p21-activated Kinase1 (PAK1) can promote ERK activation in a kinase independent manner

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Running title: PAK1 in Erk signaling

Capsule

Background: The interaction of PAK1 and Raf-Mek-Erk cascade is unclear.

Results: Overexpression of a kinase-dead mutant form of PAK1 increased phosphorylation of Mek1/2 and Erk. Hyperactivated Rac1 induced the formation of a triple complex of Rac1, PAK1 and Mek1, independent of the kinase activity of PAK1.

Conclusion: PAK1 activated Mek-Erk cascade in a kinase independent manner.

Significance: PAK1 might be a scaffold to facilitate interaction of c-Raf and Mek1.

Summary

PAK1 plays an important role in proliferation and tumorigenesis, at least partially by promoting Erk phosphorylation of c-Raf (Ser 338) or Mek1 (Ser 298). We observed now that overexpression of a kinase-dead mutant form of PAK1 increased phosphorylation of Mek1/2 (Ser 217/221) and Erk (Thr 202/Tyr 204) although phosphorylation of b-Raf (Ser445) and c-Raf (Ser 338) remained unchanged. Furthermore, increased activation of the PAK1 activator Rac1 induced the formation of a triple complex of Rac1, PAK1 and Mek1, independent of the kinase activity of PAK1. These data suggest that PAK1 can stimulate MEK activity in a kinase independent manner, probably by serving as a scaffold to facilitate interaction of c-Raf.

Key words

Mek, Erk, PAK1, Raf
Introduction

PAK1 is a member of the p21-activated kinases (PAKs) family of serine/threonine kinases, which regulate cell morphology, motility, survival, and proliferation (1,2). Important activators of PAK1 are the small GTPases Rac1 and Cdc42. In their active, membrane-bound form, they are able to recruit PAK1 to the cell membrane and induce a conformational shift, leading to the activation of the kinase function of PAK1 and autophosphorylation. Via the kinase function PAK1 was described to phosphorylate different molecules, which then mediate the biological effects of PAK1. In addition, PAK1 was shown to activate Akt activation in a kinase-independent manner by aiding the recruitment of Akt to the membrane (3).

One of the pathways regulated by Pak is the Raf-Mek-Erk cascade, which is important for proliferation. Raf phosphorylates Mek1/2 at Ser 217/221, respectively, which is necessary and sufficient to activate Mek. Activated Mek then activates Erk by phosphorylation at Thr202 and Tyr204 (4).

Previous studies have demonstrated that Mek1 and c-Raf (also known as Raf-1) are two direct Pak substrates (5). Pak phosphorylation of c-Raf (Ser338) should directly correspond to c-Raf activation, while phosphorylation of Mek1 at serine 298 is facilitating the signal transduction from Raf to Mek, thus indirectly promoting Mek1 activation. Slack et al. reported that Mek1 was phosphorylated by PAK1/2 at position Ser298, and it is thought that this phosphorylation is essential for transmitting mitogenic signals (6). More recently, we found that in primary mouse keratinocytes PAK1 regulates Erk activation by controlling phosphorylation of Mek1/2 at Ser217/221 without changing phosphorylation of c-Raf at serine 338 or of B-Raf at serine 445. Pak2 regulates of Mek1 at Ser298, but this is neither required nor sufficient for Erk activation in vitro (7). Yet, we did not elucidate, how PAK1 in this system might contribute to increased Mek1/2 (Ser 217/221) phosphorylation.

Using cancer cell lines as a model, we report now that PAK1 can promote Erk activation in a kinase independent manner, probably by recruiting Mek to the membrane, which facilitates the interaction with Raf. By this scaffold
function, PAK1 can contribute to cell proliferation and tumor formation.
EXPERIMENTAL PROCEDURES

Reagents and antibodies
Human PDGF was purchased from Sigma (SI P8147). The following primary antibodies were used: PAK1, c-Raf, Mek1, p-b-Raf (Ser 445), p-c-Raf (Ser 338), p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr 204), (all from Cell Signaling Technology), and tubulin (Abcam, Cambridge, MA, USA).

Cell Culture and transient transfection
HeLa, SW480, HT-29, IEC-6, and NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum. The cells were plated at 3×10^5 cells/well in 6-well plate in DMEM plus 10% calf serum. PAK1 wt cDNA or PAK1 K299R (PAK1 mutant) cDNA was cloned into Myc tagged pcDNA3.0 vector. Cells were then transiently transfected with Myc tagged PAK<sup>wt</sup>, Myc tagged PAK<sup>mutant</sup>, or high cycling Rac1 vector using Fugene 6 transfection reagent (Roche). shRNA target PAK1 3′-non coding area was purchased from Shanghai GenePharma Company, China (Lot No. A05925). shRNA target constitutive PAK1 was purchased from Shanghai GenePharma Company, China (Lot No. A05927).

Subcellular fractionation
Cell membrane protein was collected by using Plasma Membrane Protein Extraction Kit (Abcam, ab65400). In brief, cells were washed with cold PBS and suspended in the Homogenize Buffer Mix in an ice-cold Dounce homogenizer. Homogenates were centrifuged at 700g for 10 min at 4°C. The resulting supernatants (cytosol) were collected and the pellets were resuspended in Upper and Lower Phase Solution. The lysates were again centrifuged at 1,000g for 5 min, and the pellets (membrane) were collected. The distributions of proteins in the cytosol and membrane fractions were analyzed by western blot.

Immunoprecipitation and Western blot
Immunoprecipitation and Western blot were carried out as described previously (9). In brief, 48h after transfection, cells were washed by cold PBS and lysed with 0.5 ml of IP lysis buffer for 0.5 h at 4°C. The whole cell lysates were incubated with control rabbit normal IgG (Santa Cruz Biotechnology), PAK1 antibody, HA or the myc antibody (all from Cell signaling) at 4°C for 1 h. Pre-equilibrated protein G-agarose
beads (Roche Applied Science) were then added, collected by centrifugation after 1 h of incubation, and then gently washed with the lysis buffer. The precipitates were washed with ice-cold lysis buffer. To elute the bound proteins, washed precipitates were boiled in SDS sample buffer. Protein samples were detected by indicated antibodies using Western blot according to standard protocols. Western blot results were quantified using TotalLab TL100 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) and tubulin was used to normalize for different protein amounts.

**Immunofluorescent staining**
Hela cells were transfected with indicated plasmids for 48h. For PDGF stimulation, cells were serum starved for 4 h and then left unstimulated or stimulated with PDGF (20 ng. ml⁻¹) for 15 min. Cells were then fixed with 4% formaldehyde for 15 min at room temperature, after incubation with 2% bovine serum albumin (BSA) in PBS for 30 min to block non-specific antibody binding. Endogenous Mek1 was detected using an anti-Mek1 mouse monoclonal antibody. After washing with PBS, the cells were stained with Alexa 488-conjugated anti-rabbit IgG. Pictures were taken by confocal microscope.

**Proliferation assay**
Cells were treated as indicated reagents and plasmids. Proliferation was tested by crystal violet staining as described previously (7). Cell numbers were then determined by crystal violet staining. In brief, cells were seeded in 96-well plates for 2 days. Fixed with 70% ethanol, cells were stained with 0.5% crystal violet in 20% methanol for 15 min at room temperature. After washed with PBS, cell-bound crystal violet was extracted with 10% SDS. Absorbance at 490nm was measured using a microplate reader (Bio-rad).

**Statistics**
Averages are shown with standard deviation. Significance was calculated by Student’s t-test.
Results

PAK1 regulates cells proliferation independent of kinase activity

PAK1 has been reported to be hyperactivated in various cancer tissues to promote cancer cells proliferation (8). However, unexpectedly, after we transfected two colon cancer cell lines, SW480 and HT29, and HeLa cells with wild type PAK1 (PAK1wt) or kinase-negative PAK1 (K298R; PAK1K298R) plasmids, we observed an increase of p-PAK1/2 (Ser144/141) and p-Erk1/2(Thr 202/Tyr 204) by Western blot. Using crystal violet assay, expression of PAK1wt and PAK1K298R effectively promoted cell proliferation. Inhibition of Mek phosphorylation by PD98059 at 50µM significantly decreased the level of p-Erk1/2(Thr 202/Tyr 204), but not p-PAK1/2 (Ser144/141), consistent with previous observations in keratinocytes (7), and reduced the proliferation induced by platelet-derived growth factor (PDGF), PAK1wt, or PAK1K298R (Fig. 1A, B). Similar results were obtained with the normal intestinal epithelial cell line IEC-6 and with NIH3T3 fibroblasts (Fig. 1C), suggesting that PAK1 promotes cell proliferation through Mek1 in a kinase-independent manner.

PDGF increases binding of PAK1 and Mek1

PDGF was reported to stimulate cell proliferation partially by Rac1-dependent activation of the Mek-Erk pathway (9). To examine the interaction of PAK1 and Mek1 in this condition, Hela, SW480, HT-29, IEC-6 and NIH3T3 cells were incubated with PDGF. Increased binding of Mek1 and PAK1 was observed in response to PDGF (Fig. 2). We next tested, whether PAK1 promotes the association of Raf and Mek1. Indeed, expression of PAK1wt or PAK1K298R greatly increased the amount of Flag tagged Mek1, which co-immunoprecipitated with HA-tagged Raf (Fig. 3A). Furthermore, knockdown of endogenous PAK1 by shRNA reduced the amount of endogenous Mek1 that co-immunoprecipitated with Raf, regardless of incubation of PDGF, in NIH3T3 cells (Fig. 3B). Our results indicate that PAK1 might regulate cell proliferation by promoting interaction of Raf and Mek1, inducing Mek1 phosphorylation. Yet, this activation did not require the PAK1 kinase activity.

PAK1 enhances phosphorylation of Mek1 in a kinase independent manner
To test the importance of the kinase function of PAK1 for Mek phosphorylation in cancer cells, we transfected SW480 and HT29, HeLa, as well as IEC-6 and NIH3T3 cells with PAK1wt or PAK1K298R plasmids. Overexpression of myc-tagged PAK1wt resulted in increased levels of p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204), indicating increased activation of both Mek1/2 and Erk. Neither b-Raf, nor c-Raf phosphorylation was altered in cells transfected with PAK1wt. Similar changes were observed in cells transfected with PAK1K298R, indicating that PAK1 can activate Mek1/2 in a kinase independent manner. Loss of kinase activity of PAK1K298R was confirmed by blotting for pPAK1/2 (Ser144/141), which was increased after transfection with PAK1wt, but unchanged after transfection with PAK1K298R, while ectopic expression of both PAK1 forms was comparable (Fig. 4A, B).

To show the importance of endogenous Pak1 for the activation of Erk in HeLa cells, we next depleted endogenous PAK1 by shRNA against PAK1 3’-non coding area (Fig. 5A). Indeed, knockdown of PAK1 resulted in decreased levels of p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204). We then tried to rescue the phenotype by transfection of PAK1wt and PAK1K298R into PAK1 knock down cells. Since the Pak1 expression vectors did not contain the sequence targeted by the shRNA, they were insensitive to the Pak1 knockdown. We observed increased p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204) by both PAK1wt and PAK1K298R in a dose-dependent manner. Similarly, transfection with PAK1wt and PAK1K298R could rescue the decrease of p-Mek1/2 (Ser 217/221) and p-Erk1/2 (Thr 202/Tyr 204) due to the knock down of endogenous PAK1 in NIH3T3 cells (Fig. 5B). These data corroborate that Pak1 can activate Mek-Erk signaling in a kinase independent manner.

**PAK1 recruits Mek1 onto the membrane**

It was reported earlier that PAK1 activates Akt in a kinase independent manner by recruiting Akt to the cell membrane (3). To investigate, whether a similar mechanism could be involved in the kinase independent Mek1/2 activation of PAK, we checked whether Pak1 could bind to Mek while at the cell membrane. Pak1 can be indirectly...
associated with the cell membrane by binding to GTP-bound, active Rac1 or Cdc42. If this interaction is stable, it might increase the amount of membrane associated Pak, which can bind to the activated Pak, resulting in a triple complex of Rac-GTP, Pak1, and Mek. To probe for the existence of such triple complexes, we transfected the three cancer cell lines with a myc-tagged, high cycling form of Rac1, which is preferentially in the GTP-bound state. Immunoprecipitation of Rac1 by antibodies against myc pulled down Pak1, but also Mek1, suggesting the formation of a triple complex of activated Rac1, Pak1, and Mek1 at the cell membrane (Fig. 6).

If MEK is recruited to the cell membrane via Pak1 bound to membrane associated Rac1, we would expect an increase of Mek1 in the membrane fraction by overexpression of Pak1. Exactly this we observed in HeLa cells overexpressing Pak1wt, but also in those overexpressing the kinase-dead mutant form Pak1K298R (Fig. 7A). Growth factor stimulation induced translocation of endogenous Mek1 (Fig. 7B) from the cytosolic to plasma membrane fractions, and this process was inhibited by repression of endogenous Pak1. Decrease of Pak1 expression by shRNA knockdown, reduced translocation of Mek1 to the cell membrane (Fig. 7B). Moreover, immunofluorescent staining revealed that expression of Pak1wt and Pak1K298R effectively promoted translocation of Mek1 to the membrane fraction (Fig. 8). Similarly, stimulation of growth factor PDGF induced translocation of endogenous Mek1 (Fig. 8) from the cytosolic to plasma membrane, and this process was inhibited by Pak1 shRNA, suggesting that endogenous Pak1 is crucial for membrane translocation of Mek1 stimulated by growth factors.

As a result, Rac1 mediated recruitment of Mek to the membrane will increase the local concentration of Mek. This might facilitate the MEK1 dependent phosphorylation and activation of Raf, which is recruited to the membrane by membrane associated, activated Ras (Fig. 9).
Discussion

PAK1 is widely upregulated and hyperactivated in several human cancers, such as breast cancers, neurofibromatosis and colorectal cancers (8,10-11). Although it was shown by several groups that PAK1 is regulating Erk dependent proliferation, the molecular details of this process are not entirely clear. Our data suggest that PAK1 might contribute to Erk activation also in a kinase independent manner, most possibly by facilitating Mek recruitment to the cell membrane. About 30% of human tumors show hyperactivation of MAPK pathway represented by high phosphorylation levels of Erk1/2 (12). Many in vitro studies have implicated that PAK1 regulates both c-Raf and Mek1 activity by direct phosphorylating these proteins at Ser338 and Ser298, respectively (3). Depletion of PAK1 expression decreased the activities of Erk and Akt, thereby inhibiting cell proliferation, migration/invasion and survival, suggesting PAK1 may act as a key molecular for transmitting signals from Ras and PI3K by activating downstream Mek/Erk and Akt pathways (13). We previously found that Rac1-controlled PAK1 activation is crucial to allow activated c-Raf to interact with Mek1/2 to mediate Erk-dependent hyperproliferation in keratinocytes both in vivo and in vitro (7).

We report now that PAK1 is able to promote Mek1/2 phosphorylation in a kinase-independent manner, suggesting a scaffold function for PAK1 in addition to its kinase function. Already earlier it was suggested that PAK1 has a kinase independent scaffold function for the activation of Akt by recruiting Akt to the membrane. We demonstrate in this study that PAK1 bound to membrane bound to membrane associated, activated can recruit Mek to the cell membrane. This will increase the local concentration of Mek at the cell membrane and by this mechanism promote Mek phosphorylation by activated Raf, which is indirectly associated to the cell membrane by binding to Ras. This recruitment will be important in those cases, where normal diffusion of Mek to the cell membrane is not sufficient to saturate activated Raf. The lower the expression of Mek and the higher the activation of Raf, the more important the scaffold function of Pak is supposed to be. Future experiments should address the relative importance of the kinase independent Pak function in vivo by xenotransplantation of cancer cells.
transfected with wild type and kinase dead PAK1.
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Figure legends

Figure 1: PAK1 regulates cells proliferation through Mek1 activity independent of its kinase activity. (A) SW480, HT-29 and Hela cells were treated with PDGF (20 ng/ml), and/or Mek inhibitor PD985402 (50 µM), or transfected with indicated plasmids and analyzed for growth by crystal violet staining 2 days later (n=6; *P<0.01). (B) Hela cells were transfected with PAK1<sup>wt</sup> or PAK1<sup>K299R</sup> plasmid for 18 h. Cell lysates were immunoblotted with antibodies to p-b-Raf (Ser 445), p-c-Raf (Ser 338), p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr 204). Expression of PAK1 mutants was confirmed by immunoblotting with antibodies to PAK1, Myc and p-PAK1/2 (Ser 144/141). Representative blots from three independent experiments are shown. (C) IEC-6 and NIH3T3 cells were treated with PDGF (20 ng/ml), and/or Mek inhibitor PD985402 (50 µM), or transfected with indicated plasmids and analyzed for growth by crystal violet staining 2 days later (n=6; *P<0.01).

Figure 2: PDGF mediated binding of PAK1 and Mek. Co-immunoprecipitation of Mek1 and PAK1 in Hela, SW480, HT-29, NIH3T3 and IEC-6 cell lysates. Cells were serum-starved for 4 h and then left unstimulated or stimulated with PDGF (20 ng. ml<sup>-1</sup>) for 30 min. Cell lysates were immunoprecipitated with antibodies to Myc and then immunoblotted with antibodies to Myc or Mek1. Western blotting was carried out with indicated antibodies.

Figure 3: PAK1 bridged the interaction of Raf and Mek. (A) Hela cells were transfected with the indicated plasmids for 18 h. Cell lysates were immunoprecipitated with antibodies against HA and immunoblotted with antibodies against Flag or c-Raf. (B) NIH3T3 cells were transfected with the shRNA against PAK1 for 48h. Then cells were serum-starved for 4 h and then left unstimulated or stimulated with PDGF (20 ng. ml<sup>-1</sup>) for 0, 15, 30 min. Cell lysates were immunoprecipitated with antibodies against Raf and immunoblotted with antibodies against Mek1 or c-Raf.

Figure 4: PAK1 regulated Mek1 activity independent of its kinase activity. (A) Hela, SW480, HT-29, NIH3T3 and IEC-6 cells were transfected with PAK1<sup>wt</sup> or PAK1<sup>K299R</sup> plasmid for 18 h. Cell lysates were immunoblotted with antibodies to p-b-Raf (Ser 445), p-c-Raf (Ser 338), p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr 204). Expression of PAK1 mutants was confirmed by immunoblotting with antibodies
to PAK1, Myc and p-PAK1/2 (Ser 144/141). Representative blots from three independent experiments are shown. (B) Quantification of Western blot analyses (n=3; *P<0.01).

**Figure 5:** PAK1 regulated Mek1 phosphorylation independent of its kinase activity. (A) Hela cells were transfected with shRNA against endogenous PAK1 (PAK1 KD), and PAK1 WT or PAK1 PAK1 K299R plasmid at different concentration for 18 h. Cell lysates were immunoblotted with antibodies to p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr 204). Each blot was quantified and analyzed (n=3; *P<0.01). (B) NIH3T3 cells were transfected with shRNA against endogenous PAK1 (PAK1 KD), and PAK1 WT or PAK1 K299R plasmid at the concentration of 15μg for 18 h. Cell lysates were immunoblotted with antibodies to p-Mek1/2 (Ser 217/221), p-Erk1/2 (Thr 202/Tyr 204). Each blot was quantified and analyzed (n=3; *P<0.01).

**Figure 6:** Association of Mek1 and PAK1. Immunoprecipitation of Mek1 and PAK1 in SW480, HT-29, Hela, NIH3T3 and IEC-6 cell lysates. Cells were transfected with the indicated plasmids for 36 h. Cell lysates were immunoprecipitated with antibodies to Myc and then immunoblotted with antibodies to Myc, PAK1 or Mek1.

**Figure 7:** PAK1 recruit Mek1 onto the membrane and promotes its phosphorylation in a kinase independent manner. (A) Hela cells were transfected with shRNA against PAK1 (PAK1 KD), and PAK1 WT or PAK1 PAK1 K299R plasmid for 48h. Cells were subjected to subcellular fractionation and the amounts of Mek1 in the membrane or cytosolic were analyzed by immunoblotting with antibodies to Mek1. (B) NIH3T3 cells were transfected with shRNA against endogenous PAK1 (PAK1 KD) for 48h. Then cells were serum-starved for 4 h and then left unstimulated or stimulated with PDGF (20 ng ml⁻¹) for 0, 15 and 30 min. Cells were subjected to subcellular fractionation and the amounts of Mek1 in the membrane or cytosolic were analyzed by immunoblotting with antibodies to Mek1.

**Figure 8:** PAK1 recruit Mek1 onto the membrane and promotes its phosphorylation in a kinase independent manner. Hela cells were transfected with shRNA against endogenous PAK1 (PAK1 KD), and PAK1 WT or PAK1 PAK1 K299R plasmid for 48h. Cells were serum-starved for 4 h and then left unstimulated or stimulated with PDGF (20 ng ml⁻¹) for 15 min. Cells were fixed and stained with antibodies to Mek1. Scale bar, 20 μm.
**Figure 9:** Activated Rac1 recruits PAK1 and Mek1 to the cell membrane, where Mek is phosphorylated by activated Raf bound to membrane associated Ras.
Figure 3

A

| Flag-Mek1 | Myc-PAK1 | HA-Raf | IP: HA | Total extract | IP: Raf | Total extract |
|-----------|----------|--------|--------|---------------|--------|---------------|
| -         | -        | -      | Flag-Mek1 | - | Raf | - |
| -         | +        | +      | Flag-Mek1 | - | Raf | - |
| wt        |          |        |         |   |     |   |
| mutant    |          |        |         |   |     |   |

B

|          | Con | PAK1 KD |
|----------|-----|---------|
| +PDGF    | 0   | 15      | 30      |
| Mek1     |     |         |         |
| Raf      |     |         |         |
|          | 0   | 15      | 30 min  |
| Mek1     |     |         |         |
| Raf      |     |         |         |
Figure 4

A

|          | Myc | PAK1 | p-PAK1/2 (Ser144/141) | p-b-RAF (Ser445) | p-c-RAF (Ser338) | p-MEK1/2 (Ser217/221) | p-ERK1/2 (Thr202/Tyr204) | tubulin |
|----------|-----|------|------------------------|-------------------|-------------------|------------------------|---------------------------|---------|
| Con      |     |      |                        |                   |                   |                        |                           |         |
| PAK1     |     |      |                        |                   |                   |                        |                           |         |
| K298R    |     |      |                        |                   |                   |                        |                           |         |

B

|          | PAK1 | p-PAK1/2 (Ser144/141) |
|----------|------|------------------------|
| Relative band density |
| con | PAK1 wt | PAK1 mutant |
|     |       |             |
|     |       |             |
|     |       |             |

|          | p-b-RAF (Ser445) | p-c-RAF (Ser338) |
|----------|-------------------|-------------------|
| Relative band density |
| con | PAK1 wt | PAK1 mutant |
|     |       |             |
|     |       |             |
|     |       |             |

|          | p-MEK1/2 (Ser217/221) | p-ERK1/2 (Thr202/Tyr204) |
|----------|------------------------|---------------------------|
| Relative band density |
| con | PAK1 wt | PAK1 mutant |
|     |       |             |
|     |       |             |
|     |       |             |
Figure 5

A

PAK1

p-MEK1/2 (Ser217/221)

p-ERK1/2 (Thr202/Tyr204)

tubulin

B

PAK1

p-MEK1/2 (Ser217/221)

p-ERK1/2 (Thr202/Tyr204)

tubulin

Relative band density

Con PAK1 KD 5µg 10µg 15µg PAK1 wt

Relative band density

Con PAK1 KD 5µg 10µg 15µg PAK1 wt
Figure 6

|       | Hela       | HT-29     | SW480     | NIH3T3    | IEC-6     |
|-------|------------|-----------|-----------|-----------|-----------|
| myc   |            |           |           |           |           |
| MEK1  |            |           |           |           |           |
| PAK1  |            |           |           |           |           |

Figure 7

A

| Membrane | PAK1 KD |
|----------|---------|
| Con      | PAK1 wt| PAK1 K298R| PAK1 KD |
| MEK1     |        |           |         |
| Beta-integrin |     |           |         |
| tubulin  |        |           |         |

B

| Membrane | Cytosol |
|----------|---------|
| +PDGF    | 0 15 30 | 0 15 30  |
| PAK1 KD  | 0 15 30 | 0 15 30  |
| MEK1     |        |           |         |
| Beta-integrin |     |           |         |
| tubulin  |        |           |         |
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