RhoA effector Rho kinase (ROCK) influences coordinated epithelial cell motility by the formation of aberrant protrusions at the migrating front and by basal accumulation of F-actin aggregates [40]. Thus, the reduced RhoA activity by km23-1KD would also be expected to diminish the activity of RhoA effectors that are also known to mediate the reduced or uncoordinated cell migration of metastatic cancer cells.

WASP is one of the downstream targets of Cdc42, another member of the Rho family of small GTPases. It is known to interact directly with the Arp2/3 (actin-related protein 2/3) complex, one of the two machines that promotes actin polymerization in all eukaryotic cells [29]. Since we identified both WASP and Arp2/3 in our TAP-MS analyses, although Arp2/3 was identified with only one peptide (so not included in Table 2), it is possible that the association of km23-1 with WASP/Arp2/3 might be one of mechanisms that contributes to the cell migration of HCCCs through promoting actin polymerization. In addition, STK25 also referred to as SOK1 (Sterile 20 (Ste20)-like/oxidant stress-response kinase 1)/YSK1 (yeast Sps1/Ste20-related kinase 1) belongs to the GCKIII subfamily of the Ste20 proteins known to be involved in cell adhesion and migration [41]. For example, recent studies have shown that SOK1 promotes cells migration in MCF-7 cells [41]. Thus, the interaction of km23-1 with STK25 might represent a novel signaling pathway involved in tumor metastasis.

Since cell migration is one of the hallmarks of metastatic disease, the identification and analysis of migration promoting proteins is crucial for understanding the mechanisms underlying metastasis formation [1]. Previous results have shown that two functional activities of ITPKA increase invasive migration of tumor cells, and as such, ITPKA has become a novel promising target for a therapy directed against invasion and metastasis formation of primary tumors [42,43]. Therefore, our findings regarding km23-1 interaction with ITPKA and with the other actin-modulating proteins, as well as km23-1's ability to regulate RhoA activity and cell migration, implicate it for the first time as a novel target for anti-metastatic therapy.

Acknowledgments

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