Published in final edited form as:

Cell Signal. 2014 February ; 26(2): 343–351. doi:10.1016/j.cellsig.2013.11.012.

Phosphorylation of KIBRA by the extracellular signal-regulated kinase (ERK) - ribosomal S6 kinase (RSK) cascade modulates cell proliferation and migration

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Abstract

In mammals, KIBRA is defined as a memory performance-associated protein. The physiological function and regulation of KIBRA in non-neuronal cells is much less understood. Recent studies have identified KIBRA as a novel regulator of the Hippo signaling pathway, which plays a critical role in tumorigenesis by inhibiting cell proliferation and promoting apoptosis. We recently reported that KIBRA is phosphorylated by the mitotic kinases Aurora and cyclin-dependent kinase 1 during mitosis. In this current study, we show that KIBRA is also phosphorylated by the ERK (extracellular signal-regulated kinases)-RSK (p90 ribosomal S6 kinases) cascade. We demonstrated that ERK1/2 phosphorylate KIBRA at Ser⁵⁴⁸ in cells as well as *in vitro*. Moreover, we found that RSK1/2 specifically phosphorylates KIBRA at two highly conserved sites (Thr⁹²⁹ and Ser⁹⁴⁷) *in vitro* and in cells. RSK-mediated phosphorylation is required for KIBRA binding to RSK1, but not RSK2. Surprisingly, KIBRA knockdown impaired cell migration and proliferation in breast cancer cells. By using inducible-expression cell lines, we further show that phosphorylation of KIBRA by ERK1/2 and RSK1/2 is required for proper cell proliferation and RSK-mediated phosphorylation also modulates KIBRA's migratory activity in MDA-MB-231 breast cancer cells. Our findings uncover unexpected results and a new mechanism through which KIBRA regulates cell migration and proliferation.

Keywords

KIBRA; ERK1/2; RSK1/2; phosphorylation; proliferation; migration

1. Introduction

KIBRA (expression enriched in kidney and brain), also called WW and C2 domain containing 1 (WWC1), contains two WW domains at the N-terminus and a C2 domain in the

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C-terminal region [1]. KIBRA was originally identified as a memory performance and cognition-associated protein [2]; this function was recently confirmed by genetic models in mice [3] and studies in humans [4–8]. Increasing evidence also links the *KIBRA* locus to Alzheimer's disease [9–11]. While the role of KIBRA in neurons is well defined, its physiological function in non-neuronal cells is relatively less understood. We and others recently demonstrated that KIBRA is a regulator of the Hippo pathway in both *Drosophila* [12–14] and mammalian cells [15], suggesting a function of KIBRA in epithelial cell growth. Previous studies also indicated that KIBRA is involved in other biological processes including cell polarity, mitosis and cell migration. For example, KIBRA positively regulates directional migration in podocytes in conjunction with the polarity protein PALS1-associated tight junction (PATJ) and synaptopodin [16]. This positive regulation in migration was further demonstrated in NRK cells [17]. However, KIBRA was shown to be a negative regulator in cell motility in immortalized breast epithelial cells [18]. Despite the established role of KIBRA in cell migration, it is unknown how KIBRA is regulated during this process.

The Ras-activated MAPK/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling pathway plays a central role in diverse cellular processes [19]. MEK1/2 kinases phosphorylate and activate the mitogen-activated protein kinases (MAPKs) ERK1/2. Once activated, ERK1/2 transduce the extracellular signals to intracellular proteins by phosphorylating their substrates, including the 90 kDa ribosomal S6 kinase (RSK) family. The human RSK kinase family comprises four highly related serine/threonine isoforms (RSK1-4) [20]. The RSK kinases are the major downstream effectors of the RAS/MAPK signaling pathway and modulate various biological responses, including cell proliferation, survival, growth, and cell motility/invasiveness [20,21]. Interestingly, KIBRA was shown to be required for collagen-induced ERK signaling activity via interactions with discoidin domain receptor tyrosine kinase 1 [22], providing a potential connection between MEK-ERK signaling and KIBRA.

KIBRA is a phospho-protein and we previously reported that KIBRA is phosphorylated by the mitotic kinases Aurora and cyclin dependent kinase 1 (CDK1) during mitosis [23,24]. During the process of identifying the kinases for KIBRA shift/phosphorylation, we noticed that MEK-ERK inhibitors can significantly enhance the mobility of KIBRA on SDS-PAGE gels. In this report, we further characterize the phosphorylation of KIBRA. We show that the MEK-ERK-RSK cascade is also able to phosphorylate KIBRA and, importantly, this phosphorylation is required for KIBRA-mediated cell migration and proliferation in MDA-MB-231 cells. Thus, our study reveals an unrecognized mechanism through which KIBRA regulates cell migration and proliferation in breast cancer cells.

2. Materials and methods

2.1. Expression constructs

The human KIBRA constructs have been previously described [15]. HA-RSK1 plasmid was purchased from Addgene (#13841) [25]. Point mutations were generated by the QuickChange Site-Directed PCR mutagenesis kit (Stratagene, La Jolla, CA, USA) and verified by sequencing.

2.2. Cell culture and transfection

HEK293T, HeLa, MDA-MB-231, and MCF-7 cell lines were maintained as described [24]. All the transient overexpression transfections were performed using Attractene (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. U0126 and PD0325901 (MEK-ERK inhibitors) were from LC Laboratories (Woburn, MA, USA). BI-D1870 (RSK

inhibitor) was from ENZO Life Sciences (Farmingdale, NY, USA). All other chemicals were either from Sigma or Thermo Fisher (Waltham, MA, USA).

2.3. Establishment of Tet-On-inducible cell lines

The siRNA-resistant KIBRA cDNA (siR-KIBRA) and KIBRA phosphorylation-deficiency mutants were cloned into the Tet-All retroviral-vector, which contains both reverse tetracycline-controlled transactivator (rtTA) and tetracycline response elements (TRE) promoter. The original backbones for the Tet-All vector were pRetroX-Tight and pRetro-rtAT-Advanced from Clontech Laboratories (Mountain View, CA, USA). Modified MDA-MB-231 cells were generated by stably knocking down endogenous KIBRA and transfecting with Tet-All-siR-KIBRA (or various mutants), so siRNA-resistant KIBRA could be expressed in a doxycycline-dependent manner. Virus packaging, infection, and resistance selection were done as described [15]. Cells were maintained in medium containing Tet system-approved fetal bovine serum (Clontech Laboratories).

2.4. Immunoprecipitation, Western blot analysis and metabolic labeling

Immunoprecipitation, Western blotting and metabolic labeling assays were done as previously described [24]. [^{32}P]Orthophosphate was purchased from MP Biomedicals (Solon, OH, USA).

2.5. Recombinant protein purification

The GST-tagged proteins were bacterially expressed and purified on GSTrap FF affinity columns (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's instructions.

2.6. In vitro kinase assay

1–2 μg of GST-KIBRA was incubated with 100 ng recombinant kinase (SignalChem, Richmond, BC, Canada) in kinase buffer [24] in the presence of 5 μCi $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (3000 Ci/mmol, PerkinElmer, Waltham, MA, USA). MEK1, ERK1, ERK2, RSK1, and RSK2 active kinases were purchased from SignalChem. The samples were resolved by SDS-PAGE, transferred onto PVDF (Millipore, Billerica, MA, USA) and visualized by autoradiography followed by Western blotting.

2.7. Antibodies

Mouse monoclonal antibodies against human KIBRA have been described [24] and were used for Western blot analysis throughout the study. Rabbit polyclonal antibodies against KIBRA were used for immunoprecipitation of endogenous KIBRA [1]. The phospho-specific antibodies against KIBRA Ser⁵⁴² and Ser⁵⁴⁸ have been described [23]. Rabbit polyclonal phospho-specific antibodies against KIBRA Thr⁹²⁹ and Ser⁹⁴⁷ were generated and purified by AbMart (Shanghai, China). FLAG, HA and Myc antibodies were from Sigma. Anti- β -actin, anti-ERK1/2, anti-GFP, Anti-RSK1, and anti-RSK2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-glutathione S-transferase (GST), anti-His, anti-Mst1, anti-Mst2, anti-Lats1, and anti-Lats2 antibodies were from Bethyl Laboratories (Montgomery, TX, USA). Anti-NF2 was from Sigma. Anti-phospho-Thr²⁰²/Tyr²⁰⁴ ERK1/2, anti-phospho-Ser¹²⁷ YAP, anti-phospho-Mst1/2, and anti-phospho-Lats1/2 antibodies were from Cell Signaling Technology (Danvers, MA, USA).

2.8. Cell proliferation and migration assay

Cell proliferation assays were performed as described in [26]. 3,000 cells were seeded in each well of a 24-well plate in triplicate. *In vitro* cell migration assays were assessed using the Transwell system (Corning Inc., Corning, NY, USA) according to the manufacturer's

instructions. The migratory cells were stained with ProLong Gold Antifade Reagent with DAPI. The photos were taken under 10X lens. 5,000 cells were seeded for each Transwell. The relative migrating rate was calculated by the number of cells migrating through the membrane, divided by the number of the cells that migrated in the control group.

2.9. Statistical analysis

Statistical significance was performed using a two-tailed, unpaired Student's *t*-test.

3. Results

3.1. Inhibition of MEK-ERK signaling impairs KIBRA phosphorylation

KIBRA is a phospho-protein and runs as a smear or multiple bands on SDS-polyacrylamide gels. We previously reported that KIBRA is phosphorylated at multiple sites by Aurora and CDK1 kinases during mitosis [23,24]. During our experiments, we found that the mobility shift of KIBRA is also MEK-ERK signaling activity dependent under normal culture conditions. As shown in Figure 1A, inhibition of MEK1/2 kinases by U0126 or PD0325901 greatly enhanced the mobility of KIBRA (probably due to reduced phosphorylation levels of KIBRA) on an SDS-gel in various cancer cell lines. Metabolic labeling indicated that ³²P incorporation into KIBRA is significantly reduced in the presence of U0126, suggesting that KIBRA phosphorylation is partially MEK-ERK signaling dependent (Fig. 1B).

We also examined the response of other major Hippo pathway members to the inhibition of MEK signaling. Neither the activity (revealed by phospho-antibody on Western blots) nor the total levels of Mst1/2 was affected upon treatment with U0126 or PD0325901 (Fig. 1C). Total and phospho-YAP levels were not altered by these inhibitors either. Interestingly, phosphorylation/activity of Lats1/2 kinases was slightly increased without affecting the total level or mobility shift (Fig. 1C).

3.2. ERK1/2 kinases phosphorylate KIBRA in vitro

U0126 and PD0325901 inhibit MEK1/2 and subsequent ERK1/2 kinases (Fig. 1A). We previously showed that MEK kinase failed to phosphorylate KIBRA [23]. Thus, we tested whether KIBRA is a suitable substrate for ERK1/2 kinases. *In vitro* kinase assays demonstrated that GST-KIBRA-M (amino acids 428-835) is robustly phosphorylated by ERK1 (Fig. 2A) and ERK2 (Fig. 2B) kinases. GST-KIBRA-N (amino acids 1-453) and -C (amino acids 832-1119) are not phosphorylated by ERK1 (Fig. 2A). ERK2 does not phosphorylate the N-terminus of KIBRA and only weakly phosphorylates C-terminus of KIBRA (Fig. 2B).

We previously reported that CDK1 phosphorylates Ser⁵⁴² in a highly conserved SP cluster (S⁵³⁵PRSSLSS⁵⁴²PS⁵⁴⁴PPCS⁵⁴⁸P) in the central region of KIBRA and the phospho-status of this SP cluster modulates the mobility shift of KIBRA [23]. Since ERK1/2 and CDK1 kinases share the minimal proline-directed phospho-consensus, we tested the possibility that ERK1/2 also phosphorylate this SP region. Mutating all four serines (4SA: S535A/S542A/S544A/S548A) to alanines (which are nonphosphorylatable) completely abolished the phosphorylation stimulated by ERK1 (Fig. 2C) or ERK2 (Fig. 2D), suggesting that ERK1/2 kinases potentially phosphorylate this SP cluster. Mutating Ser⁵⁴² or Ser⁵⁴⁴ to alanine moderately reduced ERK2-mediated phosphorylation of KIBRA (Fig. 2E). Interestingly, mutating Ser⁵⁴⁸ to alanine completely abolished the ERK2-mediated phosphorylation of KIBRA-M (Fig. 2E, last lane). The mobility shift of GST-KIBRA-M is consistent with the phospho-level of KIBRA (Fig. 2E). KIBRA-M with S535A mutation does not alter the ³²P incorporation stimulated by ERK2 (Fig. 2F). These data indicate that ERK1/2 directly

phosphorylate KIBRA and Ser⁵⁴⁸ is required for ERK-mediated phosphorylation of KIBRA *in vitro*.

3.3. ERK1/2 phosphorylate KIBRA at Ser⁵⁴² and Ser⁵⁴⁸ *in vitro*

By using phospho-specific antibodies we recently generated [23], we further confirmed that ERK1/2 phosphorylate KIBRA at Ser⁵⁴² (Fig. 3A, 3D) and Ser⁵⁴⁸ (Fig. 3B, 3E). Mutating relevant serine to alanine abolished the phosphorylation signal, further confirming the specificity of these phospho-antibodies (Fig. 3A, 3B, 3D, and 3E). Mutating Ser⁵⁴² to alanine has no effect on phosphorylation at Ser⁵⁴⁸; however, mutating Ser⁵⁴⁸ to alanine completely blocked the phosphorylation at Ser⁵⁴² induced by ERK1/2 (Fig. 3C, 3F). The mobility retardation of GST-KIBRA is also evident. Consistent with Figure 2E, mutating Ser⁵⁴⁸, but not Ser⁵⁴², to alanine blocked the mobility upshift caused by ERK1/2-mediated phosphorylation (Fig. 3A–3F, lower part of each panel). Together, these results suggest that ERK1/2 phosphorylate KIBRA at Ser⁵⁴² and Ser⁵⁴⁸ and that phosphorylation of Ser⁵⁴⁸ is required for further phosphorylation at Ser⁵⁴² *in vitro*. We recently demonstrated that CDK1 failed to phosphorylate KIBRA at Ser⁵⁴⁸ [23], indicating that Ser⁵⁴⁸ is a specific phosphorylation site for ERK1/2 although both CDK1 and ERK1/2 recognize the same consensus sequence.

3.4. RSK1/2 phosphorylate KIBRA at Thr⁹²⁹ and Ser⁹⁴⁷ *in vitro*

The p90 ribosomal kinases (RSK) are the major transducers downstream ERK1/2 kinases [21]. We further tested whether RSK1/2 also phosphorylate KIBRA. *In vitro* kinase assays demonstrated that the C-terminal part of KIBRA was robustly phosphorylated by RSK1 (Fig. 4A) and RSK2 (Fig. 4B). GST-KIBRA-N and –M are not suitable substrates for RSK1/2 (Fig. 4A, 4B). Sequence analysis revealed a highly conserved region containing two potential RSK phosphorylation consensus sequences [21] (Fig. 3C). Mutating one of the potential sites (Thr⁹²⁹ or Ser⁹⁴⁷) to alanine partially reduced the ³²P incorporation into KIBRA-C and combined mutations (T929A/S947A) largely abolished the ³²P incorporation induced by RSK1/2, suggesting that RSK1/2 potentially phosphorylate KIBRA at Thr⁹²⁹ and Ser⁹⁴⁷ *in vitro* (Fig. 4D, 4E).

We have also generated phospho-specific antibodies against Ser⁹⁴⁷ and Thr⁹²⁹. Using these phospho-specific antibodies, we demonstrated that RSK1/2 strongly phosphorylates KIBRA at Ser⁹⁴⁷ (Fig. 4F) and Thr⁹²⁹ (Fig. 4G) *in vitro*. Interestingly, a number of previous proteomic studies have identified KIBRA Thr⁹²⁹ and Ser⁹⁴⁷ as phosphorylation sites in cells [27–29]. Taken together, our data indicate that the ERK-RSK cascade phosphorylates KIBRA at multiple sites *in vitro* and possibly *in vivo* as well.

3.5. The ERK-RSK signaling phosphorylates KIBRA at Ser⁵⁴⁸, Thr⁹²⁹, and Ser⁹⁴⁷ in cells

To explore whether Ser⁵⁴², Ser⁵⁴⁸, Thr⁹²⁹ and Ser⁹⁴⁷ are also phosphorylated within cells by the ERK-RSK signaling, we transfected KIBRA into cells, treated the cells with MEK-ERK inhibitors, and determined levels of phosphorylation by phospho-antibodies. U0126 or PD0325901 treatment significantly decreased the phospho-signal of Ser⁵⁴⁸ and Ser⁹⁴⁷, but not Ser⁵⁴² and Thr⁹²⁹ (Fig. 5A). We further examined KIBRA phosphorylation in cells in the presence of ectopic RSK1 or its kinase inactive mutant. Overexpression of wildtype RSK1, but not the kinase inactive form, greatly stimulated phosphorylation of KIBRA at both Thr⁹²⁹ and Ser⁹⁴⁷ (Fig. 5B). Mutating Thr⁹²⁹ and Ser⁹⁴⁷ to alanines (KIBRA2A) abolished the phosphorylation induced by RSK1, confirming the specificity of the phospho-antibodies (Fig. 5B). Conversely, BI-D1870 (an inhibitor for RSK kinases [30]) treatment significantly reduced the phosphorylation of KIBRA at Thr⁹²⁹ and Ser⁹⁴⁷ (Fig. 5C). Taken together, these results suggest that ERK1/2 phosphorylate KIBRA at Ser⁵⁴⁸ and that Thr⁹²⁹ and Ser⁹⁴⁷ of KIBRA are phosphorylated by RSK1/2 kinases in cells.

3.6. Phosphorylation is required for KIBRA to associate with RSK1

We next explored whether RSK forms a complex with KIBRA. To do so, we performed immunoprecipitation with transfected plasmids and found that wildtype KIBRA, but not the non-phosphorylatable mutant (KIBRA2A), associates with RSK1, indicating that RSK-mediated phosphorylation of KIBRA is required for associating with RSK1 (Fig. 5D). We are also able to detect the interaction between transfected KIBRA and endogenous RSK2, however, surprisingly, RSK-mediated phosphorylation of KIBRA is not required for binding to RSK2 (Fig. 5E). Interaction between KIBRA and ERK1/2 kinases was not detectable in our hands (data not shown).

3.7. Phosphorylation of KIBRA by ERK-RSK signaling modulates cell migration and proliferation

ERK and RSK are well established regulators in various cellular processes including cell proliferation, growth, survival, cell motility, and invasion [20,21]. We further examined the biological significance of the ERK-RSK-mediated KIBRA phosphorylation in cell proliferation and migration. To do so, we established a doxycycline-inducible complementation system in MDA-MB-231 breast cancer cells (Fig. 6A). The endogenous KIBRA has been stably knocked down in these cells and siRNA-resistant KIBRA [15] (and its phosphorylation deficiency mutants) were re-expressed in a doxycycline-inducible manner (Fig. 6A). We previously reported that KIBRA induces the phosphorylation of Hippo-YAP signaling [15]. However, the functional consequence of this observation was not known. Surprisingly, KIBRA knockdown significantly impaired proliferation (Fig. 6B) and migration (Fig. 6C and 6D) in MDA-MB-231 cells. Importantly, these defects were largely rescued by re-expression of wildtype KIBRA. However, expression of either KIBRA3A (S542A/S544A/S548A) or KIBRA2A (T929A/S947A) failed to rescue the proliferation defects caused by KIBRA knockdown, suggesting that ERK or RSK-mediated phosphorylation is required for KIBRA in regulating proper cell proliferation (Fig. 6B). RSK-mediated phosphorylation is also required for KIBRA to modulate cell migration as KIBRA2A failed to rescue the reduced migration in KIBRA-knockdown cells (Fig. 6C and 6D). However, ERK-mediated phosphorylation of KIBRA is not involved in cell migration (Fig. 6C and 6D). Thus, phospho-regulation of KIBRA by ERK and RSK is required for proper cell proliferation and migration in MDA-MB-231 breast epithelial cancer cells.

4. Discussion

KIBRA is a phospho-protein and its phosphorylation is regulated by several kinases including PKC ζ [31], Aurora kinases [24], and CDK1 [23]. In this study, we identified a novel mechanism whereby ERK-RSK-mediated phosphorylation of KIBRA modulates cell proliferation and migration in breast cancer cells. KIBRA (WWC1) is one of the members of the WWC family, which comprises additional WWC2 and WWC3 paralogs in addition to WWC1 [32]. Among the WWC family members, KIBRA is the most studied one, although its function and regulation are still not well understood. Little is known about the physiological function, expression (patterns/levels) and regulation of WWC2 and WWC3. Interestingly, the phosphorylation sites Ser⁹⁴⁷ and Thr⁹²⁹ also exist in both WWC2 and WWC3 and Ser⁵⁴⁸ is conserved in WWC2. In future studies, it will be worthwhile to investigate whether ERK-RSK-mediated phosphorylation also occurs in WWC2 and/or WWC3 in cells and, if so, whether this phosphorylation functions redundantly with KIBRA (WWC1) phosphorylation. Our phospho-antibodies may be helpful in exploring this direction.

Recent studies showed that the Hippo pathway is regulated by EGFR-RAS-ERK signaling in response to mitogens. EGFR regulates Yorkie/YAP activity through MAPK-mediated

phosphorylation of Ajuba LIM protein Jub/WTIP [33]. Interestingly, a previous study also showed that KIBRA is required for collagen-induced MEK-ERK activation [22]. It is at present not known whether collagen can induce KIBRA phosphorylation in a MEK-ERK-dependent manner and, if so, whether this phosphorylation is required for mediating the activation of MEK-ERK signaling. Nevertheless, these observations imply a molecular link between Hippo and RAS-MAPK signaling pathways. In the current study, we showed that the MEK-ERK-RSK signaling also associates with KIBRA and regulates KIBRA activity in cell proliferation and migration properties. Since ERK-RSK-mediated phosphorylation of KIBRA does not impact Hippo activity (S.Y. and J.D., unpublished observations) and Hippo-YAP activity is not altered upon MEK-ERK inhibition (Fig. 1), it seems the ERK-RSK-KIBRA cascade regulates cell proliferation and migration in a Hippo-independent manner. Therefore, it will be interesting to explore the downstream signaling or effector mediating the ERK-RSK-KIBRA axis and such studies should further provide the underlying mechanisms of KIBRA-regulated cell proliferation and/or migration.

RSK family kinases have been shown to control/regulate diverse cellular processes [21]. One of the established roles for RSK is regulating cell motility and invasiveness/metastasis. RSK is the key effector of RAS-ERK-stimulated cell motility and invasion [34]. More recently, a genome-wide RNAi screen has identified RSK as a common effector in epithelial cell migration stimulated by diverse migratory stimuli [35]. Accordingly, overexpression of RSK1 or RSK2 is sufficient to promote cell motility in melanoma cells [36] and invasion/metastasis in human head and neck squamous cell carcinoma cells [37], respectively. Further experiments are required to examine whether KIBRA is a mediator of RSK-driven migration and invasion in human cancer cells. Furthermore, the correlation between RSK expression/activity and the phosphorylation levels of KIBRA needs to be investigated in tumor samples from human cancer patients.

We recently showed that KIBRA is a positive regulator of Hippo pathway activity in mammalian cells [15], which suggests that KIBRA functions as a growth suppressive protein as it does in *Drosophila* [12–14]. Consistent with these observations, a recent study showed that KIBRA knockdown enhanced migration and invasion of immortalized breast epithelial cells [18]. However, our current study shows that KIBRA knockdown significantly reduces cell proliferation and migration in MDA-MB-231 cells (Fig. 6), which contrasts to the study in immortalized non-cancerous cells [18]. Interestingly, several studies have also demonstrated that KIBRA positively regulates cell migration and proliferation [16,17,38,39]. Furthermore, KIBRA plays a positive role in the collagen-induced activation of the ERK1/2 kinases in breast epithelial cells [22]. A very recent study showed that high expression of KIBRA in gastric cancer is correlated with lymphatic and venous invasion [40]. Thus the tumor suppressive function of KIBRA may be cell-type specific and in many cases KIBRA could function as a positive regulator of cell proliferation, cell motility and invasion. More systematic and detailed investigations are required for clarity of this controversy. Our study is consistent with these latter studies, supporting a positive role of KIBRA in proliferation and migration in breast cancer cells.

5. Conclusions

In conclusion, the results show that KIBRA is phosphorylated on multiple sites by the ERK-RSK kinase cascade. KIBRA knockdown impairs proliferation and migration in MDA-MB-231 breast cancer cells. ERK-RSK phosphorylation of KIBRA is required for proper cell proliferation and RSK-mediated phosphorylation also positively modulates KIBRA's migratory activity. Our findings uncover an unrecognized mechanism through which KIBRA regulates cell migration and proliferation.

Acknowledgments

We thank Dr. Joyce Solheim for critical reading and comments on the manuscript. This work was supported in part by National Institutes of Health grant number 8 P20 GM103489 (to J.D.).

Abbreviations

ERK1/2	extracellular signal-regulated kinases 1/2
RSK1/2	p90 ribosomal S6 kinases 1/2
MAPK	mitogen-activated protein kinases
CDK1	cyclin-dependent kinase 1
WWC	WW and C2 domain containing proteins

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Highlights

- ERK-RSK cascade phosphorylates KIBRA *in vitro* and in cells.
- KIBRA knockdown impairs cell proliferation and migration.
- ERK-RSK phosphorylation of KIBRA modulates cell proliferation and migration

Fig. 1. Phosphorylation of KIBRA is MEK-ERK signaling dependent

(A) MDA-MB-231, HeLa, and MCF-7 cells were treated with DMSO, U0126 (10 μ M), or PD0325901 (20 μ M) for 2 h and the total cell lysates were probed with the indicated antibodies.

(B) MCF-7 cells were metabolically labeled in the presence of 32 P for 2 h as described [24] in the presence or absence of U0126. Endogenous KIBRA was immunoprecipitated and the immunoprecipitated products were separated by SDS-PAGE and transferred onto PVDF membrane, followed by autoradiography and Western blot analysis.

(C) MCF-7 cells were treated as in (A) and the total cell lysates were analyzed with the indicated antibodies.

Fig. 2. ERK kinases phosphorylate KIBRA *in vitro*

(A, B) GST-KIBRA-N, M, and -C proteins were used as substrates for *in vitro* kinase assays with purified ERK1 (A) or ERK2 (B) kinases. The products were separated by SDS-PAGE and transferred onto PVDF membrane, followed by autoradiography and Western blot analysis.

(C, D) GST-KIBRA-M and GST-KIBRA-M-4SA proteins were used as substrates for *in vitro* kinase assays with purified ERK1 (C) or ERK2 (D) kinases. 4SA: S535A/S542A/S544A/S548A.

(E, F) GST-KIBRA-M and various mutated proteins were used as substrates for *in vitro* kinase assays with ERK2 kinase. The 32 P incorporation was revealed by autoradiography. Western blot shows the substrate loading.

Fig. 3. ERK kinases phosphorylate KIBRA at Ser⁵⁴² and Ser⁵⁴⁸ *in vitro*
(A–C) *In vitro* kinase assays with ERK1 kinase without ³²P. The samples were probed with phospho-specific antibodies against KIBRA Ser⁵⁴² or KIBRA Ser⁵⁴⁸.
(D–F) *In vitro* kinase assays with ERK2 without ³²P. The samples were probed with phospho-specific antibodies against KIBRA Ser⁵⁴² or KIBRA Ser⁵⁴⁸.

Fig. 4. RSK1/2 kinases phosphorylate KIBRA at Thr⁹²⁹ and Ser⁹⁴⁷ in vitro

(A, B) GST-KIBRA-M, N, and -C proteins were used as substrates for *in vitro* kinase assays with purified RSK1 (A) or RSK2 (B) kinases. The ³²P incorporation was revealed by autoradiography. Western blot shows the substrate loading.

(C) Sequence alignment of a highly conserved region encompassing two RSK consensus sequences in KIBRA.

(D, E) GST-KIBRA-C and various mutated proteins were used as substrates for *in vitro* kinase assays with RSK1 (D) or RSK2 (E) kinases. The ³²P incorporation was revealed by autoradiography. Western blot shows the substrate loading.

(F, G) *In vitro* kinase assays with RSK1/2 kinases without ³²P. The samples were probed with phospho-specific antibodies against KIBRA Ser⁹⁴⁷ (F) or KIBRA Thr⁹²⁹ (G), and subsequent GST.

Fig. 5. RSK1/2 associate with and phosphorylate KIBRA in cells

(A) MCF-7 cells were transfected with FLAG-KIBRA. At 48 h post-transfection, cells were treated with DMSO or MEK-ERK inhibitors for 2 h and lysed and immunoprecipitated with FLAG antibody. The immunoprecipitates and total cell lysates before immunoprecipitation were probed with the indicated antibodies.

(B) MCF-7 cells were transfected with various DNAs as indicated. At 48 h post-transfection, exogenous KIBRA was immunoprecipitated. The immunoprecipitates and total protein lysates before immunoprecipitation were subjected to Western blot analysis with the indicated antibodies. wt: wild type; kd: kinase dead (K103R/K456R).

(C) MCF-7 cells were transfected with HA-KIBRA. At 48 h post-transfection, cells were treated with DMSO or BI-D1870 (10 μ M) for additional 4 h. Exogenous KIBRA was immunoprecipitated with HA antibody and subjected to Western blot analysis with the indicated antibodies.

(D) MCF-7 cells were transfected with various DNAs as indicated. Immunoprecipitation and Western blots were done as in (A). 2A: T929A/S947A

(E) MCF-7 cells were transfected with FLAG-KIBRA or FLAG-KIBRA2A as indicated. Immunoprecipitation and Western blots were done as in (A). 2A: T929A/S947A.

Fig. 6. Phospho-regulation of KIBRA modulates cell proliferation and migration

(A) Characterization of Tet-On-inducible MDA-MB-231 cells expressing Vector, wildtype KIBRA (WT), KIBRA2A (T929A/S947A) or KIBRA3A (S542A/S544A/S548A) mutant. The cell lines were maintained under doxycycline-free medium and treated with doxycycline (Dox, 0.2 μ g/ml for wildtype KIBRA and 1 μ g/ml for other lines) as indicated for 48 h. Total protein lysates were subjected to Western blot analysis with the indicated antibodies. WT: wild type.

(B) The proliferation rate was determined of the cell lines in (A) in the presence of Dox.

(C, D) The cell lines in (A) were induced by Dox for 48 h and cell migration effect was determined in the presence of Dox. Representative photos (C) are shown for migrated cells and relative migrating rate is shown in (D). Data (B and D) were from at least three independent experiments and expressed as mean $\pm$ s.e.m. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (t-test).