

Essential Role of KIBRA in Co-activator Function of Dynein Light Chain 1 in Mammalian Cells*

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Recently dynein light chain 1 (DLC1), a cytoskeleton signaling component, has been shown to interact with and transactivate estrogen receptor- α (ER), leading to increased expression of ER target genes and growth stimulation of breast cancer cells. However, the molecular mechanism by which DLC1 regulates the ER pathway remains poorly understood. To gain insights into the putative mechanism, here we set out to identify novel DLC1-interacting proteins. We identified KIBRA, a WW domain- and a glutamic acid stretch-containing protein, as a DLC1-binding protein and showed that it interacts with DLC1 both *in vitro* and *in vivo*. We found that KIBRA-DLC1 complex is recruited to ER-responsive promoters. We also found that KIBRA-DLC1 interaction is mandatory for the recruitment and transactivation functions of ER or DLC1 to the target chromatin. Finally we found that KIBRA interacts with histone H3 via its glutamic acid-rich region and that such interaction might play a mechanistic role in conferring an optimal ER transactivation function as well as the proliferation of ligand-stimulated breast cancer cells. Together these findings indicate that DLC1-KIBRA interaction is essential for ER transactivation in breast cancer cells.

Breast cancer is one of the most common malignancies among women. Despite several advances in diagnosis and therapy, breast cancer remains a significant public health problem with high morbidity and mortality. Overwhelming data from clinical and tissue culture model systems suggest that the estrogen pathway plays a crucial role in the development of female breast cancer (1, 2). Estrogen receptor α (ER)³ belongs to a superfamily of ligand-activated transcription factors whose transcriptional activities are influenced by numerous signaling pathways. ER consists of an N-terminal hormone-independent transcriptional AF1 domain, a central DNA-binding domain

with two zinc fingers, and a C-terminal ligand-binding domain with a hormone-dependent transcriptional AF2 domain (3). The classic mechanism of ER activation involves ligand binding leading to the receptor dimerization and direct interaction with estrogen response elements (EREs) in the promoter regions of target genes (4, 5). ER also regulates transcription through non-classic response sites involving other transcription factors, such as Sp1, c-Jun, and AP-1, probably via protein-protein interactions (6).

Recently we identified DLC1 as an ER-interacting protein. DLC1, an 8-kDa component of the cytoplasmic dynein complex, is highly conserved among species and widely expressed in a number of tissues. In addition to playing an essential role in dynein motor function, DLC1 interacts with a number of proteins of diverse functions. DLC1 is a physiologically interactive substrate of Pak1, and their interaction is essential for the cell survival functions of both Pak1 and DLC1 (7). Furthermore DLC1 up-regulation and its phosphorylation by Pak1 promote the growth of ER-positive breast cancer cells. Conditional up-regulation of DLC1 facilitates estrogen-induced ER transactivation, growth stimulation, and anchorage-independent growth of breast cancer cells (8). We discovered that DLC1 is an important mediator of the transactivation functions of ER. In addition, DLC1 expression promoted recruitment of DLC1-ER complex to the ER target gene pS2, whereas DLC1 down-regulation compromised the transactivation function of ER (8).

To better understand the molecular mechanism by which DLC1 regulates the ER pathway, we performed glutathione S-transferase (GST)-DLC1 pulldown assay using lysates from exponentially growing ZR-75 breast cancer cells. Precipitated proteins were resolved onto a gel. Sequencing of the proteins in individual bands revealed the presence of a novel DLC1-interacting protein termed KIBRA. KIBRA was originally identified as a dendrin-interacting protein expressed predominantly in kidney and brain (9, 10). It contains two N-terminal WW domains, an internal C2-like domain, and a C-terminal region rich in glutamic acids. The WW domain of KIBRA consists of compact protein domains ranging from 35 to 40 amino acids that bind to proline-rich motifs in the target molecules; such domains are found in both cytoplasmic and nuclear proteins (11). Although WW domain-containing proteins have been implicated in a variety of human diseases (12), the functional importance of such proteins (e.g. KIBRA) for the interaction with DLC1 remains unknown.

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³ The abbreviations used are: ER, estrogen receptor α ; KIBRA, kidney and brain; E₂, 17 β -estradiol; siRNA, short interfering RNA; DLC1, dynein light chain 1; luc, luciferase; ERE, estrogen response element; GST, glutathione S-transferase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ChIP, chromatin immunoprecipitation; PR, progesterone receptor; DCC, charcoal-stripped serum.

Here we show that KIBRA directly binds to DLC1 and that it is a downstream mediator of the regulation of ER transactivation by DLC1. We also show that KIBRA by itself is a co-activator of ER. These co-activator functions of KIBRA and DLC1 are dependent on interactions between the two proteins that involve histone H3 via the glutamic acid-rich region of KIBRA. Together our findings reveal a previously unknown connection between KIBRA, DLC1, and ER responsiveness and the existence of a regulatory pathway between histone H3-interacting KIBRA and ER via DLC1 that optimally stimulates the growth of breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Reagents—Human breast cancer cells and other cancer cell lines were maintained in Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal bovine serum. MCF-7/DLC-1 Tet-On cells expressing T7-DLC1 were maintained in 5% tetracycline-free serum in RPMI 1640 medium (8). DLC1 was induced by adding 10 $\mu\text{g}/\text{ml}$ of doxycycline for 24 h. Antibodies against DLC1, T7, Cyclin D1, BCL-2, and Myc tag were purchased from BD Biosciences, Novagen (Milwaukee, WI), Santa Cruz Biotechnology, Dako, and Neomarkers (Fremont, CA), respectively.

Identification of New Binding Partners of DLC1—Immunoprecipitation experiments to identify novel DLC1-interacting partners were performed by adding GST-DLC1 to the total cell lysates from ZR-75 cells. The cells were lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40, 1 \times protease inhibitor mixture, and 1 mM sodium vanadate). The experiment was performed by adding equal amounts (35 μg) of GST and GST-DLC1 proteins immobilized on GST beads (Amersham Biosciences) to an equal amount (7 mg) of ZR-75 cell lysate. The new binding partners were then isolated by incubating the mixture overnight at 4 °C and washing them three times with the Nonidet P-40 lysis buffer. The proteins were eluted with 2 \times SDS buffer. The precipitates were separated by SDS-PAGE and stained with Coomassie Blue dye. Several distinct bands were cut from the gel and digested, the peptides were then sequenced, and the sequences were mapped against known proteins and DNA sequences.

Transfection, Cell Growth, and Protein Assays—Stable cell lines were generated by transfecting MCF-7 cells with pcDNA3.1 or pcDNA-T7-KIBRA. After 48 h, cells were selected in medium containing G418 (500 $\mu\text{g}/\text{ml}$). Transient transfection studies were performed using a FuGENE 6 kit (Roche Applied Science) in accordance with the manufacturer's instructions. β -Galactosidase reporter activity was used as a measure of transfection efficiency. For cell growth assays, cells were grown in phenol red-free medium supplemented with 3% charcoal-stripped serum in 6-well plates with or without 1 nM estradiol. Cells were trypsinized and counted with a Coulter counter at indicated time points. For Western blotting and immunoprecipitation studies, cell lysates in Nonidet P-40 lysis buffer were resolved on 18% Tricine gels (for DLC1) and 8% gels for all other proteins of interest.

Subcellular Protein Extraction—ZR-75 cellular components were sequentially extracted using a widely adopted

biochemical fractionation and sequential extraction procedure as described earlier (22) as "soluble" (with Nonidet P-40 buffer), "cytoskeletal/nucleoplasm-associated" (with Triton X-100), and "chromatin-associated" (with DNase treatment) protein fractions. The presence of KIBRA in individual fractions was confirmed by Western blot analysis with a KIBRA-specific antibody (9).

Northern Blotting and RNA Interference—Northern blotting analysis was performed as described previously (13). SMART POOL siRNA for DLC1 was synthesized by Dharmacon (Lafayette, CO). For knockdown of KIBRA expression, four sets of siRNAs (Qiagen Biosciences, Germantown, MD) targeting human KIBRA sequences were used. Of these four sets, set 3 worked the best.

The sequences for set 3 were sense r(CCGCUCACCU-UUGCUGACU)d(TT) and antisense $-\text{r}(\text{AGUCAGCAAAG-GUGAGCGG})\text{d}(\text{TT})$. A commercially available nonspecific random siRNA was used as a control (Dharmacon). Transfection of siRNA was performed as described previously (14).

Cloning of KIBRA Deletion Constructs—T7-KIBRA and GST-tagged KIBRA were generated in pcDNA3.1C and PGEX vectors, respectively. To map the binding sites of KIBRA on histone H3 we generated deletion constructs of KIBRA. To generate T7-KIBRA ΔEEE mutant, the codon for leucine, GAA at 838, was mutated to a stop codon such as TAA using pcDNA-KIBRA as a template by site-directed mutagenesis kit (Stratagene, Cedar Creek, TX).

GST Pulldown Assays and Far Western Analysis—*In vitro* transcription and translation of the histone H3 and KIBRA proteins were performed using the TNT T7 quick coupled transcription/translation system (Promega, Madison, WI) as described previously (15). Far Western analysis for histones was performed as described earlier (22).

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation (ChIP) assays were performed as described previously (8). Formaldehyde was added at a final concentration of 1% directly to the tissue culture medium. The cells were washed twice with cold phosphate-buffered saline (pH 7.4) containing protease inhibitor mixture (Roche Applied Science), lysed with buffer containing 1% SDS, and sonicated. Equal amounts of DNA were used for each sample and diluted with chromatin dilution buffer containing 0.01% SDS, 1% Triton X-100, and protease inhibitor mixture. For input DNA, 1% of the chromatin solution was kept aside before immunoprecipitation. Chromatin solutions were immunoprecipitated with anti-T7 antibody at 4 °C overnight. Beads bound with immunoprecipitates were washed on a rotating platform before eluting the antibody-bound chromatin by incubation with 400 μl of 1% SDS containing 0.1 M NaHCO_3 . The eluate and the input chromatin were heated at 65 °C for 6 h to reverse the formaldehyde cross-link in the presence of 200 mM NaCl, and RNA was removed by the addition of RNase. The samples were then digested with proteinase K and subjected to phenol-chloroform extraction. The supernatant was ethanol-precipitated and resuspended in 30 μl of water. PCR analysis was performed with primers for the pS2 gene, which encompasses the ERE (pS2), or 1 kb upstream of this element to serve as negative control (pS2 upstream), 3 μl of sample DNA, and 1 μl of input DNA. The PCR was restricted to 30 cycles.

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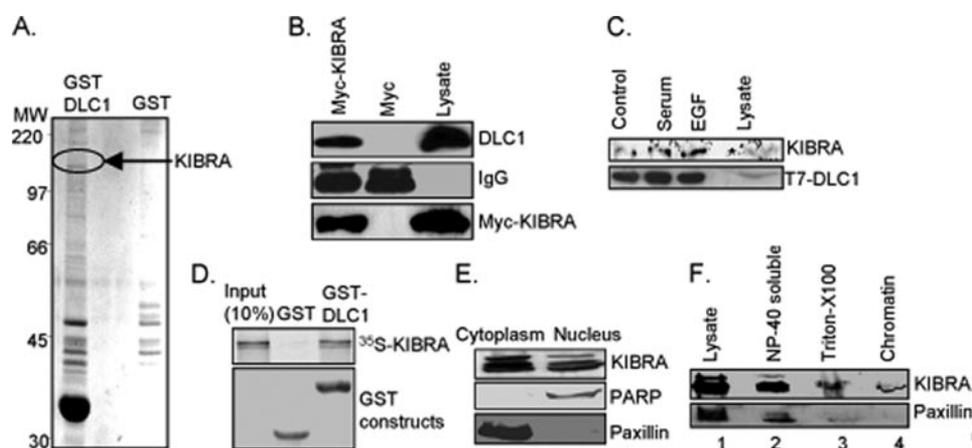


FIGURE 1. KIBRA interacts with DLC1 both *in vitro* and *in vivo*. *A*, identification of new binding partners. KIBRA was identified from a band at ~120 kDa by peptide sequencing. *B*, KIBRA and DLC1 interact *in vivo*. Total cellular lysates from MCF-7 cells transfected with Myc-KIBRA were immunoprecipitated with Myc antibody after which Western analysis was performed for DLC1 and Myc-KIBRA. *C*, total cellular lysates from MCF-7/DLC1 Tet-On cells treated with epidermal growth factor (EGF)/serum for 1 h were immunoprecipitated with T7-antibody after which Western analysis was performed for T7-DLC1 and endogenous KIBRA. *D*, interaction of KIBRA with DLC1 *in vitro*. GST pull-down assay shows the association of GST-DLC1 with *in vitro* translated [³⁵S]KIBRA. *E*, KIBRA is localized in nuclear and cytoplasmic compartments. Nuclear and cytoplasmic extracts were made from ZR-75 cells and probed with antibodies against KIBRA, poly(ADP-ribose) polymerase (PARP), and paxillin. *F*, proteins from different subcellular locations of ZR-75 cells were isolated by sequential extraction and were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis with KIBRA-specific antibody. NP-40, Nonidet P-40.

sequences of the pS2 promoter primers used in this study were 5'-GAATTAGCTTAGGCCTAGACGGAATG-3' and 5'-AGGATT-TGCTGATAGACAGAGACGAC3'. For the element upstream of the ER element in the pS2 gene promoter, the primers were 5'-CTCCCTCT-CAGGCCTCT-3' and 5'-TTCCCTGGTGTGTCAAGTG-3', and for the progesterone receptor gene element, the primers were 5'-GGC-TTTGGGCGGGGCCTCCCTA-3' and 5'-TCTGCTGGCTCCGTACT-GCGG-3'.

RESULTS

Identification of KIBRA as a DLC1-interacting Protein—To better understand the cellular functions of DLC1 in ER-responsive breast cancer cells, we set out to identify novel DLC1-interacting proteins using the modified

proteomic strategy. This analysis identified several previously known DLC1-binding partners as well as several new DLC1-binding proteins, including a previously identified cytoplasmic protein named KIBRA with no assigned function (Fig. 1A) (GenBankTM accession number NP_056053) (9).

KIBRA Interacts with DLC1—To demonstrate an *in vivo* interaction of KIBRA and DLC1 in breast cancer cells, we transiently transfected Myc-tagged KIBRA and control cytomegalovirus vector in MCF-7 cells. Immunoprecipitation of cell lysates with an anti-Myc monoclonal antibody followed by immunoblotting with a DLC1 monoclonal antibody showed specific interaction of Myc-KIBRA and endogenous DLC1 (Fig. 1B). Similarly cell lysates from well characterized MCF-7/T7-DLC1 clones (8) were immunoprecipitated with T7-DLC1 and analyzed by Western blotting with T7 or KIBRA antibody (9). Interestingly KIBRA-DLC1 interaction was enhanced upon stimulation with epidermal growth factor or serum (Fig. 1C). Together these results show that KIBRA interacts with DLC1 *in vivo*. To determine the direct interaction between KIBRA and DLC1, we next examined the ability of *in vitro* translated [³⁵S]KIBRA protein to bind with a GST-DLC1 fusion protein. KIBRA interacted efficiently with GST-DLC1 but not with GST alone in GST pull-down assays (Fig. 1D). Upon careful analysis of the protein sequence of KIBRA, we detected a nuclear localization signal (amino acids ³⁶¹KWTQGEVEQLEMAR³⁷⁶). Based on this information, we wished to determine the localization of KIBRA in the cell. Biochemical fractionation of breast cancer cells showed that, although KIBRA was predominantly found in the cytoplasmic fraction, a substantial amount of KIBRA was also present in the nuclear fraction (Fig. 1E).

Differential extraction of subcellular proteins and immunoblotting with KIBRA-specific antibody showed that KIBRA was present in almost all of the subcellular fractions. These results

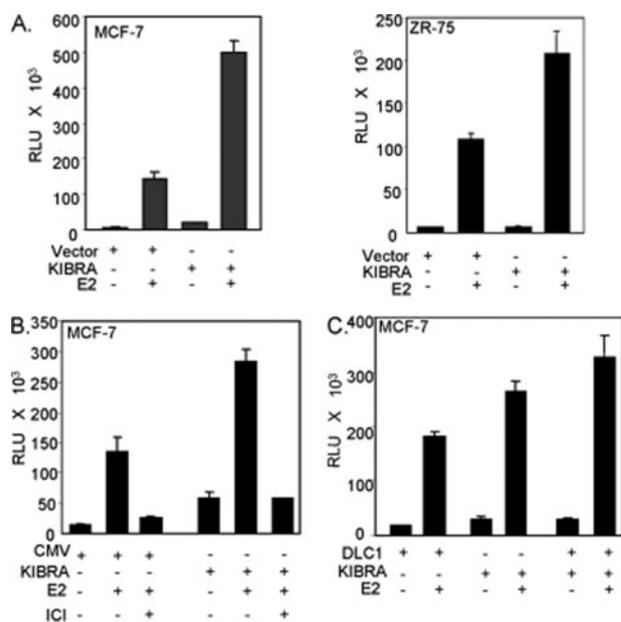


FIGURE 2. KIBRA overexpression potentiates basal as well as E₂-mediated stimulation of ERE-luc activity. *A*, MCF-7 and ZR-75 cells were co-transfected with KIBRA or cytomegalovirus (CMV) along with ERE-luc and treated with E₂ for 24 h, and ERE-luc activity was measured. *B*, MCF-7 cells were co-transfected with KIBRA or cytomegalovirus along with ERE-luc. After 24 h, cells were treated with 10⁻⁹ M E₂ or E₂ and 10⁻⁸ M ICI182780 (ICI), and luc activity was measured after 24 h. *C*, KIBRA potentiated ER transactivation functions of DLC1. MCF-7 cells were co-transfected with DLC1 and/or KIBRA or pcDNA along with ERE-luc and treated with E₂ for 24 h, and ERE-luc activity was measured. RLU, relative light units.

Our pS2 gene primers amplified the region inclusive of the ER-responsive element from -463 to -159. The pS2 upstream primers amplified the region from -1953 to -1651. The PR gene primers amplified the region including the ERE from +90 to +310. PCR products (304, 302, and 220 bp, respectively) were resolved on a 2% agarose gel and visualized with ethidium bromide. The

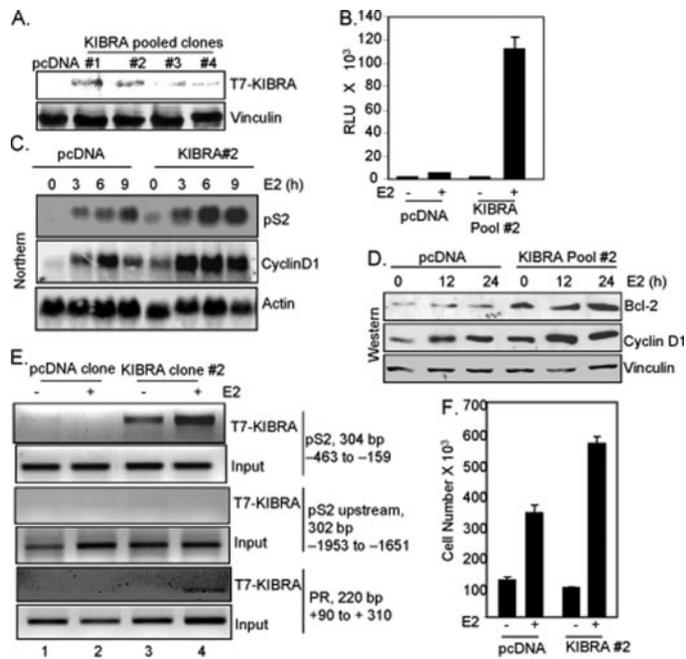


FIGURE 3. KIBRA induced ER target genes by its recruitment to the target DNA. *A*, total cellular lysates were obtained from MCF-7 clones stably expressing pcDNA and KIBRA, and expression of T7-tagged KIBRA was analyzed by Western blotting using anti-T7 epitope monoclonal antibody. Vinculin was used as a loading control. *B*, pcDNA- and KIBRA-expressing clones were cultured in 5% DCC serum for 48 h, transfected with ERE-luc, and treated with or without E_2 (10^{-9} M) for 24 h, and ERE-luc activity was measured. *C*, Cyclin D1 and pS2 gene expression was also analyzed by Northern blotting. *D*, MCF-7 stable pooled clones expressing pcDNA and KIBRA were treated with or without E_2 (10^{-9} M) for indicated time points, total protein and RNA were made, and Western blot analysis was performed for Cyclin D1 and BCL-2. *E*, MCF-7 stable pooled clones expressing pcDNA and KIBRA were treated with or without E_2 (10^{-9} M) for 1 h. *Upper panel*, PCR analysis of the 304-bp pS2 promoter fragment associated with T7-KIBRA. PCR analysis of the input DNA is shown. *Middle panel*, PCR analysis of the 302-bp pS2 promoter fragment 1 kb upstream of the ERE in the pS2 promoter serves as negative control. PCR analysis of the input DNA is shown. *Lower panel*, PCR analysis of the 220-bp PR promoter fragment associated with T7-KIBRA. PCR analysis of the input DNA is shown. *F*, equal numbers of pcDNA- and KIBRA-expressing cells were cultured in 5% DCC serum for 48 h and treated with or without E_2 (10^{-9} M) for 5 days, and the cell number was determined. RLU, relative light units.

demonstrated that KIBRA could be localized in the nuclear compartment and thus raised the possibility that it may be involved in transcriptional modulation. At least two KIBRA bands of different mobility could be detected in the Western blot analysis (Fig. 1*F*) presumably due to different phosphorylated forms of KIBRA. Of interest, KIBRA existed as a single band in the chromatin fraction (Fig. 1*F*, lane 4). Further *in vivo* labeling with orthophosphoric acid revealed that KIBRA is a phosphoprotein (data not shown).

KIBRA Potentiates ER Transactivation—The observed interaction of KIBRA with DLC1 and the fact that DLC1 interacts with ER (8), raised the possibility that KIBRA might be involved in the ER pathway. To explore this notion, we examined the ability of KIBRA to influence the transcription of an ERE-luciferase reporter system in MCF-7 and ZR-75 breast cancer cells. Co-expression of KIBRA potentiated the ability of ligand-activated ER to stimulate ERE-driven transcription (Fig. 2*A*). Pretreatment of MCF-7 cells with the pure antiestrogen compound ICI182780 completely blocked this potentiating effect of estrogen (Fig. 2*B*). The expression of KIBRA alone also had a

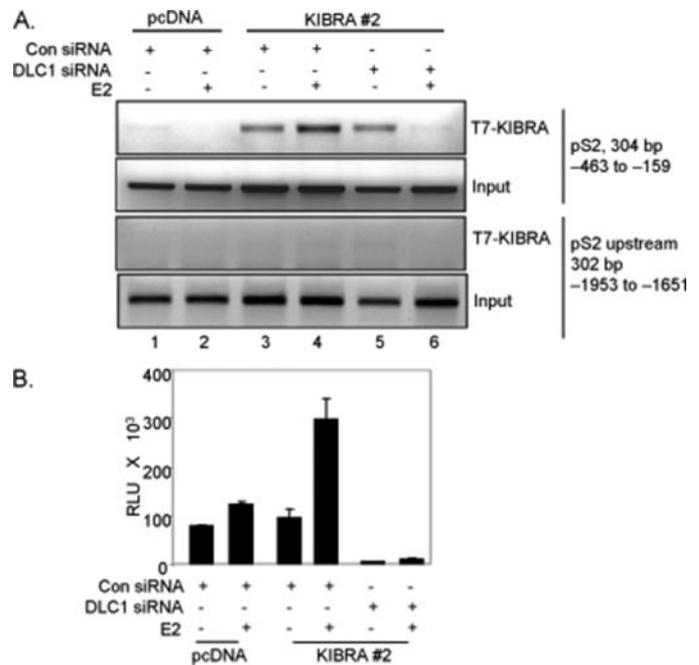


FIGURE 4. DLC1 is required for ER transactivation functions of KIBRA. *A*, pcDNA- and KIBRA-expressing clones were transfected with control or DLC1-specific siRNA. The cells were harvested 48 h after transfection of the siRNA. *Upper panel*, PCR analysis of the 304-bp pS2 promoter fragment associated with T7-KIBRA. PCR analysis of input DNA is shown. *Lower panel*, PCR analysis of the 302-bp pS2 promoter fragment 1 kb upstream of the ERE in the pS2 promoter serves as negative control. PCR analysis of the input DNA is shown. *B*, pcDNA- and KIBRA-expressing clones were cultured in 5% DCC serum for 24 h, transfected with control or DLC1-specific siRNA, and treated with or without E_2 (10^{-9} M) for 24 h, and ERE-luc activity was measured. RLU, relative light units; Con, control.

modest but reproducible stimulatory effect on reporter activity in the absence of estrogen. Because we initially identified KIBRA as a DLC1-interacting protein, we next examined whether DLC1 and KIBRA could cooperate in stimulating the transactivating ability of ER. Using MCF-7 breast cancer cells, we found that DLC1 enhanced the stimulatory effect of KIBRA on ERE-luciferase activity (Fig. 2*C*).

KIBRA Interacts with ER-responsive Chromatin—To determine the biological effects of KIBRA upon the ER pathway, we generated pooled clones of MCF-7 cells overexpressing T7-KIBRA or control pcDNA (Fig. 3*A*). We examined the effect of estrogen on the transactivation activity of ER in these stable clones and as observed previously (Fig. 2*A*) found that KIBRA promoted the ability of ER to stimulate ERE-luciferase activity in these clones (Fig. 3*B*).

To confirm the potential involvement of KIBRA in the expression of ER target genes, we performed Western blot analysis for estrogen-responsive genes Cyclin D1 and BCL-2 and Northern blot analysis of Cyclin D1 and pS2 gene product using mRNA isolated from MCF-7/T7-KIBRA and MCF-7/pcDNA clones. The results showed that KIBRA enhanced both basal and estrogen-induced levels of Cyclin D1, BCL-2, and pS2 (Fig. 3, *C* and *D*), thus implicating KIBRA in the potentiation of estrogen-sensitive transcription and consequently the steady-state expression of the ER target genes. To further elucidate the role of KIBRA in ER target gene expression, we next determined whether KIBRA is recruited to the pS2 promoter using ChIP

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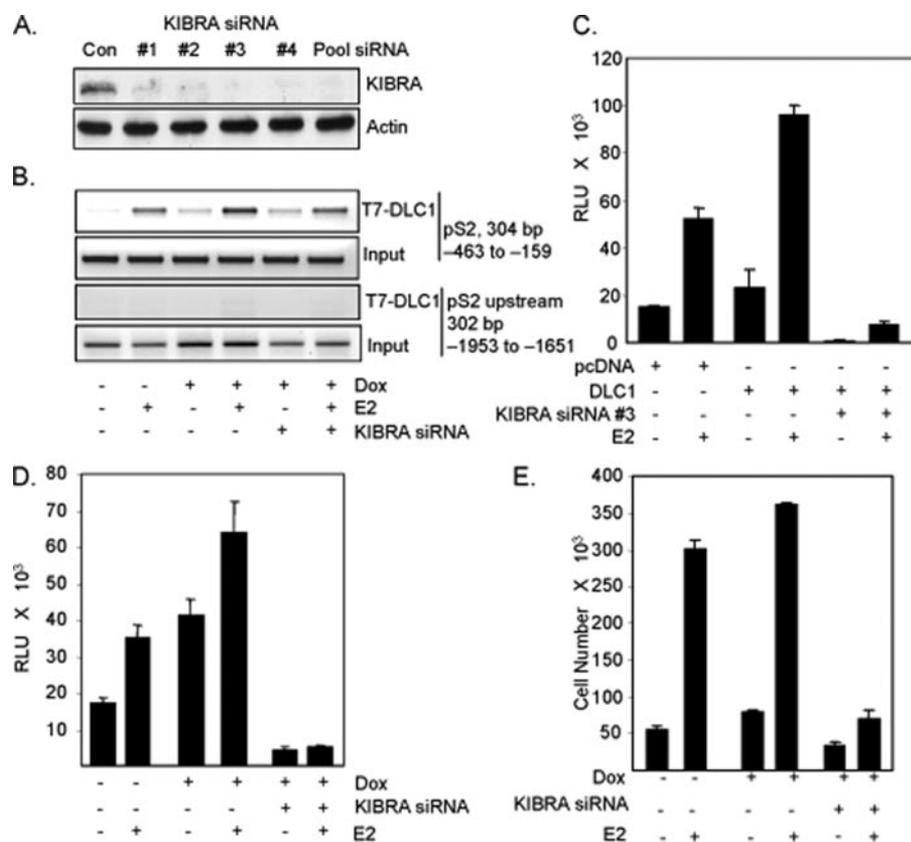


FIGURE 5. KIBRA is essential for ER transactivation functions of DLC1. *A*, Western blot showing efficacy of different sets of siRNAs targeting human KIBRA in MCF-7 cells. *B*, MCF-7/DLC1 cells were transfected with control or KIBRA-specific siRNA. The cells were stimulated with doxycycline (Dox) 48 h after transfection of the siRNA and harvested 24 h after induction. *Upper panel*, PCR analysis of the 304-bp pS2 promoter fragment associated with T7-DLC1. PCR analysis of input DNA is shown. *Lower panel*, PCR analysis of the 302-bp pS2 promoter fragment 1 kb upstream of the ERE in the pS2 promoter serves as negative control. PCR analysis of the input DNA is shown. *C*, MCF-7 cells transfected with pcDNA or DLC1 were treated with control or KIBRA-specific siRNA and treated with or without E₂ (10⁻⁹ M) for 24 h, and ERE-luc activity was measured. *D*, MCF-7/DLC1 cells were treated with or without doxycycline, transfected with control or KIBRA-specific siRNA, then transfected with ERE-luc reporter gene, and treated with or without E₂ (10⁻⁹ M) for 24 h, and ERE-luc activity was measured. *E*, equal numbers of MCF-7/DLC1 cells were cultured in 5% DCC serum for 24 h, treated with or without doxycycline, transfected with control or KIBRA-specific siRNA, and treated with or without E₂ (10⁻⁹ M) for 5 days, and the cell number was determined. RLU, relative light units; Con, control.

assays. PCR analysis of coimmunoprecipitated DNA revealed that T7-KIBRA was recruited to the pS2 gene chromatin at the estrogen response element and not to the chromatin 1 kb upstream and that this specific recruitment was enhanced upon estrogen stimulation of the KIBRA-overexpressing cells (Fig. 3E). To show KIBRA recruitment to other estrogen-inducible genes, we performed a PCR analysis of the progesterone receptor gene and were able to show the same recruitment pattern for the ERE on PR. The observed promotion of ER target gene expression in estrogen-stimulated cells has functional implications because estrogen stimulated the growth of MCF-7/KIBRA cells more effectively than it stimulated the growth of MCF-7/pcDNA cells (Fig. 3F). Together these observations suggested a role for KIBRA in promoting the expression of ER target genes as well as the proliferation of ligand-stimulated breast cancer cells.

KIBRA Regulation of ER Transactivation Functions Requires DLC1—Because we identified KIBRA as a DLC1-interacting protein and because co-expression of DLC1 further elevated the levels of ERE-driven transcription by KIBRA (Fig. 2C), we

hypothesized that DLC1 might be involved in the action of KIBRA on the ER pathway. To explore this concept, we first knocked down the endogenous DLC1 with specific siRNA and performed a ChIP assay in the KIBRA-overexpressing cells. We found that KIBRA from such cells was unable to interact with pS2 gene chromatin upon estrogen stimulation (Fig. 4A). To show that the failure of KIBRA recruitment to the target chromatin, by knocking down DLC1, translates into a reduced ER transactivation activity, we next performed a luciferase assay under the same conditions. Interestingly we found that down-regulation of DLC1 expression drastically reduced the potentiating effect of KIBRA on ER transactivation under both basal and estrogen-stimulated conditions (Fig. 4B). Together these observations suggested an essential role for DLC1 in the ability of KIBRA to stimulate the transactivating function of ER.

KIBRA Plays an Essential Role in DLC1-mediated ER Transactivation—Because DLC1 promotes the transactivation functions of ER (8) and because DLC1 interacts with KIBRA, we hypothesized that KIBRA might also be important in mediating the effects of DLC1 upon ER transactivation function. To

investigate this notion, we used KIBRA-specific siRNA to knock down the expression of endogenous KIBRA. We screened four KIBRA-specific siRNAs for their ability to knock down KIBRA expression and identified the most effective as siRNA set 3 (Fig. 5A). To delineate the molecular basis for the role of KIBRA in the function of DLC1, we performed ChIP analysis on MCF-7/DLC1 Tet-On cells (8) in which the endogenous KIBRA expression has been knocked down. As expected, DLC1 overexpression was accompanied by increased recruitment of T7-DLC1 to the pS2 promoter in the DLC1-stable clones compared with non-induced cells (Fig. 5B, lanes 3 and 4 versus lanes 1 and 2) and not to the chromatin 1 kb upstream of the ERE in the pS2 promoter. Interestingly, however, the increased recruitment of T7-DLC1 to the pS2 promoter was lost in estrogen-treated cultures (compare lane 6 with lane 4) after knocking down KIBRA, suggesting an obligatory role for KIBRA in the DLC1 regulation of ER-targeted gene expression. To show a functional consequence of the derecruitment of DLC1 by knocking down KIBRA, we performed a luciferase assay under the same experimental conditions. The results

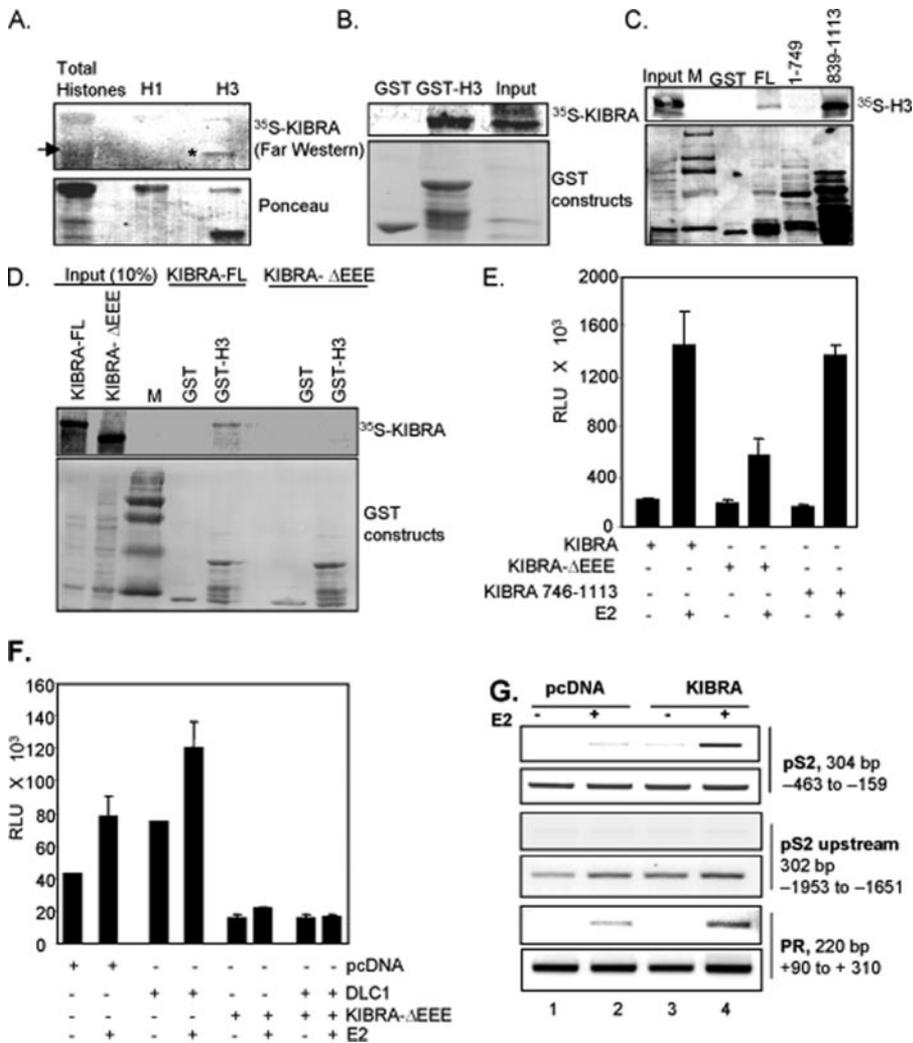


FIGURE 6. KIBRA interacts with histone H3. A, KIBRA interacts with histone H3 in far Western analysis. Membrane-immobilized total histones and purified H1 and H3 were probed with ³⁵S-labeled KIBRA. B, [³⁵S]KIBRA was subjected to GST pull-down assay using GST-tagged histone H3. C, [³⁵S]histone H3 interacts with the glutamic acid-containing region of KIBRA in GST pull-down assay. D, interaction of GST-H3 with full-length KIBRA (KIBRA-FL), not with KIBRA-ΔEEE. E, MCF-7 cells were co-transfected with KIBRA, KIBRA-ΔEEE, KIBRA-(746–1113), and pcDNA along with ERE-luc and treated with E₂ for 24 h, and ERE-luc activity was measured. F, glutamic acid-rich region of KIBRA is essential for ER transactivation functions of DLC1. MCF-7 cells were co-transfected with DLC1 and/or KIBRA-ΔEEE or pcDNA along with ERE-luc and treated with E₂ for 24 h, and ERE-luc activity was measured. G, MCF-7 stable pooled clones expressing pcDNA and KIBRA were treated with or without E₂ (10⁻⁹ M) for 24 h, and double ChIP analysis was performed using acetylated histone H3 and H4 antibodies in which we first immunoprecipitated the acetylated histones and reimmunoprecipitated the eluted complex with T7 antibody to precipitate T7-KIBRA-bound DNA. Upper panel, PCR analysis of the 304-bp pS2 promoter fragment associated with T7-KIBRA. PCR analysis of the input DNA is shown. Middle panel, PCR analysis of the 302-bp pS2 promoter fragment 1 kb upstream of the ERE in the pS2 promoter serves as negative control. PCR analysis of the input DNA is shown. Lower panel, PCR analysis of the 220-bp PR promoter fragment associated with T7-KIBRA. PCR analysis of the input DNA is shown. FL, full length; M, molecular weight markers; RLU, relative light units.

KIBRA Regulates ER Transactivation Function via Its Histone-interacting Motif—Because both KIBRA and DLC1 stimulated ligand-induced ER transactivation function (Figs. 2 and 5) and assisted each other in their ER-modulating activity and because both of these molecules interact with the active chromatin, we next sought the potential unifying mechanism by which DLC1 and KIBRA participate in the transactivation activity of ligand-activated ER. Closer examination of the C-terminal region of KIBRA comprising amino acids 839–1113 revealed the presence of a 29-amino acid glutamic acid-rich region (amino acids ⁸⁴⁵EETSENEAVAE⁸⁷³EEEEVEEGEDVFT⁸⁷³). Because proteins with glutamic acid-rich regions have been shown to interact with histones (16, 17), this raised the possibility that KIBRA might interact with histones. To test this hypothesis, we used purified native histones, resolved them along with purified histones H1 and H3 by SDS-PAGE, and then subjected them to far Western analysis using [³⁵S]KIBRA as a probe. This analysis revealed a high affinity interaction between KIBRA and histone H3 (Fig. 6A). The KIBRA probe also interacted with a histone band corresponding to purified native histone H3 (Fig. 6A, lane 1). Results from GST-histone pull-down assays further confirmed the ability of [³⁵S]KIBRA to interact strongly with the GST-histone H3 (Fig. 6B). Histone H3 bound to KIBRA in a region that mapped to amino acids 839–1113, and this binding ability was lost upon deletion of the glutamic acid-rich region of KIBRA (Fig. 6, C and D). We also found that [³⁵S]histone H3 bound to GST-KIBRA in a region that mapped

to amino acids 30–57 in the histone H3 head region (data not shown), a region previously implicated in active chromatin remodeling (18) and stabilizing interactions of co-activator complex with the putative target chromatin (19). Because DLC1 interacted with KIBRA and KIBRA in turn interacted with histone H3 via its C-terminal glutamic acid-rich region-containing amino acids 746–1113 (Fig. 6, C and D), we examined the importance of the histone-binding region of KIBRA in stimulating ER transactivation activity of KIBRA. We found that amino acids 839–1113, but not the region without glutamic acids, effectively stimulated ER transactivation activity (Fig. 6E). In addition, results from transient transfection experiments with MCF-7 cells also showed that co-expres-

showed that elimination of KIBRA impaired the ability of DLC1 to stimulate both basal and estrogen-induced ER transactivation function (Fig. 5, C and D). To evaluate the biological functionality of these molecular effects of KIBRA, we next showed that down-regulation of KIBRA expression also had a profound effect on the proliferation of MCF-7/DLC1 Tet-On cells (Fig. 5E). This in turn supported the notion that KIBRA might be essential for conferring optimal estrogen responsiveness on breast cancer cells. Taken together, these findings suggested that KIBRA might be required not only for the binding and recruitment of ER/DLC1 to the promoter of pS2 but also for ER function.

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sion of KIBRA lacking the glutamic acid-rich region failed to stimulate ER transactivating functions of DLC1 (Fig. 6F). These observations suggested a role of glutamic acid-rich region in conferring ER transactivation functions. Taken together, these findings suggest an important role of histone H3-interacting regions of KIBRA in the ER transactivation functions of KIBRA and DLC1.

Because the co-activator function of KIBRA on ER transactivation function could be effectively blocked by DLC1 knockdown (Fig. 4), we hypothesized that KIBRA needs to interact with DLC1 to induce optimal ER transactivation activity. Because DLC1 has been shown to interact with ER, we also hypothesized that DLC1-ER interaction might stabilize DLC1-KIBRA complexes to allow a more efficient interaction of KIBRA with histone H3. As a consequence the DLC1-KIBRA complex is positioned more on active chromatin conformation on the ER target gene. To determine whether histone H3 interaction has any mechanistic role in the co-activator function of KIBRA, we performed double ChIP analysis using acetylated histone H3 and H4 antibodies in which we first immunoprecipitated the acetylated histones and reimmunoprecipitated the eluted complex with T7 antibody to precipitate T7-KIBRA-bound DNA. We found that in the acetylated histone precipitate T7-KIBRA was also associated with the same DNA after estrogen stimulation (Fig. 6G). Taken together, these results suggest that the DLC1-KIBRA complex might utilize the glutamic acid-rich region of KIBRA to interact with histone H3 and thus stabilize the interactions of ER-multiprotein complexes with the putative target chromatin.

DISCUSSION

Emerging data suggest a role for DLC1 in the transactivation functions of ER and in ligand-induced growth stimulation of breast cancer cells (8). This study was undertaken to understand better the mechanism of the co-activator function of DLC1. Using a GST pulldown assay, we identified a new DLC1-interacting protein called KIBRA, which contains a WW domain and glutamic acid-rich region with a nuclear localization signal. Because DLC1 interacts with ER and the results of our present study show that DLC1 also interacts with KIBRA, we conclude that KIBRA may contribute to the functionality of the ER pathway. Indeed we found that KIBRA is recruited to ERE sites in ER-responsive genes and potentiates ER transactivation activity upon ligand stimulation and that these responses of KIBRA depend on the presence of DLC1. Furthermore we found that the ligand-induced transactivation activity of ER or its potentiation by DLC1 also requires KIBRA. Because KIBRA and DLC1 both interact with each other and because DLC1 interacts with ER, it is possible that KIBRA stabilizes transcriptionally active ER complexes in the ligand-stimulated breast cancer cells. Our view is supported by the observation that selective depletion of the endogenous DLC1 by a specific siRNA compromised the ligand-induced recruitment of KIBRA complexes to the pS2 promoter chromatin and had no significant inhibitory effect on the basal recruitment of KIBRA to the pS2 promoter.

Our observation that the glutamic acid-rich region of KIBRA interacted with histone H3 and that this interaction was essential

for the transactivation-promoting activity of KIBRA is novel and relevant because it provides clues about the potential role of histone H3 in ensuring the proper regulatory interaction of KIBRA-DLC1-ER complexes with target chromatin. Previous studies have shown the presence of glutamic acid-rich regions in various nuclear proteins (*i.e.* PELP1, high mobility group chromatin proteins-1 and -2, nucleoplasmin, prothymosin α , and nucleolin) (20–22) and implicated such regions in the structural modifications of chromatin via core histones. The presence of a 29-amino acid glutamic acid-rich region in KIBRA, its localization in the nucleus, and its ability to interact with histone H3 suggest that KIBRA-mediated ER transactivation involves KIBRA-histone H3 interactions. The blockage of ER-mediated transcriptional activation by a KIBRA mutant lacking the histone H3-binding region supports this hypothesis. Numerous studies have shown that histones play an essential regulatory role in gene expression. Histone acetylation and deacetylation have been identified as potential mechanisms by which nuclear receptor co-activators modify chromatin structure (23). Formation of a complex between KIBRA and histone H3 might lead to the opening of chromatin and the subsequent recruitment of KIBRA-DLC1 complexes to chromatin. This view is supported by our findings that ligand stimulation of breast cancer cells distinctly enhanced the amount of acetylated histone H3-associated KIBRA that bound to the pS2 gene (Fig. 6G). These findings suggest a model wherein KIBRA may interact simultaneously with histone H3 and DLC1 while assisting in the recruitment of DLC1-ER complexes to the chromatin of ER-targeted genes. In summary, our findings suggest that KIBRA, a DLC1-interacting protein, interacts in a regulatory fashion with histone H3 and that these regulatory interactions play a role in ER-mediated transactivation function.

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