

Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes

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Summary

We identified dynein light chain 1 (DLC1) as a physiologic substrate of p21-activated kinase 1 (Pak1). Pak1-DLC1 interaction plays an essential role in cell survival, which depends on Pak1's phosphorylation of DLC1 on Ser88. Pak1 associates with the complex of DLC1 and BimL, a proapoptotic BH3-only protein, and phosphorylates both proteins. Phosphorylation of BimL by Pak1 prevents it from interacting with and inactivation of Bcl-2, an antiapoptotic protein. Overexpression of DLC1 but not DLC1-Ser88Ala mutant promotes cancerous properties of breast cancer cells. DLC1 protein level is elevated in more than 90% of human breast tumors. The regulation of cell survival functions by Pak1-DLC1 interaction represents a novel mechanism by which a signaling kinase might regulate the cancerous phenotypes.

Introduction

The p21-activated kinases (Paks), an evolutionarily conserved family of serine/threonine kinases, are essential for a variety of cellular functions, including cell morphogenesis, cell motility, cell survival, angiogenesis, and mitosis (Bokoch, 2003; Kumar and Vadlamudi, 2002). At present, the Pak family consists of six members, Pak1 through Pak6. Pak1 has been identified as one of the targets of the activated Rho GTPases Cdc42 and Rac1, which stimulate Pak autophosphorylation and activity (Manser et al., 1994). Stimulation of Pak1 activity results in several phenotypic changes reminiscent of those produced by Cdc42 and Rac1 (Sells et al., 1997; Vadlamudi et al., 2000; Li et al., 2002; Thiel et al., 2002). Paks are widely expressed in numerous tissues and are activated by a number of polypeptide factors and extracellular signals in both a GTPase-dependent manner via Rac1 or Cdc42 and a GTPase-independent manner via its localization to membrane/focal adhesion (Bokoch et al., 1996; Bagrodia and Cerione, 1999; Zhao et al., 2000). Paks are also activated by lipids (Bokoch et al., 1998), tyrosine kinases (McManus et al., 2000; Bagheri-Yarmand et al., 2001), novel substrates such as filamin (Vadlamudi et al., 2002), and G proteins (Lian et al., 2001).

The activation of Pak1 by diverse signals leads to its autophosphorylation at multiple sites, including threonine 423

(T423), within the activation loop of the kinase (Daniels and Bokoch, 1999). Expression of an activated Pak1 mutant (T423E) triggers the dissolution of stress fibers and focal adhesion complexes, the formation of lamellipodia (Manser et al., 1997), and reorganization of actin cytoskeleton. Some of the effects of Pak1 on the actin cytoskeleton appear to be independent of Pak1 kinase activity but dependent on protein-protein interactions (Turner et al., 1999). However, kinase activity is important for directional motility (Sells et al., 2000). Nonetheless, Pak1 mutants with defective GTPase binding sites or kinase activity retain the ability to induce the formation of membrane ruffles and filopodia (Sells et al., 1997; Frost et al., 1998). Pak1 also stimulates LIM kinase (LIMK) activity and, in turn, increases phosphorylation and inactivation of cofilin, leading to reduction in the depolymerization of actin filaments (Edwards and Gill, 1999; Edwards et al., 1999). Pak1 has been shown to influence cell survival by phosphorylating Bad (Schurmann et al., 2000), and overexpression of Paks in cancer cells increases cell migration potential and anchorage-independent growth and causes abnormalities in mitosis (Banerjee et al., 2002; Li et al., 2002). However, the molecular mechanisms by which Pak1 regulates cell growth and cell survival functions remain elusive.

Dynein light chain 1 (DLC1), an 8 kDa component of the cytoplasmic dynein complex (Hirokawa, 1998), is highly conserved among species, is widely expressed in a number of

SIGNIFICANCE

There is increasing evidence that p21-activated kinase 1 (Pak1) and dynein light chain 1 (DLC1), both components of the cytoskeleton system, target different pathways to support cell survival. However, no DLC1 function has been associated with its phosphorylation, and no signaling kinase has been shown to phosphorylate DLC1. We found that DLC1 is a physiologic interacting substrate of Pak1 and that the Pak1-DLC1 interaction is essential to its cell survival functions. We have discovered that deregulation of DLC1, but not its Pak1 phosphorylation mutant, confers cell survival, anchorage independence, and tumorigenesis in nude mice and that DLC1 is widely overexpressed in human breast tumors. Thus, DLC1 can function as a tumor promoter and requires active Pak1 signaling.

tissues, and is localized predominantly in the cytoplasm. In addition to playing an essential role in dynein motor function, DLC1 interacts with a number of proteins with diverse functions. For example, DLC1 associates with neuronal nitric oxide synthase (nNOS) and inhibits its activity and proapoptotic function in neuronal cells; hence, DLC1 has also been referred to as protein inhibitor of NOS (PIN) (Jaffrey and Snyder, 1996). DLC1 also interacts and interferes with the proapoptotic Bcl-2 family protein BimL (Puthalakath et al., 1999; Puthalakath and Strasser, 2002). Although the functional role of DLC1 in cell survival has been observed, no function of DLC1 has been assigned to its phosphorylation, and no upstream signaling kinase has been shown to phosphorylate DLC1 and influence these functional outcomes.

To identify novel Pak1 binding proteins, we performed a yeast two-hybrid screen of the mammary gland cDNA expression library using Pak1 N-terminal amino acids 1–270 as a bait. We identified DLC1 as a Pak1-interacting protein. We showed that DLC1 is a physiologic, interacting substrate of Pak1. The Pak1-DLC1 interactions play an essential role in the cell-survival functions of both Pak1 and DLC1, and the underlying mechanism involves Pak1's phosphorylation of DLC1 on serine 88 (Ser88). Unexpectedly, we discovered that DLC1 expression is commonly elevated in human breast tumors and that deregulation of DLC1, but not DLC1 mutant lacking Ser88, promotes the tumorigenic potential of breast cancer cells. The regulation of cell survival and tumorigenic functions by Pak1-DLC1 interaction represents a novel mechanism by which a signaling kinase might control these essential processes in cancer cells.

Results

Pak1 interacts with DLC1

A yeast two-hybrid screening of the mammary gland cDNA expression library was performed to identify Pak1-interacting proteins using the Pak1 N-terminal amino acids 1–270 as bait. This bait contains several protein binding motifs that differ from one Pak family member to another. This analysis resulted in the identification of a substantial number of known Pak1 binding proteins (i.e., Cdc42, Rac1, Pix, and Nck) and several previously uncharacterized Pak1-interacting proteins. Sequence analysis of several isolates from one of the novel interacting clones was identical to that of DLC1 (GenBank accession number NM_003746). The specificity of the Pak1-DLC1 interactions was verified by cotransfection of Pak1 and DLC1 plasmids into the yeast cells (Figure 1A). Cotransfection of DLC1 with the Pak1 N-terminal amino acids 1–270 but not control vector enabled the transformed colonies to grow in medium lacking adenosine, histidine, leucine, and tryptophan (AHLT) and reacted positively in β -galactosidase assay (Figure 1A).

To verify the specificity of the Pak1-DLC1 interaction, we next evaluated the ability of *in vitro* translated Pak1 protein to bind with the DLC1-GST fusion protein. The DLC1-GST fusion protein, but not GST, efficiently interacted with 35 S-labeled full-length Pak1 protein (Figure 1B, left). Conversely, *in vitro* translated 35 S-labeled DLC1 protein specifically interacted with the GST-Pak1 (Figure 1B, right). The *in vivo* interaction of the endogenous DLC1 with Pak1 was confirmed by immunoprecipitation of Pak1 and DLC1 using lysates from MCF-7 breast cancer cells treated with or without epidermal growth factor (EGF), and results show that Pak1 interacted with DLC1 upon ligand

stimulation (Figure 1C). Similarly, immunoprecipitation of the endogenous DLC1 along with a catalytically active Pak1 (HA-tagged T423E Pak1) further confirmed the existence of Pak1-DLC1 interaction *in vivo* (Figure 1D). In brief, these findings strongly suggested that Pak1 interacts with DLC1 in a physiologically relevant setting.

DLC1 interacts with Pak1 via its C-terminal region

We next mapped the DLC1 binding site in Pak1 using the GST fusion domains of Pak1 (Figure 2A). Results showed that the Pak1 amino acids (aa) 132–270, but not aa 1–132, efficiently interacted with DLC1. Furthermore, the Cdc42/Rac interactive binding (CRIB) domain (aa 53–132), the Pak1 autoinhibitory domain (aa 75–149), and the kinase domain of Pak1 (aa 270–545) did not interact with DLC1, suggesting that the binding sites for DLC1 are localized within the aa 150–270 of Pak1. Earlier studies showed that the Pak1 aa 150–270 region contains the PIX binding site (a noncanonical proline-rich region, aa 192–203) (Daniels et al., 1999). Therefore, to further examine whether the DLC1 binding region in Pak1 overlaps with the PIX binding site, we created additional fine deletions in this region (Figure 2B). GST protein containing the PIX binding domain (aa 182–203) showed no interaction with DLC1, suggesting that the Pak1 binding site does not overlap with the PIX binding site. However, GST fusion containing Pak1 aa 203–270 showed distinct binding to DLC1, implying that the DLC1 binding site in Pak1 is localized to the 67 amino acid region spanning aa 203–270 of Pak1.

Next, using a series of DLC1 deletion constructs, we identified the minimal region of DLC1 required for its interaction with Pak1. As illustrated in Figure 2C, deletion of N-terminal regions had no effect on Pak1 binding, whereas deletion of the C-terminal 22 amino acids (aa 67–89) completely abolished Pak1 binding (Figure 2C, lanes 6 and 7). GST fusion of DLC1 C-terminal 19 amino acids alone was sufficient for Pak1 binding (Figure 2C, lane 5), suggesting that the Pak1 binding site in DLC1 is in the C terminus.

DLC1 is a physiological substrate of Pak1

To determine whether DLC1 is a substrate of Pak1, we explored the possibility that DLC1 could be phosphorylated *in vivo* by physiologic signals, such as EGF, that activate Pak1 (Kumar and Vadlamudi, 2002). MCF-7 cells were metabolically labeled with [32 P]orthophosphoric acid and stimulated with EGF, and DLC1 was immunoprecipitated with an anti-DLC1 antibody. The autoradiogram showed a substantial increase in the level of DLC1 phosphorylation in EGF-treated cells (Figure 3A). To examine the specific role of Pak1 in growth factor-mediated phosphorylation of DLC1, we used an siRNA specific to Pak1 (Wang et al., 2002) to selectively knock down its expression. Introduction of Pak1 siRNA in MCF-7 cells resulted in 70% reduction in the level of Pak1 protein and was accompanied by a 60% reduction in the ability of EGF to stimulate DLC1 phosphorylation compared with its activity in control cells (Figure 3A, lane 3). To independently validate these findings, we repeated the experiment using a Pak1 autoinhibitory fragment, Pak1 aa 83–149, a well-established inhibitor of Pak1 (Vadlamudi et al., 2002). MCF-7 cells were transiently transfected with T7-tagged DLC1 with or without the Pak1 inhibitory fragment. As shown in Figure 3B, EGF treatment rapidly stimulated DLC1 phosphorylation in a Pak1-sensitive manner, and it was substantially blocked by coexpression of Pak1 autoinhibitory fragment aa 83–149. Next,

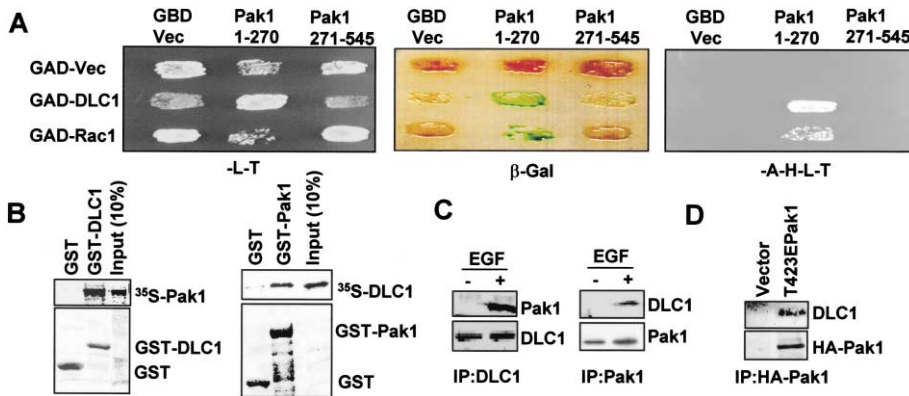


Figure 1. Identification of DLC1 as a Pak1 binding protein

A: Yeast cells were cotransfected with control GAD vector or GAD-DLC1. GAD-Rac1 along with GBD vector, or GBD-Pak1 (aa 1–270) or GBD-Pak1 (aa 271–545). Cotransformants were plated on selection plates lacking leucine and tryptophan (LT) or adenosine, histidine, leucine, and tryptophan (AHLT). Growth was recorded after 72 hr. For β-galactosidase assay, filter lift assays were performed (middle). Blue color indicates specific interaction of two proteins. Rac1 was used as a positive control.

B: Pak1 and DLC1 interaction in the GST pull-down assays. DLC1 or Pak1 cDNAs were translated in vitro, and ³⁵S-labeled proteins were incubated with either GST-Pak1 or GST-DLC1 and analyzed by SDS-PAGE and autoradiography.

C: In vivo interaction of Pak1 with endogenous DLC1. MCF-7 cells were serum starved for 48 hr and treated with epidermal growth factor (EGF) for 30 min, and cell lysates were immunoprecipitated with either Pak1 or DLC1 antibody. DLC1 association was analyzed by Western blotting with Pak1 or DLC1 antibody.

D: Activated Pak1 interacts with DLC1. Tet on-vector or Tet on-HA-Pak1-T423E expressing MCF-7 cells grown in 10% serum were lysed, and lysates containing equal amounts of protein were immunoprecipitated with HA antibody and immunoblotted with antibodies against DLC1 and Pak1.

we examined whether expression of catalytically active Pak1 (Pak1T423E) would mimic EGF-mediated DLC1 phosphorylation. MCF-7 cells were cotransfected with T7-tagged DLC1 and myc-tagged constitutively active T423E Pak1 or kinase dead Pak1 K299R and labeled with [³²P]orthophosphoric acid. Immunoprecipitation of DLC1 with an anti-T7 monoclonal antibody showed increased phosphorylation of DLC1 in cells expressing myc-T423E Pak1 but not in cells expressing myc-K299R Pak1

(Figure 3C), suggesting a role of Pak1 in the phosphorylation of DLC1 in vivo.

To map the Pak1 phosphorylation site in DLC1, we performed in vitro kinase assays using GST-DLC1 as a substrate. The BAD, a well-known Pak1 substrate, was used as a positive control. The Pak1 enzyme efficiently phosphorylated GST-DLC1 as well as BAD (Figure 3D). We next mapped the Pak1 phosphorylation site in DLC1 using various deletion constructs of DLC1. GST fusion containing the C-terminal 19 amino acids of DLC1 alone was efficiently phosphorylated by Pak1 (Figure 3E, lane 5), suggesting the presence of a putative phosphorylation site in the C-terminal region. Analysis of the C-terminal 19 amino acids sequence revealed one serine at amino acid 88. Deletion of two C-terminal amino acids of DLC1 (aa 88 and aa 89) completely abolished the ability of Pak1 to phosphorylate DLC1 (Figure 3E, lane 7). To confirm these findings, we substituted Ser88 to alanine (DLC1 Ser88A). As expected from the deletion studies, Pak1 failed to phosphorylate DLC1 Ser88A, confirming that Pak1 phosphorylates DLC1 on Ser88 (Figure 3F). To verify that Ser88 is the only DLC1 site that is phosphorylated by growth factors in vivo, we performed a transient transfection experiment with wild-type and Ser88A mutants of DLC1 (Figure 3G). Mutation of Ser88 on DLC1 to alanine completely abolished growth factor-mediated phosphorylation of DLC1. Collectively, these observations confirmed that DLC1 is a physiologic substrate of Pak1 and that Ser88 on DLC1 is the Pak1 phosphorylation site.

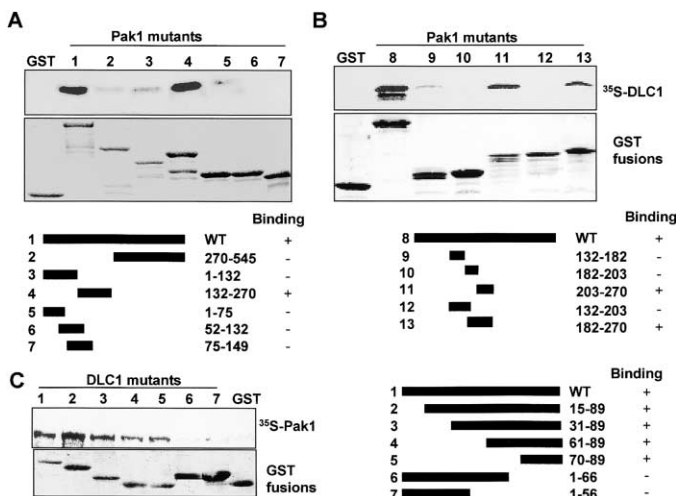


Figure 2. Mapping of interaction domains of Pak1 and DLC1

A: Identification of Pak1 domains that interact with DLC1. GST-Pak1 fusion proteins containing wild-type (aa 1–545), kinase domain (aa 271–545), N-terminal domain (aa 1–132), PIX binding domain (aa 182–203), Nck binding domain (aa 1–75), Cdc42/Rac-interactive binding (CRIB) domain (aa 52–132), and autoinhibitory domain (aa 75–149) were incubated with in vitro translated DLC1 (aa 1–89), and binding was analyzed by GST pull-down assay.

B: GST pull-down assay in lanes 9–13 shows various deletions of Pak1 in the region of 132–270.

C: Identification of the DLC1 region involved in the binding of Pak1. GST fusions of various lengths of DLC1 were used in the GST pull-down assay using ³⁵S-labeled Pak1 (amino acids 1–545).

DLC1 regulation of cell growth

To further delineate the potential effects of DLC1 on the biology of breast cancer cells, we next examined the status of DLC1 expression in a number of breast cancer cell lines. Breast cancer cells contained a higher level of DLC1 than nontumorigenic breast cancer cell lines, such as MCF10A and HC11 (Figure 4A). Because the ZR75 cells showed lower levels of DLC1 expression than other breast cancer cell lines used here, we next established stable ZR75 pooled clones expressing T7-tagged wild-type DLC1 or mutant DLC1^{del88–89} or control vector (Figure 4B). In general, these clones contained DLC1 levels 2- to 4-fold higher than the endogenous level of DLC1. Because both Pak1

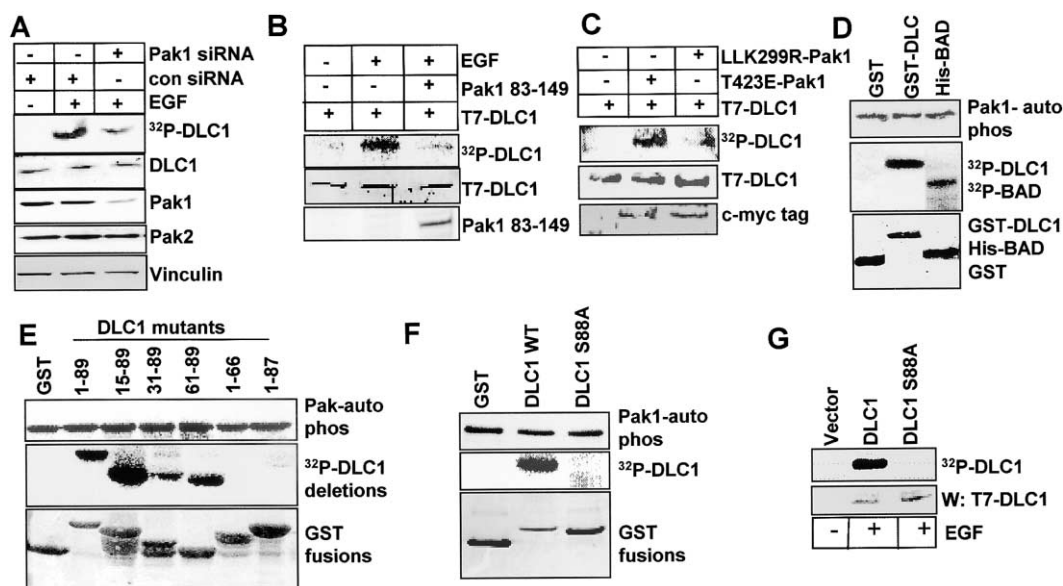


Figure 3. Pak1 phosphorylation of DLC1

A: MCF-7 cells were transfected with Pak1-specific or control siRNA, metabolically labeled with [32 P]orthophosphoric acid, and treated with or without EGF. Endogenous DLC1 was immunoprecipitated, and the phosphorylation status of DLC1 was analyzed by autoradiography. Total lysates were analyzed for Pak1, Pak2, and vinculin by Western blotting.

B: MCF-7 cells were transfected with T7-DLC1 with or without Pak1 autoinhibitory domain (Pak1 aa 83–149). Cells were metabolically labeled with [32 P]orthophosphoric acid and treated with or without EGF (1 nM) for 1 hr; then the phosphorylation of T7-DLC1 was analyzed by autoradiography.

C: MCF-7 cells were cotransfected with wild-type T7-DLC1 and T423E-Pak1 or K299R-Pak1. Cells were metabolically labeled with [32 P]orthophosphoric acid. T7-DLC1 was immunoprecipitated, and the phosphorylation status was visualized by autoradiography.

D: In vitro kinase assay using GST-DLC1 as a substrate and purified bacterially expressed Pak1 as an enzyme. GST was used as negative control, and His-BAD was used as a positive control.

E: In vitro Pak1 kinase assay using GST fusion of DLC1 fragments containing the indicated serial deletions of DLC1.

F: In vitro Pak1 kinase assay using DLC1 wild-type or mutant DLC1S88A as substrates.

G: MCF-7 cells transfected with wild-type DLC1 or mutant DLC1S88A were labeled in vivo with [32 P]orthophosphoric acid, serum starved, treated with EGF for 60 min, and immunoprecipitated; then phosphorylation status of DLC1 or its mutant was visualized by autoradiography.

and DLC1 are both shown to have a role in cell growth and survival (Puthalakath et al., 1999; Chang et al., 2000; Schurmann et al., 2000), we next examined whether Pak1 and DLC1 interaction have a role in cell growth and cell survival pathways. Interestingly, deregulated DLC1 expression increased the proliferation rate of the ZR75 cells (Figure 4C). The increased proliferation of the ZR75 cells could have reflected either an increased proliferation rate or protection of the cells from apoptosis. Flow cytometry analysis of serum-starved ZR75 clones revealed that deregulation of wild-type DLC1, but not mutant DLC1^{del88-89}, was accompanied by increased G1→S progression of breast cancer cells. Interestingly, ZR75 cells expressing DLC1^{del88-89} exhibited a significant increase in the number of cells in the pre-G0 phase of the cell cycle (Figure 4D). Consistent with a potential role of DLC1 in the G1/S transition, we found an increased expression of cyclin E in the clones with deregulated DLC1 but not in control pcDNA clones (Figure 4E). Further analysis using CDK2 phospho-specific antibody revealed a substantial enhancement of CDK2 phosphorylation in DLC1 expressing clones. In addition, biochemical analysis of the cyclin E immunocomplex confirmed the presence of elevated CDK2 kinase activity in the DLC1 clones, as well as increased phosphorylation of pRb at Ser795, a known CDK2/cyclin E phosphorylation site (Figure 4E). These findings provided clues about a potential role of Pak1-mediated DLC1 phosphorylation in optimal cell survival.

Interference with this Pak1-DLC1 pathway might trigger spontaneous apoptosis.

An essential role of DLC1 in Pak1-mediated cell survival

Earlier studies have shown that Pak family kinases protect murine NIH3T3 cells from ultraviolet (UV)-induced apoptosis (Jakobi et al., 2001). Using this well-established model system, we next investigated the potential role of Pak1-DLC1 interaction in protecting NIH3T3 cells against UV-induced apoptosis. NIH3T3 cells were cotransfected with RFP-tagged DLC1 or the mutant Ser88A DLC1 and catalytically active Pak1 (myc-T423E Pak1) or kinase-dead Pak1 (myc-K299LL Pak1), and cells were exposed to UV irradiation. After 10 hr, apoptosis was quantitated by scoring the integrity of the nuclear membrane in cells expressing both RFP (red) and myc-epitope tags to detect DLC1 and Pak1, respectively (Figure 5A). Results indicated that UV-induced apoptosis was substantially reduced in cells expressing DLC1 and catalytically active Pak1 but not in Ser88A DLC1 or kinase-dead Pak1 or the combination of these (Figure 5A). Interestingly, overexpression of active Pak1 could not protect the cells transfected with the mutant Ser88A DLC1. Similarly, overexpression of DLC1 failed to protect the NIH3T3 cells overexpressing the kinase-dead Pak1 from UV-induced apoptosis (Figure 5A).

As an additional evidence of a role of DLC1 in the cell survival and a broader significance of our findings in human cells in

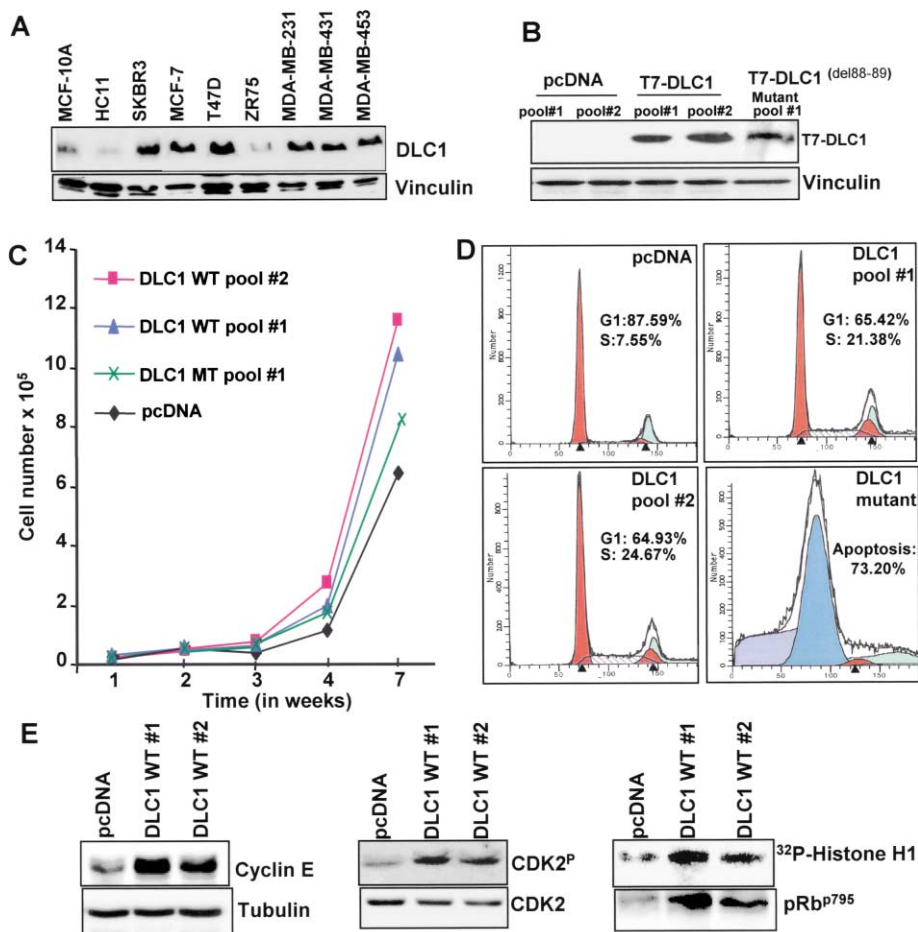


Figure 4. DLC1 expression and cell growth

A: Expression of DLC1 in a number of normal and breast cancer cells analyzed by Western blot analysis.

B: Expression of T7-DLC1 wild-type or T7-DLC1^{del88-89} in stable clones analyzed by Western blot analysis.

C: Equal number of ZR75 stable cells expressing DLC1 wild-type or DLC1^{del88-89} mutant were cultured. At indicated intervals, cells were trypsinized and counted using a Coulter Counter to determine cell proliferation. Each clone was plated in triplicate, and the experiment was repeated twice with similar results.

D: Cell cycle status of DLC1 wild-type, mutant, and pcDNA clones as analyzed by flow cytometry.

E: pcDNA and DLC1 stable clones were analyzed by Western blotting for the levels of cyclin E (left), for the levels of phospho CDK2 (middle) and the activity of CDK2 by immunoprecipitation followed by in vitro kinase assay using histone H1 as substrate, and also by Western blotting of total lysate using phospho pRb Ser795-specific antibody (right).

general, we next downregulated Pak1 in IMR-90 human fibroblasts by transiently expressing GFP vector or GFP vector expressing Pak1-siRNA. In some cultures, cells were cotransfected with GFP-tagged dominant active Pak1-T423E along with control or DLC-specific siRNA (Figure 5B). After 48 hr, cells were exposed to UV (100 J/m²). The onset of apoptosis was monitored by scoring the nuclear integrity using lamin A/C antibody. GFP staining was used to localize the positive transfected cells. We found that as expected, expression of activated Pak1 increased the cell survival after UV exposure, while knockdown of the expression of DLC1 resulted in a substantial increase in the ratio of apoptotic cells after UV damage. Interestingly, the noticed apoptotic response due to lack of DLC1 could not be rescued by activated Pak1 (Figure 5C). These findings suggest that Pak1-DLC1 interactions play an important role in their survival functions.

Pak1-DLC1 interaction promotes cell survival by blocking BimL's apoptotic functions

Because DLC1 is known to interact and sequester proapoptotic BimL in the cytoplasm (Puthalakath et al., 1999) and because Pak1 phosphorylates DLC1 in the region that interacts with BimL (Puthalakath et al., 1999), we next examined whether Pak1 phosphorylation of BimL interferes with BimL apoptotic functions. Initially, we examined the effect of Pak1 phosphorylation on the ability of DLC1 to interact with BimL. GST-DLC1 was

phosphorylated with Pak1 enzyme and subjected to the GST pull-down assay with ³⁵S-labeled BimL. Our results suggest that unphosphorylated but not phosphorylated DLC1 efficiently interacts with BimL, raising the possibility that Pak1 phosphorylation might interfere with the ability of DLC1 to interact with BimL (Figure 6A). To confirm these observations, we next mutated the Pak1 phosphorylation site Ser88 either to alanine or glutamic acid. GST pull-down assays showed that a Ser88 to alanine mutation has no effect on BimL binding to DLC1 (Figure 6B). However, a Ser88 to glutamic acid mutation, which mimics the phosphorylated form of DLC1 due to the addition of negative charge, abolished DLC1 interaction with BimL. Similarly, deletion of the C-terminal amino acids 88 and 89, which contain Ser88, also abolished BimL binding (Figure 6C). These results support the earlier view that DLC1 interacts with BimL via the C-terminal region (Puthalakath et al., 1999) and now offers an additional regulatory role of the Pak1 phosphorylation of DLC1.

In normal physiologic conditions, BimL is sequestered by DLC1, and DLC1-BimL dimers are released upon stimulation of cells with apoptotic stimuli. The DLC1-BimL dimers then interact with Bcl-2 and thus inhibit cell survival functions of Bcl-2 (Puthalakath et al., 1999; Puthalakath and Strasser, 2002). Therefore, we next explored whether Pak1 recognizes DLC1-BimL dimers. Results from the GST pull-down studies demonstrated that Pak1 recognizes DLC1 as well as DLC1 bound to BimL (Figure 6D). We then examined whether apoptotic signals

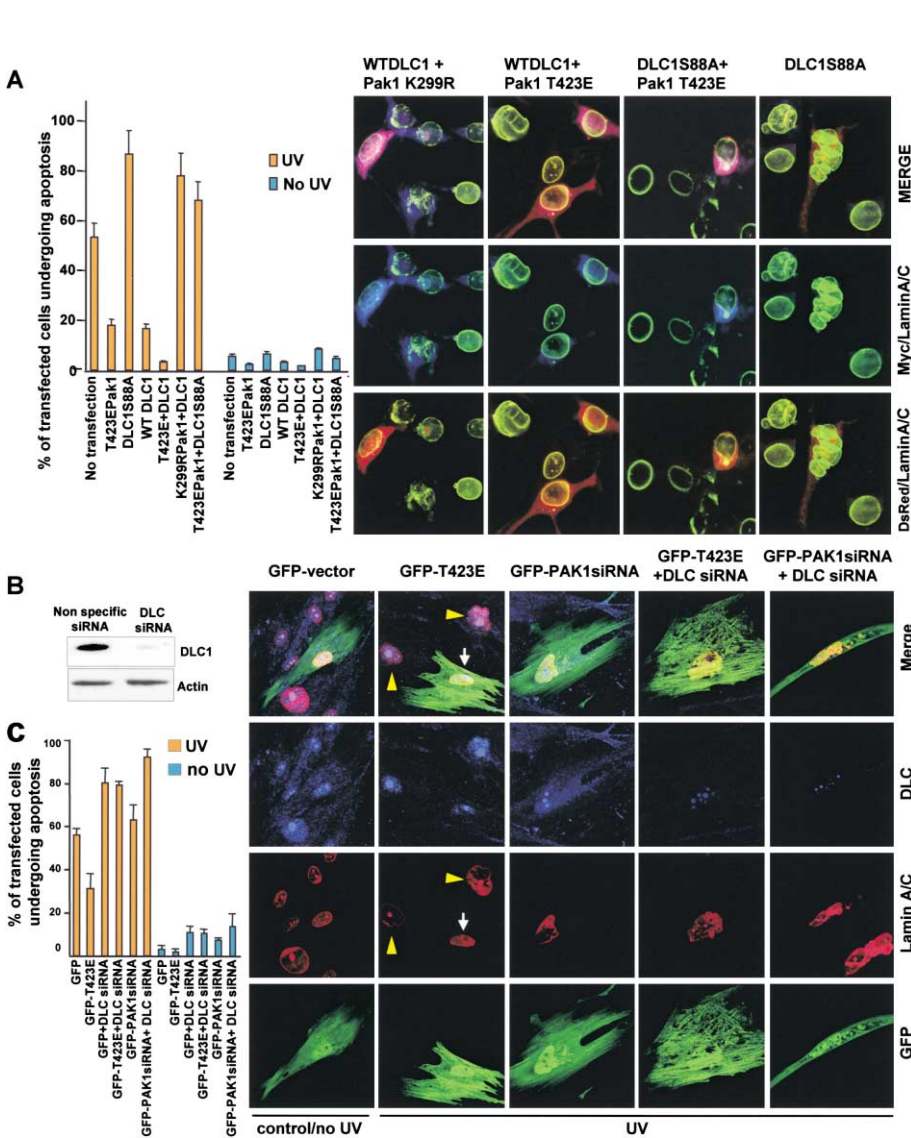


Figure 5. Requirement of DLC1 and Pak1 interaction for cell survival

A: Immunofluorescence staining of NIH3T3 cells cotransfected with various combinations of DLC1 wild-type or DLC1S88A mutant and various Pak1 constructs, exposed to UV radiation 100 mJ/s and after 10 hr, costained for myc-tag (for Pak1 constructs, blue), RFP-DLC1 wild-type, or mutants (DLC1 constructs, red) and for lamin A/C (green). Cells expressing the transfected plasmids were identified using appropriate filter settings and then characterized for their nuclear integrity using morphologic features identified by lamin A/C staining. For example, a smooth nuclear membrane was scored as nonapoptotic as opposed to membrane blebbing or rupture, which was scored as apoptotic. Nuclei committed to enter mitosis were not scored in either of these groups because they were easily identified for their specific "pulverized" pattern of lamin A/C staining. Ten randomly selected fields were analyzed for each condition. The experiment was repeated twice with similar results.

B: Functionality of DLC siRNA was analyzed by Western analysis.

C: Immunofluorescence staining of IMR90 human fibroblast cells cotransfected with GFP-tagged T423E Pak1 or GFP-tagged Pak1 siRNA or DLC1 siRNA or in combination. After 48 hr, cells were exposed to UV radiation 100 mJ/s and after 10 hr, costained for GFP-Pak1 T423E (green), GFP-Pak1 siRNA (green), or endogenous DLC1 (blue) and for lamin A/C (red). Apoptotic cells were scored as described in (A). Yellow arrow points to an apoptotic cell and white arrow points to a cell that was protected by expression of active Pak1.

such as UV and survival signals such as EGF modulate the status of phosphorylation of DLC1 and BimL. UV treatment alone did not induce phosphorylation of DLC1 or BimL; rather, a slight decrease in the phosphorylation was observed compared to control (Figure 6E). Interestingly, the addition of survival factors such as EGF followed by UV treatment resulted in a substantial increase in the phosphorylation of both DLC1 and BimL. Transfection of cells with Pak1 inhibitory domain (Pak1 aa 83–149) effectively inhibited the noticed upregulation of BimL phosphorylation by EGF under condition of UV treatment (data not shown), suggesting an additional regulatory role of Pak1 in BimL phosphorylation. In support of this notion, we next found that Pak1 could indeed directly phosphorylate BimL (Figure 6F). We then examined whether Pak1 phosphorylates BimL when present as a dimer with DLC1 and when attached to the dynein complex via dynein intermediate chain (DIC) as a trimeric complex (DIC-DLC1-BimL) using *in vitro* Pak1 kinase assay. Results show that Pak1 preferentially phosphorylates BimL present in the dimeric form (DLC1-BimL) compared to BimL present in trimeric form (DIC-DLC1-BimL) (Figure 6G).

Physiologic cell survival signals are known to downregulate

the levels of BimL expression via its phosphorylation and thus counteract proapoptotic functions of BimL (Ley et al., 2003; Luciano et al., 2003; Reginato et al., 2003; Seward et al., 2003). Because growth factors activate Pak1 (Kumar and Vadlamudi, 2002) and because Pak1 phosphorylated BimL in this study, we next examined the effect of Pak1 activation on the status of BimL expression using a well-established Tet-inducible MCF-7 model system (Vadlamudi et al., 2000). Treatment of cells with doxycycline induced the levels of constitutively active Pak1 in a time-dependent manner. Analysis of BimL expression under this condition showed a decrease in the expression of BimL levels with an increase in the expression of active Pak1 (Figure 6H). As a positive control, we analyzed the status of cyclin D1 expression, which is known to be upregulated by Pak1 (Balasenthil et al., 2004). As expected, cyclin D1 expression was upregulated with an increase in active Pak1 levels. To examine whether Pak1 kinase activity indeed is involved in the downregulation of BimL, we transfected activated Pak1 (T423E Pak1) or GTPase inactive kinase-dead Pak1 (LLK299R) into MCF-7 cells, and the expression of BimL was analyzed after 48 hr. Our results showed a decrease in the level of BimL expression in cells with activated

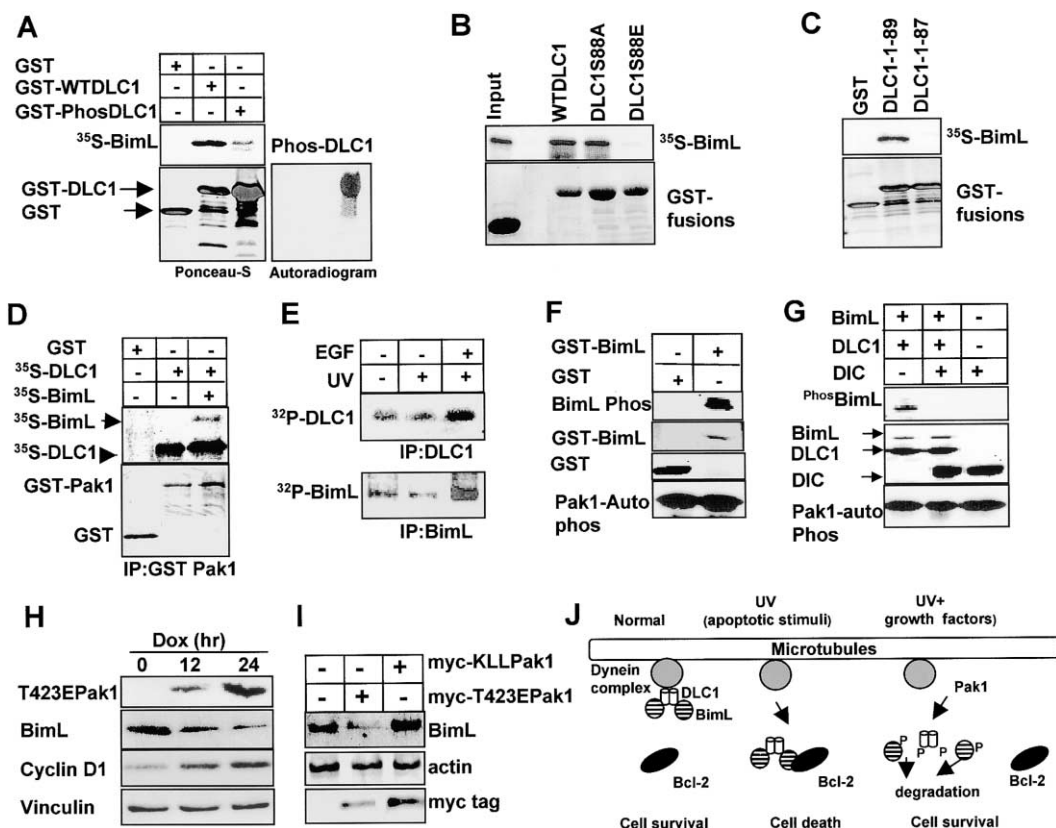


Figure 6. Pak1 phosphorylation regulates BimL and DLC1 interactions

A: Effect of Pak1 phosphorylation on the binding of DLC1 with BimL. GST-DLC1 was phosphorylated with Pak1 enzyme, and the GST pull-down assay was performed using phosphorylated GST-DLC1 or wild-type GST-DLC1. Autoradiogram showing the phosphorylation of DLC1 is shown in the right panel.

B: Ability of DLC1-Ser88A or DLC1-Ser88E mutants to interact with BimL in GST pull-down assay.

C: Ability of GST-DLC1 (aa 1-87) to interact with BimL was analyzed by GST pull-down assay.

D: Ability of Pak1 to interact with DLC1-BimL dimers. ³⁵S-labeled DLC1 and BimL were incubated with GST-Pak1, and GST pull-down assay was performed.

E: MCF-7 cells were labeled with ³²P-orthophosphoric acid, and cells were treated with UV or pretreated with EGF (100 ng/ml) followed by UV treatment. Cell lysates were immunoprecipitated with antibodies against DLC1 or BimL, and the phosphorylation status of proteins was analyzed by autoradiography.

F: Phosphorylation of GST-BimL by Pak1 in in vitro kinase assay.

G: Purified GST-BimL was incubated with purified beads bound GST-DLC1 along with or without purified GST-dynein intermediate chain proteins. After 60 min of incubation, dimeric and trimeric complexes were purified by GST-DLC1 pull-down assay and used as a substrate in in vitro Pak1 kinase assay.

H: Downregulation of BimL levels by constitutively active Pak1. MCF-7 cells expressing T423E-Pak1 under the control of Tet-regulated promoter were treated with doxycycline for 12 or 24 hr. Expression of BimL, cyclin D1, and HA-tagged T423E Pak1 was analyzed by Western blotting.

I: MCF-7 cells were transiently transfected with CMV vector or constitutively active Pak1 (CMV-myc-T423EPak1) or kinase-dead Pak1 (CMV-myc-LLK299Rpak1). After 48 hr of transfection, cell lysates were analyzed for BimL expression. Actin was used as a loading control. Myc-epitope antibody was used to monitor the expression of myc-tagged Pak1 constructs.

J: Model for Pak1 regulation of DLC1 and BimL functions. Under physiologic conditions, DLC1 sequesters BimL to the microtubules. Under apoptotic conditions, DLC1-BimL dimers are released and interact with Bcl-2. Survival factors activate Pak1, which in turn phosphorylates DLC1-BimL dimers and, thus, prevents BimL interaction with Bcl-2.

Pak1 but not in kinase-dead Pak1, suggesting Pak1 kinase activity is involved in the downregulation of BimL expression (Figure 6I). These findings suggested a mechanistic role of DLC1 Ser88 phosphorylation in conferring a survival advantage by modulating the levels of DLC1-BimL dimers, which are dissociated upon apoptotic signaling and, thus, may have a role in enhanced cell survival and, consequently, increased growth rate (Figure 6J).

Deregulation of DLC1 promotes tumorigenesis

Because DLC1 expression promoted G1→S transition as well as cell survival (Figures 4C and 4D) and because Pak1 overexpression is known to support the anchorage-independent

growth of breast cancer cells (Vadlamudi et al., 2000), we examined the ability of the ZR75/DLC1 clones to grow in an anchorage-independent manner. DLC1 wild-type but not the DLC1 mutant expression substantially enhanced the number of colonies in soft agar (Figure 7A), suggesting a role of DLC1 in anchorage-independent growth.

To examine the potential correlation of DLC1 deregulation with the progression of breast cancer to more aggressive phenotypes, we next analyzed DLC1 expression in lysates from exponentially growing cell lines derived from the MCF10AT model system. This model contains a spectrum of cell lines that allows us to examine the expression of genes during the progression of breast malignancy (Heppner et al., 2000; Santner et al., 2001).

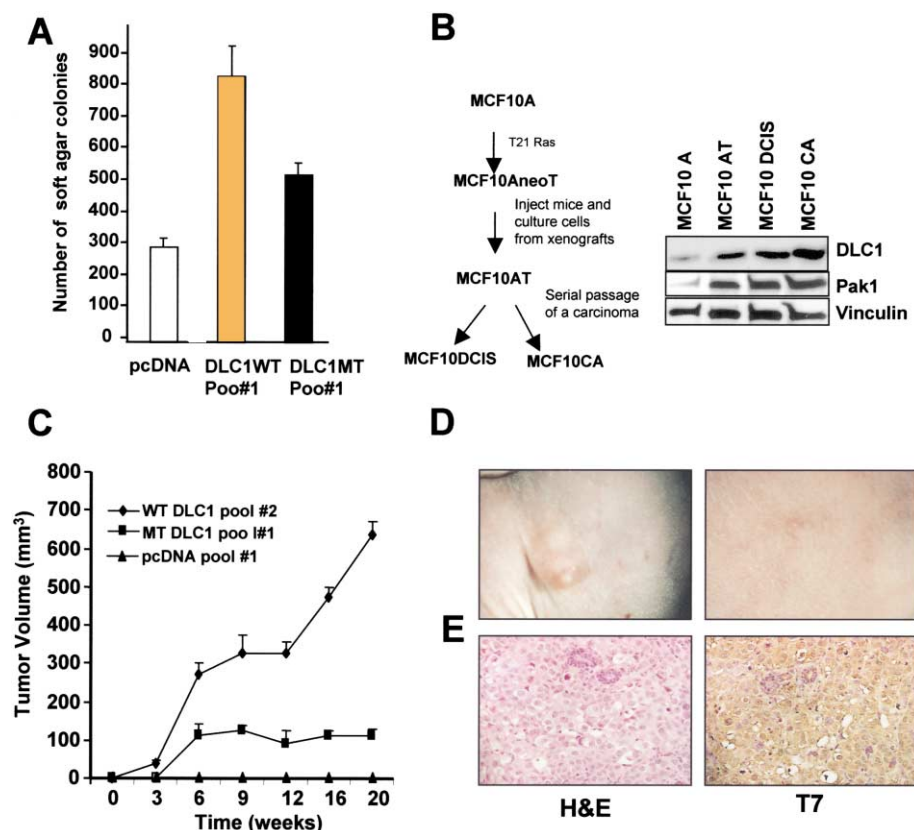


Figure 7. DLC1 deregulation potentiates anchorage-independent growth and promotes tumorigenesis

A: Effect of DLC1 wild-type or mutant overexpression on the ability of ZR75 cells to grow in an anchorage-independent manner. The anchorage-independent growth potential of DLC1-expressing clones was measured by the ability of the cells to form colonies on soft agar. Similar results were obtained in three independent experiments. Error bars represent standard error of mean.

B: Western blot analysis of DLC1 and Pak1 levels in lysates from cells derived from MCF10AT model system. MCF10A, nonmalignant human breast cancer cells; MCF10AT, weakly tumorigenic; MCF10DCIS, Comedo-type DCIS, highly proliferative, aggressive, invasive; and MCF10CA, undifferentiated carcinomas, metastatic.

C: Nude mice were injected with 5×10^6 cells stably expressing pcDNA or wild-type DLC1 (pooled clone #2) or mutant pooled clone DLC1^{del88-89}, and tumor growth was measured at weekly intervals.

D: A representative picture of DLC1-induced tumor in nude mice. Tumor growth was observed only in cells expressing wild-type DLC1 but not detected in mutant-expressing cells.

E: Morphology of tumor and expression of T7-DLC1 in tumors as evaluated by hematoxylin and eosin and anti-T7 staining, respectively.

Figure 7B shows a clearly progressive upregulation of DLC1 expression as a function of tumorigenesis. Consistent with the aggressive growth characteristics of the DLC1-expressing breast cancer cells, the ZR/DLC1 cells (pooled clone 2) exhibited increased tumorigenic potential in nude mice in the absence of any exogenous estradiol treatment (Figures 7C and 7D), suggesting that deregulation of DLC1 might be sufficient to promote tumorigenic phenotypes. Interestingly, DLC1 mutant lacking the Pak1 phosphorylation site did not exhibit any tumorigenic potential in nude mice (Figure 7C). The clonal origin of T7-DLC1-deregulated cells from tumors was confirmed by staining the cells with an anti-T7 monoclonal antibody (Figure 7E). These findings suggested the existence of a close relationship between DLC1 deregulation and its phosphorylation by Pak1 and tumorigenesis.

DLC1 upregulation in human breast cancer

We next examined the level of DLC1 in 15 paired samples of human breast tumors and adjacent normal tissues. The status of DLC1 expression was considerably higher in 14 out of 15 breast tumor specimens than in adjacent normal tissues (Figures 8A and 8B). The anti-DLC1 antibody also works well in formalin-fixed, paraffin-embedded human breast tissues, as shown in a representative DLC1 immunohistochemical (IHC) staining (Figure 8C). Further IHC examination of a tumor array containing 60 breast tumor specimens revealed widespread upregulation of DLC1 expression in 54 out of 60 breast tumors (Figure 8D). Because breast tumor specimens used here have recently been characterized for the levels of Pak1 expression (Balasenthil et

al., 2004), we found that 34 out of 60 specimens had overexpressed both Pak1 and DLC1. To examine whether DLC1 protein in tumors is phosphorylated, we have used a recently available phospho-serine-specific antibody, which specifically recognizes serine-phosphorylated proteins (Gronborg et al., 2002). We have used an affinity column of this antibody to purify serine-phosphorylated proteins from the human breast tumors and adjacent normal tissues. Elutes from columns were precipitated and then Western blotted with DLC1-specific antibody. As a proof of principle, we have analyzed six normal and six tumor samples. Results showed that five out of six tumors have more serine-phosphorylated form of DLC1 compared to control (Figure 8E). These results further support our hypothesis that increased DLC1 protein expression in tumors also translates into its more phosphorylated form. Together, results from Figures 7 and 8 suggested that deregulation of DLC1 and Pak1 might be a common event in cancer progression and that these molecules may be involved in the development and/or maintenance of the malignant phenotypes in tumors.

Discussion

In this study, we discovered that DLC1 is a Pak1-interacting protein. This conclusion is supported by the ability of Pak1 to interact with DLC1 in yeast two-hybrid, in GST pull-down, and in reciprocal immunoprecipitation assays. Furthermore, our results show that DLC1 is a physiologic substrate of Pak1. We have identified Ser88 of DLC1 as the Pak1 phosphorylation site and demonstrated an essential role of this pathway in growth factor-mediated cell survival.

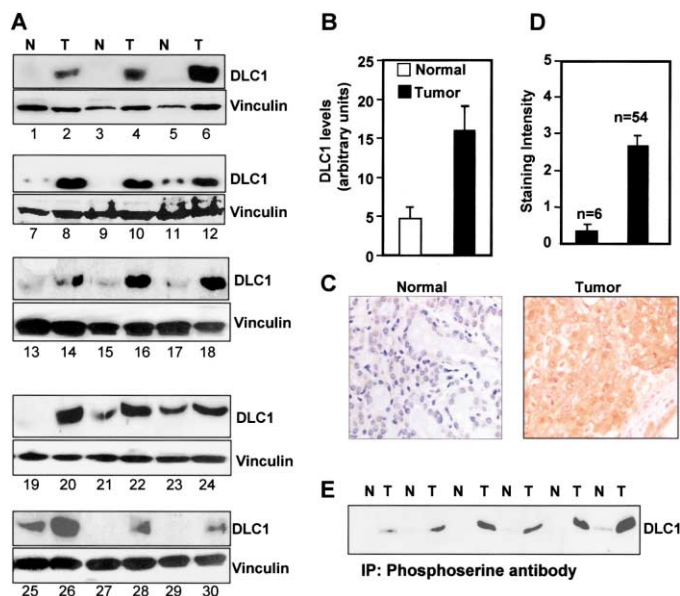


Figure 8. DLC1 deregulation in human breast tumors

A: Breast tumor lysates were analyzed by Western blot analysis for DLC1 expression (top) and subsequently reprobbed with vinculin antibody as a loading control.

B: Quantitation of DLC1 expression in normal and tumor samples.

C: Immunohistochemical analysis of DLC1 in breast tissue samples. The left panel shows normal mammary gland tissue, and the right panel shows tumor from the same patient. Tumor shows very strong nuclear and cytoplasmic DLC1 immunoreactivity.

D: Quantitation of DLC1 expression in breast tumor array.

E: Phosphorylation of DLC1 in normal and tumor samples. Serine phosphorylated proteins in normal and tumor lysates (2 mg) were purified using phosphoserine purification kit (Qiagen) as described in Experimental Procedures, and purified proteins were precipitated using trichloroacetic acid. Levels of DLC1 in the precipitate were then measured by Western blotting using DLC1-specific antibody.

DLC1 is ubiquitously expressed in different cell types and highly conserved throughout evolution. DLC1 is not only associated with myosin V (Naisbitt et al., 2000) and dynein (Hirokawa, 1998) motors, but it also binds to nNOS (Jaffrey and Snyder, 1996; Fan et al., 1998), the proapoptotic Bcl-2 family protein BimL (Puthalakath et al., 1999), the *Drosophila* mRNA localization protein Swallow (Schnorrer et al., 2000), transcriptional regulator I κ B (Crepieux et al., 1997), and the postsynaptic scaffold protein GKAP (Naisbitt et al., 2000). These observations suggest that DLC1 is a multifunctional regulatory protein. Previously structural studies have shown that DLC1 exists as a dimer and contains two identical target binding grooves on the opposite faces of the protein dimer interface (Fan et al., 2001). In this context, DLC1 has been suggested to function as a linker protein by binding two different proteins (Liang et al., 1999). The ability of DLC1 to interact with proteins with diverse cellular functions suggests that DLC1, in principle, might recruit Pak1 to other signaling proteins.

Pak1 has been shown to promote cell survival by phosphorylating and inactivating the proapoptotic functions of Bad (Schurmann et al., 2000). Overexpression of a constitutively active T423E Pak1 mutant promotes the survival of NIH3T3 murine fibroblasts. Similar to Pak1, *Drosophila* DLC1 plays a role in the

inhibition of apoptosis (Dick et al., 1996). In human cells, DLC1 interacts with BimL and promotes cell survival by inhibiting its proapoptotic activity (Puthalakath et al., 1999). In addition, cyclooxygenase 2 promotes cell survival by stimulating DLC1 expression and inhibiting the neuronal NO pathway (Chang et al., 2000). Under physiologic conditions, BimL is sequestered to dynein motor via the DIC-DLC1-BimL complex (Puthalakath et al., 1999; Puthalakath and Strasser, 2002). Apoptotic stimuli release dimer-containing DLC1-BimL, which then translocates and interacts with Bcl-2, leading to the inactivation of the anti-apoptotic function of Bcl-2 and promotion of the apoptotic function of BimL. Growth factors have been shown to increase phosphorylation and degradation of BimL and thus modulate the apoptotic functions of BimL. Although the mechanism by which growth factors regulate BimL functions remains elusive, emerging evidence suggests a role of phosphorylation in the degradation of BimL by growth factors (Ley et al., 2003; Luciano et al., 2003; Seward et al., 2003). In this context, our findings that Pak1 phosphorylates BimL and that activated Pak1 reduces BimL levels are important because they suggest that Pak1 might act as a downstream effector of growth factor-mediated modulation of BimL levels. The ability of Pak1 to recognize and phosphorylate DLC1-BimL dimer also strongly supports the possibility that Pak1 acts as a growth factor sensor to detect the DLC1-BimL dimers generated by apoptotic signals and that Pak1 might dissociate these complexes via phosphorylation.

Emerging evidence implies that Pak1 might play an important role in tumorigenesis (Kumar and Vadlamudi, 2002). Deregulation of Pak1 expression is reported in breast and ovarian cancers (Vadlamudi et al., 2000; Balasenthil et al., 2004; Schraml et al., 2003). We showed that Pak1 interacted with DLC1 and that such interactions are essential for cell cycle progression and survival functions. Accordingly, overexpression of DLC1 substantially increased the growth potential of the breast cancer cells, allowing the cells to survive in an anchorage-independent manner and exhibit increased tumorigenic potential of breast cancer cells. Interestingly, DLC1 and Pak1 expression levels were more elevated in breast tumors than in normal control specimens, raising the possibility that DLC1 has a role in tumorigenesis.

In summary, our results showed that DLC1 is an interacting substrate of Pak1, that DLC1 is overexpressed in human breast cancers, and that Pak1 phosphorylation of DLC1 on Ser88 plays a critical role in tumorigenic phenotypes of DLC1 in breast cancer cells.

Experimental procedures

Cell cultures, reagents, transfection, and cell extracts

MCF-7 breast cancer cells (Vadlamudi et al., 2000) were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 (1:1) supplemented with 10% fetal calf serum. Antibodies DLC1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences (San Jose, CA). Antibody against T7 was purchased from Novagen (Milwaukee, WI). BimL antibody was purchased from Chemicon (Melbourne, Australia). Antibodies for Pak1, phos Ser795 pRb, Phos CDK2, and siRNA against Pak1 (#6361) and control FITC siRNA (#6201) were purchased from Cell Signaling (Boston, MA). SMART POOL siRNA for DLC1 and DLC2 was synthesized by Dharmacon (Lafayette, CO). Transient transfection studies were performed using a FuGENE-6 kit (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with the manufacturer's instructions. Cell lysates were resolved on an 18% tricine gels. ZR75 cells were transfected with pcDNA3.1, pcDNA-T7-DLC1, or dsREDGFP-DLC1 via the calcium phosphate method. Forty-

eight hours after transfection, cells were selected in medium containing G418 (500 $\mu\text{g}/\text{ml}$).

Two-hybrid library screening and plasmid construction

Pak1 baits were constructed by amplifying amino acids 1–270 and amino acids 271–545 by polymerase chain reaction (PCR) and then subcloned into Gal4 binding domain vector pGBD vector (Clontech, Palo Alto, CA). A mammary gland cDNA library fused to Gal4 activation domain was purchased from Clontech and screened using N-terminal Pak1 (aa 1–270) as bait in accordance with the manufacturer's instructions. A total of 2×10^6 clones were screened. Positive interactors were verified by one-on-one transformations followed by selection of agar plates lacking adenine, histidine, leucine, and tryptophan (AHLT) and also by β -galactosidase assay. To generate epitope-tagged constructs of DLC1, the open reading frame of DLC1 was amplified by PCR and subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA) or dsRED (Clontech) vectors. Mutation of Ser88A was created by site-directed mutagenesis (Stratagene, Cedar Creek, TX). GST fusions of various DLC1 mutants were generated by PCR cloning into PGEX vector (Amersham Pharmacia Biotech, Piscataway, NJ). Pak1 GST constructs were described earlier (Vadlamudi et al., 2002).

Pak kinase assay

In vitro kinase assays using myelin basic protein (MBP) or glutathione-S-transferase (GST)-DLC1 protein (4 μg each) were performed in HEPES buffer (50 mM HEPES, 10 mM MgCl_2 , 2 mM MnCl_2 , 0.2 mM DTT) containing 1 μg of purified GST-Pak1 enzyme, 10 μCi of [γ - ^{32}P]ATP, and 25 μM cold ATP. Purified human Pak1 enzyme (#GLO110-100) was purchased from Alexis Biochemicals (San Diego, CA). His-BAD (#14-357) was purchased from Upstate Biotechnology (Waltham, MA) and used as a positive control.

GST pull-down assay

In vitro transcription and translation of the Pak1 and DLC1 proteins were performed using the TNT T7 Quick Coupled Transcription/Translation system (Promega, Madison, WI) as previously described (Vadlamudi et al., 2002).

Cell proliferation, soft agar, and tumorigenicity assays

Soft agar colony-growth assays were performed as previously described (Vadlamudi et al., 2000). Briefly, 1 ml of 0.6% DIFCO agar in DMEM supplemented with 10% fetal bovine serum and insulin was layered onto tissue culture plates. Test cells (10^4) mixed with 1 ml of 0.36% bactoagar solution in DMEM were layered on top of the 0.6% bactoagar layer. The plates were incubated at 37°C in 5% CO_2 for 21 days. For xenograft studies, 5×10^6 cells were implanted subcutaneously into the mammary fat pads of 10 nude mice as previously described (Bagheri-Yarmand et al., 2001) and allowed to grow for 20 weeks. Tumor size was then measured.

Immunofluorescence and confocal studies

The cellular location of proteins was determined using indirect immunofluorescence, as described previously (Kumar et al., 2002). Cells were incubated with primary antibodies for 2 hr, washed 3 times in PBS, and then incubated with 546-Alexa- (red), 633-Alexa- (blue), or 488-Alexa- (green)-labeled secondary antibodies (Molecular Probes, Eugene, OR). The procedure involved processing the same section for each detector (the two excitations corresponding to 546 and 488 or 633 nm) and making a pixel-by-pixel comparison.

Human samples

Tissue samples from patients who had undergone routine surgery for breast cancer were snap frozen in liquid nitrogen and stored at -80°C in the M.D. Anderson Breast Core Pathology Laboratory and have been described previously (Balasenthil et al., 2004). Purification of phosphoserine proteins in normal and tumor tissues was done using phospho protein purification kit (#37101) and phosphoserine antibody (#37430) purchased from Qiagen (Valencia, CA) as per manufacturer instructions. In brief, tissue samples were lysed in zwitterionic detergent CHAPS-, phosphatase inhibitor-, and nuclease-containing lysis buffer to disrupt protein-protein and protein-DNA complexes. Cleared cell lysates were then passed through a column containing immobilized phosphoserine antibody provided in the kit. Elutes from columns were precipitated with TCA and washed four times with acetone, and the precipitated proteins were separated on 18% tricine gels. Blots were then Western blotted with DLC1-specific antibody.

Immunohistochemistry

For immunohistochemical detection of DLC1 and T7, sections were deparaffinized with xylene and rehydrated using graded ethanol. Sections were incubated in 0.3% H_2O_2 and methanol for 30 min to inactivate endogenous peroxidase. The sections were then boiled for 10 min in 0.01 M citrate buffer and cooled for 30 min at room temperature to expose antigenic epitopes. The sections were incubated with 2% normal goat serum in 1% bovine serum albumin and PBS for 30 min and then with an anti-DLC1 antibody (1:50 dilution) or anti-T7 antibody (1:800 dilution) and incubated overnight at room temperature. The sections were washed 3 times with 0.05% Tween in PBS for 10 min and incubated with secondary antibody, developed with DAB- H_2O_2 , and counter stained with Mayer's hematoxylin.

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References

- Bagheri-Yarmand, R., Mandal, M., Taludker, A.H., Wang, R.A., Vadlamudi, R.K., Kung, H.J., and Kumar, R. (2001). Etk/Bmx tyrosine kinase activates Pak1 and regulates tumorigenicity of breast cancer cells. *J. Biol. Chem.* 276, 29403–29409.
- Bagrodia, S., and Cerione, R.A. (1999). Pak to the future. *Trends Cell Biol.* 9, 350–355.
- Balasenthil, S., Sahin, A.A., Barnes, C.J., Wang, R.A., Pestell, R.G., Vadlamudi, R.K., and Kumar, R. (2004). p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells. *J. Biol. Chem.* 279, 1422–1428.
- Banerjee, M., Worth, D., Prowse, D., and Nikolic, M. (2002). Pak1 phosphorylation on t212 affects microtubules in cells undergoing mitosis. *Curr. Biol.* 12, 1233–1239.
- Bokoch, G.M. (2003). Biology of the p21-activated kinases. *Annu. Rev. Biochem.* 72, 743–781.
- Bokoch, G.M., Wang, Y., Bohl, B.P., Sells, M.A., Quilliam, L.A., and Knaus, U.G. (1996). Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J. Biol. Chem.* 271, 25746–25749.
- Bokoch, G.M., Reilly, A.M., Daniels, R.H., King, C.C., Olivera, A., Spiegel, S., and Knaus, U.G. (1998). A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. *J. Biol. Chem.* 273, 8137–8144.
- Chang, Y.W., Jakobi, R., McGinty, A., Foschi, M., Dunn, M.J., and Sorokin, A. (2000). Cyclooxygenase 2 promotes cell survival by stimulation of dynein light chain expression and inhibition of neuronal nitric oxide synthase activity. *Mol. Cell. Biol.* 20, 8571–8579.
- Crepieux, P., Kwon, H., Leclerc, N., Spencer, W., Richard, S., Lin, R., and Hiscott, J. (1997). I kappaB alpha physically interacts with a cytoskeleton-associated protein through its signal response domain. *Mol. Cell. Biol.* 17, 7375–7385.
- Daniels, R.H., and Bokoch, G.M. (1999). p21-activated protein kinase: a crucial component of morphological signaling? *Trends Biochem. Sci.* 24, 350–355.
- Daniels, R.H., Zenke, F.T., and Bokoch, G.M. (1999). alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. *J. Biol. Chem.* 274, 6047–6050.

- Dick, T., Ray, K., Salz, H.K., and Chia, W. (1996). Cytoplasmic dynein (ddlc1) mutations cause morphogenetic defects and apoptotic cell death in *Drosophila melanogaster*. *Mol. Cell. Biol.* 16, 1966–1977.
- Edwards, D.C., and Gill, G.N. (1999). Structural features of LIM kinase that control effects on the actin cytoskeleton. *J. Biol. Chem.* 274, 11352–11361.
- Edwards, D.C., Sanders, L.C., Bokoch, G.M., and Gill, G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat. Cell Biol.* 1, 253–259.
- Fan, J.S., Zhang, Q., Li, M., Tochio, H., Yamazaki, T., Shimizu, M., and Zhang, M. (1998). Protein inhibitor of neuronal nitric-oxide synthase, PIN, binds to a 17-amino acid residue fragment of the enzyme. *J. Biol. Chem.* 273, 33472–33481.
- Fan, J., Zhang, Q., Tochio, H., Li, M., and Zhang, M. (2001). Structural basis of diverse sequence-dependent target recognition by the 8 kDa dynein light chain. *J. Mol. Biol.* 306, 97–108.
- Frost, J.A., Khokhlatchev, A., Stippes, S., White, M.A., and Cobb, M.H. (1998). Differential effects of PAK1-activating mutations reveal activity-dependent and -independent effects on cytoskeletal regulation. *J. Biol. Chem.* 273, 28191–28198.
- Gronborg, M., Kristiansen, T.K., Stensballe, A., Andersen, J.S., Ohara, O., Mann, M., Jensen, O.M., and Pandey, A. (2002). A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phosphospecific antibodies. *Mol. Cell. Proteomics* 1, 517–527.
- Heppner, G.H., Miller, F.R., and Shekhar, P.M. (2000). Nontransgenic models of breast cancer. *Breast Cancer Res.* 2, 331–334.
- Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* 279, 519–526.
- Jaffrey, S.R., and Snyder, S.H. (1996). PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science* 274, 774–777.
- Jakobi, R., Moertl, E., and Koeppl, M.A. (2001). p21-activated protein kinase gamma-PAK suppresses programmed cell death of BALB3T3 fibroblasts. *J. Biol. Chem.* 276, 16624–16634.
- Kumar, R., and Vadlamudi, R.K. (2002). Emerging functions of p21-activated kinases in human cancer cells. *J. Cell. Physiol.* 193, 133–144.
- Kumar, R., Wang, R.A., Mazumdar, A., Talukder, A.H., Mandal, M., Yang, Z., Bagheri-Yarmand, R., Sahin, A., Hortobagyi, G., Adam, L., et al. (2002). A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. *Nature* 418, 654–657.
- Ley, R., Balmanno, K., Hadfield, K., Weston, C., and Cook, S.J. (2003). Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J. Biol. Chem.* 278, 18811–18816.
- Li, F., Adam, L., Vadlamudi, R.K., Zhou, H., Sen, S., Chernoff, J., Mandal, M., and Kumar, R. (2002). p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells. *EMBO Rep.* 3, 767–773.
- Lian, J.P., Crossley, L., Zhan, Q., Huang, R., Coffey, P., Tokar, A., Robinson, D., and Badwey, J.A. (2001). Antagonists of calcium fluxes and calmodulin block activation of the p21-activated protein kinases in neutrophils. *J. Immunol.* 166, 2643–2650.
- Liang, J., Jaffrey, S.R., Guo, W., Snyder, S.H., and Clardy, J. (1999). Structure of the PIN/LC8 dimer with a bound peptide. *Nat. Struct. Biol.* 6, 735–740.
- Luciano, F., Jacquelin, A., Colosetti, P., Herrant, M., Cagnol, S., Pages, G., and Auberger, P. (2003). Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* 22, 6785–6793.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z.S., and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* 367, 40–46.
- Manser, E., Huang, H.Y., Loo, T.H., Chen, X.Q., Dong, J.M., Leung, T., and Lim, L. (1997). Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. *Mol. Cell. Biol.* 17, 1129–1143.
- McManus, M.J., Boerner, J.L., Danielsen, A.J., Wang, Z., Matsumura, F., and Mailhe, N.J. (2000). An oncogenic epidermal growth factor receptor signals via a p21-activated kinase-caldesmon-myosin phosphotyrosine complex. *J. Biol. Chem.* 275, 35328–35334.
- Naisbitt, S., Valtchanoff, J., Allison, D.W., Sala, C., Kim, E., Craig, A.M., Weinberg, R.J., and Sheng, M. (2000). Interaction of the postsynaptic density-95/guanylate kinase domain-associated protein complex with a light chain of myosin-V and dynein. *J. Neurosci.* 20, 4524–4534.
- Puthalakath, H., and Strasser, A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. *Cell Death Differ.* 9, 505–512.
- Puthalakath, H., Huang, D.C., Reilly, L.A., King, S.M., and Strasser, A. (1999). The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol. Cell* 3, 287–296.
- Reginato, M.J., Mills, K.R., Paulus, J.K., Lynch, D.K., Sgroi, D.C., Debnath, J., Muthuswamy, S.K., and Brugge, J.S. (2003). Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat. Cell Biol.* 5, 733–740.
- Santner, S.J., Dawson, P.J., Tait, L., Soule, H.D., Eliason, J., Mohamed, A.N., Wolman, S.R., Heppner, G.H., and Miller, F.R. (2001). Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res. Treat.* 65, 101–110.
- Schnorrer, F., Bohmann, K., and Nusslein, V. (2000). The molecular motor dynein is involved in targeting swallow and bicoid RNA to the anterior pole of *Drosophila* oocytes. *Nat. Cell Biol.* 2, 185–190.
- Schraml, P., Schwerdtfeger, G., Burkhalter, F., Raggi, A., Schmidt, D., Rufalo, T., King, W., Wilber, K., Mihatsch, M.J., and Moch, H. (2003). Combined array comparative genomic hybridization and tissue microarray analysis suggest Pak1 at 11q13.5-q14 as a critical oncogene target in ovarian carcinoma. *Am. J. Pathol.* 163, 985–992.
- Schurmann, A., Mooney, A.F., Sanders, L.C., Sells, M.A., Wang, H.G., Reed, J.C., and Bokoch, G.M. (2000). p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis. *Mol. Cell. Biol.* 20, 453–461.
- Sells, M.A., Knaus, U.G., Bagrodia, S., Ambrose, D.M., Bokoch, G.M., and Chernoff, J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr. Biol.* 7, 202–210.
- Sells, M.A., Pfaff, A., and Chernoff, J. (2000). Temporal and spatial distribution of activated Pak1 in fibroblasts. *J. Cell Biol.* 151, 1449–1458.
- Seward, R.J., von Haller, P.D., Aebersold, R., and Huber, B.T. (2003). Phosphorylation of the pro-apoptotic protein Bim in lymphocytes is associated with protection from apoptosis. *Mol. Immunol.* 39, 983–993.
- Thiel, D., Reeder, M., Pfaff, A., Coleman, T., Sells, M., and Chernoff, J. (2002). Cell cycle-regulated phosphorylation of p21-activated kinase 1. *Curr. Biol.* 12, 1227–1232.
- Turner, C.E., Brown, M.C., Perrotta, J.A., Riedy, M.C., Nikolopoulos, S.N., McDonald, A.R., Bagrodia, S., Thomas, S., and Leventhal, P.S. (1999). Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. *J. Cell Biol.* 145, 851–863.
- Vadlamudi, R.K., Adam, L., Wang, R.A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M.C., and Kumar, R. (2000). Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. *J. Biol. Chem.* 275, 36238–36244.
- Vadlamudi, R.K., Li, F., Adam, L., Nguyen, D., Ohta, Y., Stossel, T.P., and Kumar, R. (2002). Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat. Cell Biol.* 4, 681–690.
- Wang, R.A., Mazumdar, A., Vadlamudi, R.K., and Kumar, R. (2002). P21-activated kinase-1 phosphorylates and transactivates estrogen receptor-alpha and promotes hyperplasia in mammary epithelium. *EMBO J.* 21, 5437–5447.
- Zhao, Z.S., Manser, E., and Lim, L. (2000). Interaction between PAK and nck: a template for Nck targets and role of PAK autophosphorylation. *Mol. Cell. Biol.* 20, 3906–3917.