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KIBRA promotes prostate cancer cell proliferation and motility

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Abstract

KIBRA is a regulator of the Hippo-YAP (yes-associated protein) pathway, which plays a critical role in tumorigenesis. In the current study, we show that KIBRA is a positive regulator in prostate cancer cell proliferation and motility. We found that KIBRA is transcriptionally upregulated in androgen-insensitive LNCaPC4-2 and LNCaP-C81 cells when compared to the parental androgen-sensitive LNCaP cells. Ectopic expression of KIBRA enhances cell proliferation, migration and invasion in both immortalized and cancerous prostate epithelial cells. Accordingly, knockdown of KIBRA reduces migration, invasion, and anchorage-independent growth in LNCaP-C4-2/C81 cells. Moreover, KIBRA expression is induced by androgen signaling and KIBRA is partially required for androgen receptor (AR) signaling activation in prostate cancer cells. In line with these findings, we further show that KIBRA is overexpressed in human prostate tumors. Our studies uncover unexpected results and identify KIBRA as a tumor promoter in prostate cancer.

Keywords

AR signaling; KIBRA; prostate cancer; proliferation; motility

Introduction

KIBRA (expressed in kidney and brain, also called WWC1) is one of the members of the WWC (WW and C2 domain containing, WWC1, 2, 3) family of proteins [1,2]. KIBRA is a memory performance and cognition-associated protein [3–9], and the *KIBRA* locus has been linked to brain-related disorders such as Alzheimer's disease [10–12]. KIBRA functions as an adaptor protein to transduce its biological functions in various physiological processes via interactions with many other proteins [13]. In addition to its functions in neurons, KIBRA also has multiple roles in non-neuronal cells involving cell polarity, trafficking, mitosis, cell proliferation, and cell migration [13]. For example, KIBRA positively regulates cell migration in podocytes [14], NRK cells [15], and breast cancer cells [16], while it does the opposite in immortalized breast epithelial cells [17]. KIBRA functions as a growth

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Author contributions

JD and SS designed and wrote the paper. SS, XC, LZ, and YC performed the experiments, analyzed the data and interpreted the results. YC also provided technical support. All authors reviewed and approved the manuscript prior to submission.

suppressive regulator through the Hippo pathway in *Drosophila* [18–20]. KIBRA is phosphorylated by mitotic kinases cyclin-dependent kinase 1 (CDK1) and Aurora A during mitosis and is required for chromosome alignment [21–23].

KIBRA has been linked with human cancers in several reports. Weakened expression of KIBRA in Claudin-low subtypes of breast cancer specimens correlates with poor prognosis [17]. Moreover, downregulation of KIBRA was shown to be a contributing factor to the malignancy of acute lymphocytic B-cell leukemia [24,25]. This alteration in expression in leukemia is due to epigenetic changes in the well-defined CpG island within the promoter of the *KIBRA* locus [24,25]. Strikingly, in common epithelial cancers such as colorectal, kidney, lung, breast and prostate there is virtually no methylation detected [24]. Instead of a tumor suppressive function of KIBRA, as suggested by the above studies, many previous reports have validated KIBRA's role in positively regulating proliferation and motility [14–16,26–29] and KIBRA expression has positive clinical correlation with gastric cancer progression [30]. This duality of KIBRA's suppression or promotion of proliferation and migration may be tissue- and context-dependent, requiring further investigation before KIBRA's role can be fully deciphered. Furthermore, a role for KIBRA in prostate cancer has not been previously defined.

Prostate cancer is the most prevalent form of cancer in men in the United States and second only to lung cancer as the leading cause of cancer deaths in men [31]. Prostatectomy, usually the initial treatment, tends to be very effective for localized prostate cancer [32]. Tumor progression is initially androgen-dependent and androgen ablation therapy is at first very successful at reducing the tumor burden. Despite this early response, genetic alterations lead to the development of androgen-independent or castration-resistant prostate cancer (CRPC), which is almost always fatal [33]. This transition from androgen-dependent to androgen-independent growth is not well understood, and further insight into the mechanisms driving this process will help with developing target-driven therapeutics for the effective treatment of CRPC in the future.

We recently reported that YAP, the Hippo pathway effector, is upregulated in androgen-insensitive prostate cancer cells (LNCaP-C4-2 and C81) and confers castration-resistant growth *in vivo* [34]. During that study, we noticed that in addition to YAP, the protein levels of KIBRA were also significantly increased in LNCaP-C4-2 and LNCaP-C81 cells. Here we characterize the biological significance of KIBRA upregulation in androgen-insensitive prostate cancer cells. We show that KIBRA is a positive regulator in prostate cancer cell proliferation and motility. Moreover, increased expression of KIBRA was also observed in clinical prostate tumor samples. Thus, the current study reveals an unexpected role for KIBRA in regulating cell migration and proliferation in prostate cancer cells.

Results and Discussion

KIBRA is upregulated in androgen-insensitive prostate cancer cells

LNCaP and LNCaP-C33 cells rely on androgen to grow, while the LNCaP-C4-2 and LNCaP-C81 sublines are androgen-insensitive and can grow under androgen deprivation conditions [35,36]. We recently showed that YAP is upregulated during progression from an

androgen-sensitive to an androgen-insensitive state [34]. During that work, we indeed found that KIBRA/WWC1 protein levels were also significantly increased in LNCaP-C4-2 and LNCaP-C81 cells when compared to the parental cells (Figure 1A, B). WWC2, which is in the same protein family as KIBRA, was increased in cancer cells when compared to RWPE-1 (immortalized prostate epithelial) cells; however, no significant change was observed between LNCaP and LNCaP-C4-2 cells (Figure 1A). Quantitative reverse transcription-PCR (RT-PCR) showed that the levels of KIBRA mRNA were significantly elevated in LNCaP-C4-2 and LNCaP-C81 cells (Figure 1C). Surprisingly, WWC2 and WWC3 mRNA levels were reduced in LNCaP-C4-2 and LNCaP-C81 cells when compared to LNCaP cells (Figure 1C). KIBRA protein stability/half-life is similar in both LNCaP and LNCaP-C4-2 cells (Figure 1D). Since KIBRA is induced by YAP [37] and YAP is elevated in androgen-insensitive prostate cancer cells [34], we tested whether KIBRA expression is YAP-dependent in LNCaP-C4-2 cells. Figure 1E showed that KIBRA expression was partially reduced in LNCaP-C4-2 cells with YAP knockdown (Figure 1E). Together, these observations indicate that KIBRA expression is transcriptionally increased in androgen-insensitive prostate cancer cells compared with androgen-sensitive cells and that its expression in these cells is partially regulated by YAP.

KIBRA promotes proliferation, migration and invasion in RWPE-1 cells

To determine the role of KIBRA in prostate cancer, we first established stable cell lines expressing KIBRA in RWPE-1 cells (Figure 2A). Overexpression of KIBRA did not alter the WWC2 protein and WWC3 mRNA levels (Figure 2A). Ectopic expression of KIBRA stimulated cell proliferation (Figure 2B). Overexpression of KIBRA also induced cell migration (Figure 2C, D) and invasion (Figure 2E, F). KIBRA expression was not sufficient to cause anchorage-independent growth (neoplastic transformation) of RWPE-1 cells (Figure 2G). KIBRA regulates an epithelial-mesenchymal transition (EMT) in breast epithelial cells [17]. However, KIBRA overexpression did not alter the expression of E-cadherin (an epithelial marker) and vimentin (a mesenchymal marker) (Figure 2H), suggesting that KIBRA regulates EMT in a cell type specific manner.

KIBRA promotes proliferation, migration and invasion in LNCaP cells

Next we investigated whether KIBRA plays a similar role in prostate cancer cells. LNCaP cells with stable expression of KIBRA was established (Figure 3A). No changes of WWC2 and WWC3 expression were detected in KIBRA-overexpressing cells (Figure 3A). LNCaP-KIBRA-expressing cells proliferated significantly faster than LNCaP-vector cells (Figure 3B). Similarly, KIBRA overexpression also enhanced cell migration and invasiveness in LNCaP cells (Figure 3C–F). However, ectopic expression of KIBRA did not alter the LNCaP cells' anchorage-independent growth (Figure 3G, H).

We recently showed that YAP overexpression converted LNCaP cells from androgen-sensitive to androgen-insensitive growth [34], we also tested whether KIBRA plays a role in androgen sensitivity. Under androgen-deprivation conditions, both LNCaP-vector and LNCaP-KIBRA cells failed to divide, suggesting that enhanced expression of KIBRA was not sufficient to promote androgen-insensitive growth in LNCaP cells (Figure 3I).

KIBRA knockdown impairs migration, invasion and anchorage-independent growth in prostate cancer cells

To explore the significance of KIBRA upregulation in androgen-insensitive prostate cancer cells, we reduced KIBRA expression using a validated shRNA against KIBRA [21] in LNCaP-C4-2 and LNCaP-C81 cells (Figure 4A). In contrast to the gain of function phenotypes in LNCaP and RWPE-1 cells, knockdown of KIBRA did not alter the proliferation rates in LNCaP-C4-2 and LNCaP-C81 cells (Figure 4B). Interestingly, knockdown of KIBRA greatly reduced migration and invasion (Figure 4C–E) and anchorage-independent growth (Figure 4F) in these cells. Together, these data revealed that KIBRA is a positive regulator in prostate cancer cell proliferation and motility.

KIBRA is induced by AR signaling

Consistent with the results from RWPE-1 cells, KIBRA overexpression or knockdown did not alter the expression of E-cadherin, an epithelial marker (Figure 5A). N-cadherin and vimentin (both are mesenchymal markers) were not detectable in LNCaP or LNCaP-C4-2 cells. AR protein levels were not affected by KIBRA expression either (Figure 5A). AR signaling activity is higher in LNCaP C4-2 and LNCaP-C81 cells than in LNCaP cells [34,38]. To analyze whether KIBRA expression is regulated by AR signaling, we treated the LNCaP cells with R1881, an analog of androgen ligand, to activate the AR signaling. Interestingly, KIBRA protein levels were dramatically increased upon R1881 treatment (Figure 5B). Similar findings were also observed in androgen-insensitive cells (LNCaP-C4-2 and LNCaP-C81) (Figure 5C). However, the levels of mRNA of KIBRA were not altered in the presence of R1881 (Figure 5D, E). The expression of prostate-specific antigen (PSA), a known target of AR, was induced (Figure 5D, E). KIBRA protein stability was not significantly affected in the presence of R1881 (Figure 5F). In future studies, we will explore the underlying mechanisms through which AR signaling activation by R1881 induces KIBRA expression.

Interestingly, mRNA levels of PSA were significantly reduced in KIBRA knockdown LNCaP-C4-2 cells, suggesting that KIBRA is required for full activation of AR signaling (Figure 5G). However, it is not known how KIBRA regulates AR activity and the downstream signaling. Previous reports showed that both Lats2 [39] and YAP [39] associate with AR, and that KIBRA interacts with Lats2 [37]. Therefore, KIBRA may form a complex with AR through other binding partners to regulate AR activity though KIBRA expression did not affect the Lats1 kinase activity and YAP phosphorylation in prostate cancer cells (Figure 5H).

KIBRA is upregulated in prostate tumors

Having established the role of KIBRA in prostate cancer with cell culture systems, we wanted to determine the clinical relevance of KIBRA in prostate cancer. Publically available data demonstrated that mRNA levels of KIBRA were significantly higher in prostate tumors than normal prostate tissue (Figure 6A–D). Moreover, KIBRA protein levels were also greatly increased in prostate tumors than normal prostate tissue in another set of samples (Figure 6E).

In conclusion, the results show that ectopic expression of KIBRA promotes prostate cancer cell proliferation and motility. KIBRA knockdown impairs migration and invasion in androgen-insensitive prostate cancer cells. KIBRA expression is induced by AR signaling and is increased in prostate tumors. Our findings reveal a pro-tumor function for KIBRA in prostate cancer.

Materials and Methods

Expression constructs and cell culture

The human KIBRA cDNA and shRNA constructs have been previously described [37]. LNCaP-C4-2 cell lines with shControl or shYAP have been described in [34]. RWPE-1 and LNCaP cell lines and related media and supplements were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and the cell lines were cultured following ATCC's instructions. LNCaP-C4-2 was provided by Dr. Kaustubh Datta (University of Nebraska Medical Center) [40]. LNCaP-C33 and LNCaP-C81 cell lines were obtained from Dr. Ming-Fong Lin (University of Nebraska Medical Center) [41,42]. R1881 (an androgen analog) was purchased from PerkinElmer (Waltham, MA, USA). All other chemicals were either from Sigma (St. Louis, MO, USA) or Thermo Fisher (Waltham, MA, USA).

Generation of cell lines

Ectopic expression of empty vector (control) or KIBRA in the RWPE-1 and LNCaP cell lines was achieved by a retrovirus-mediated approach. ShRNA-mediated knockdown of KIBRA in LNCaP-C4-2 or LNCaP-C81 cells was achieved similarly. Virus packaging, infection, and resistance selection were done as described [37].

Antibodies

Mouse monoclonal antibodies against human KIBRA have been described [21] and were used for Western blot analysis throughout the study. Anti-WWC2 antibodies were purchased from Sigma. Anti-β-actin antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-YAP, anti-vimentin, and anti-E-cadherin antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-N-cadherin antibodies were provided by Dr. Keith Johnson [43]. Total cell lysate preparation and Western blotting assays were done as previously described [21].

Cell proliferation, migration, invasion, and colony assays

Cell proliferation assays were performed as described in [44]. Cells (3,000/well) were seeded in a 24-well plate in triplicate. Colony assays (to assess anchorage-independent growth in soft agar) were done in 6-well plates as we have described [45]. *In vitro* cell migration and invasion assays were assessed using the Transwell system (Corning Inc., Corning, NY, USA) and BioCoat invasion system (BD BioSciences, San Jose, CA, USA), respectively, according to the manufacturer's instructions. Cells (50,000 or 100,000) were seeded for each Transwell/Insert in duplicate and repeated twice [34]. The migratory and invasive cells were stained with ProLong Gold Antifade Reagent with DAPI [16,46] and counted under a 20X lens. The rates are the average of counts in five fields of view per Transwell/Insert.

Quantitative real time-PCR

Total RNA isolation, RNA reverse transcription, and PCR were done with SYBR green (Bio-Rad) as previously described [37]. Primer sequences were as follows: WWC2: 5'-tctggcctccagacattttt (forward); 5'-tctcacacaagcttattctcagg (reverse); WWC3: 5'-agttcgtccccaacacaatc (forward); 5'-cgcgtcttttacattgacca (reverse). Other primers used in this study were listed in [34,37].

Prostate tumor samples

Normal prostate and tumor samples/proteins were obtained from Protein Biotechnologies and the Tissue Bank at the University of Nebraska Medical Center.

Statistical analysis

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

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Abbreviations

AR	androgen receptor
CRPC	castration-resistant prostate cancer
EMT	epithelial-mesenchymal transition
PSA	prostate-specific antigen
WWC1–3	WW and C2 domain containing protein 1–3
YAP	yes-associated protein

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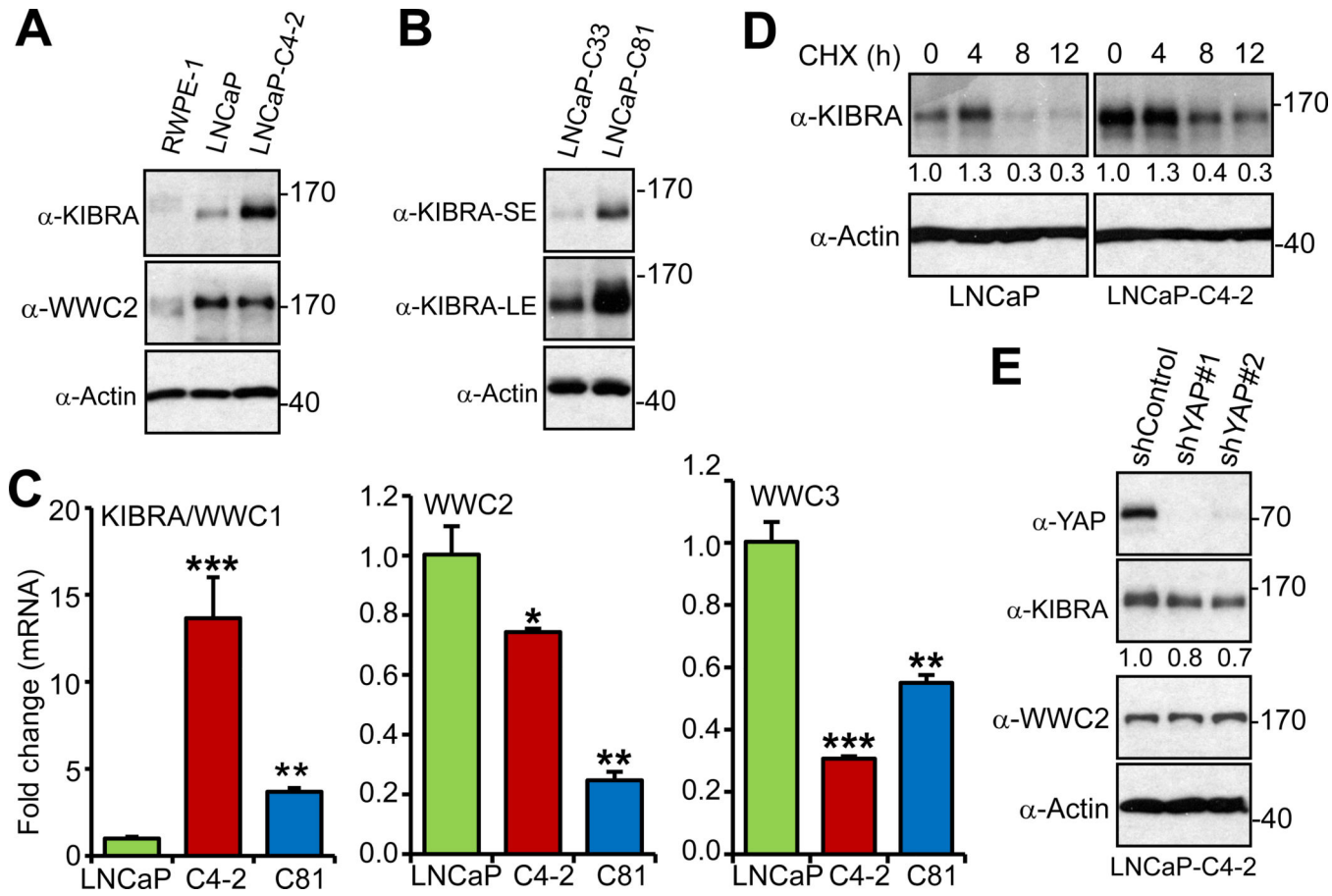

Fig.1. KIBRA is transcriptionally upregulated in androgen-insensitive prostate cancer cells

(A, B) RWPE-1, LNCaP, LNCaP-C4-2, LNCaP-C33, and LNCaP-C81 cells lines were cultured as described in 'Material and methods'. The total cell lysates were probed with the indicated antibodies. SE: short exposure; LE: long exposure.

(C) Quantitative RT-PCR of WWC1/2/3 in LNCaP and its sublines.

(D) LNCaP and LNCaP-C4-2 cells were treated with cycloheximide (CHX, 50 μg/ml) at the indicated time points and the total cell lysates were analyzed with the indicated antibodies. The relative intensity was shown from the average of three blots (Image J).

(E) LNCaP-C4-2 cell lines expressing control shRNA or shRNA against YAP were utilized to determine the indicated protein levels by Western blotting. Data were obtained from three (n=3) independent experiments (A–E) and expressed as mean ± s.e.m (C). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (t-test).

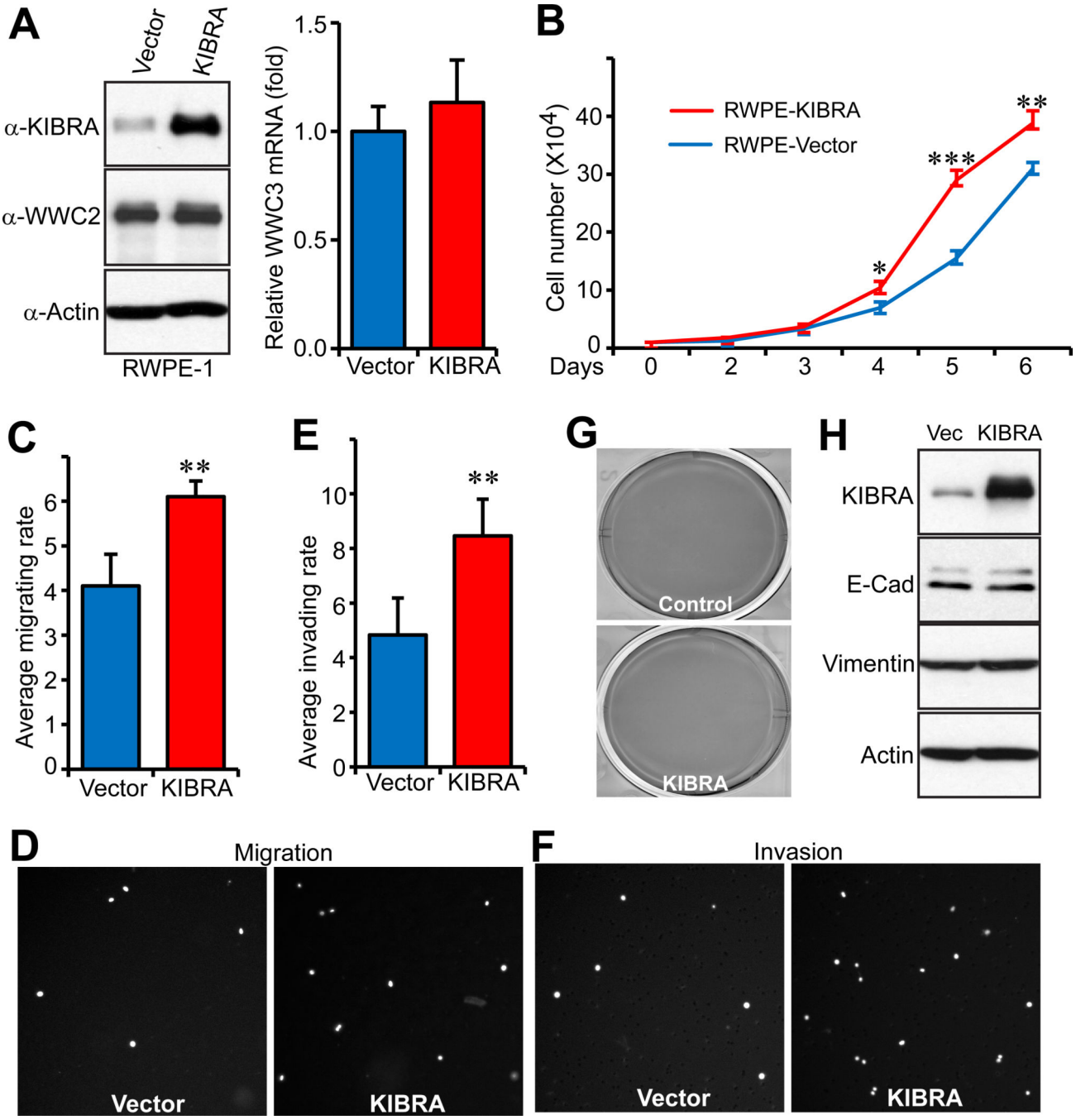

Fig.2. KIBRA promotes proliferation, migration and invasion in RWPE-1 cells

(A) Establishment of RWPE-1 cells expressing vector (control) or KIBRA. WWC2 protein and WWC3 mRNA levels were determined in these cells.

(B) The proliferation curve of the cell lines established in (A).

(C, D) Cell migration effect was determined with the cell lines in (A). Representative photos for migrating cells are shown in (D).

(E, F) Cell invasion effect was determined with the cell lines in (A). Representative photos for invading cells are shown in (F).

(G) Anchorage-independent growth (colony assay in soft agar) was determined with the cell lines established in (A). No colony was formed in RWPE-1-vector and –KIBRA cells. (H) Total cell lysates from cell lines established in (A) were probed with the indicated antibodies. Data were obtained from three (n=3) independent experiments (A–H) and expressed as mean ± s.e.m (B, C). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (t-test).

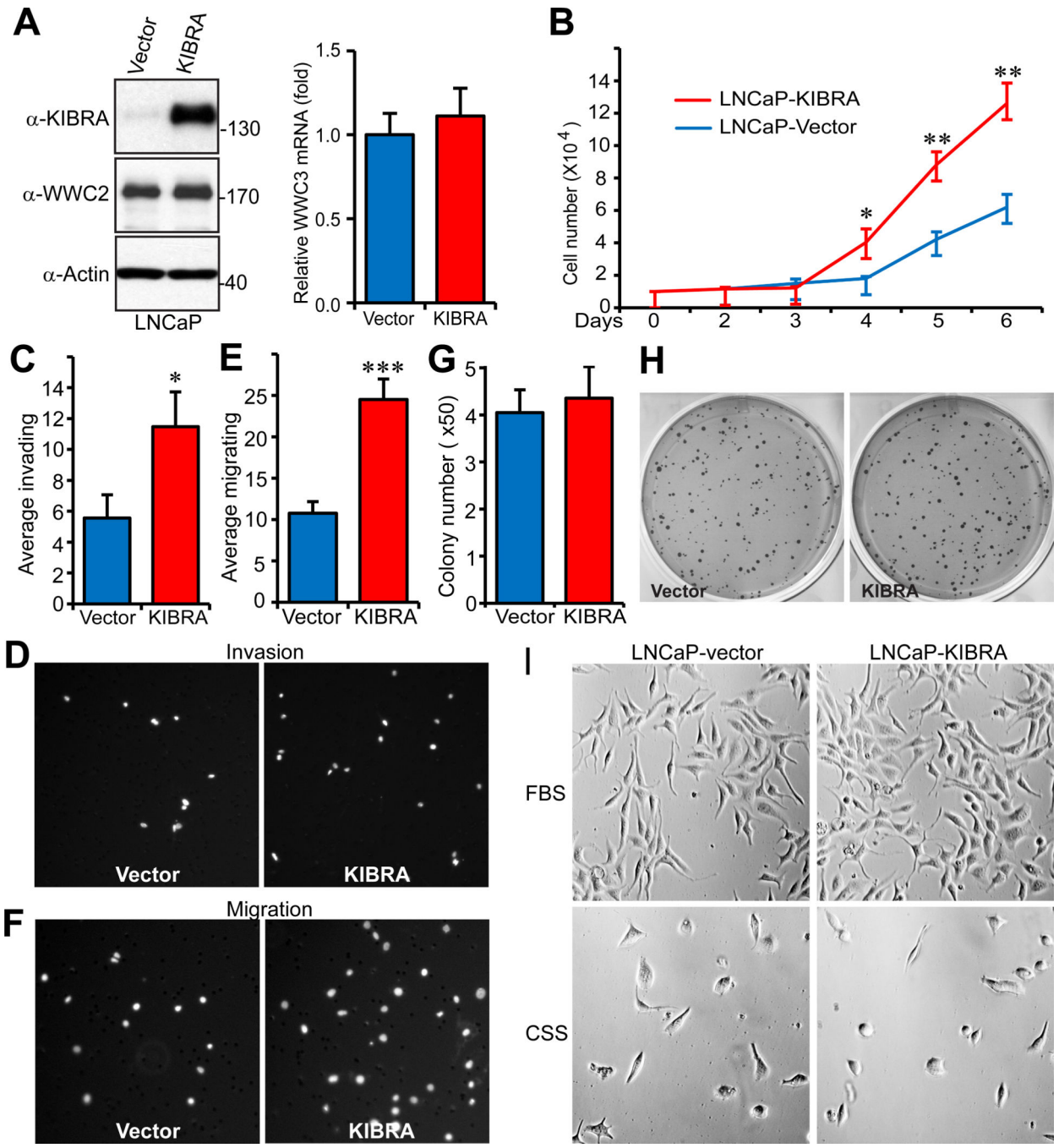

Fig.3. KIBRA promotes proliferation, migration and invasion in LNCaP cells

(A) Establishment of LNCaP cells expressing vector (control) or KIBRA. WWC2 protein and WWC3 mRNA levels were determined in these cells.

(B) The proliferation curve of the cell lines established in (A).

(C, D) Cell invasion effect was determined with the cell lines established in (A). Representative photos for invading cells are shown in (D).

(E, F) Cell migration effect was determined with the cell lines established in (A). Representative photos for invading cells are shown in (F).

(G, H) Anchorage-independent growth (colony assay in soft agar) was determined with the cell lines established in (A).

(I) Representative photos of LNCaP-vector or LNCaP-KIBRA cells that have been cultured in normal medium (FBS) or androgen deprivation medium (CSS) for 4 days. FBS: fetal bovine serum; CSS: charcoal striped serum. Data were obtained from three (n=3) independent experiments (A–I) and expressed as mean ± s.e.m (B, C, E, G). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (t-test).

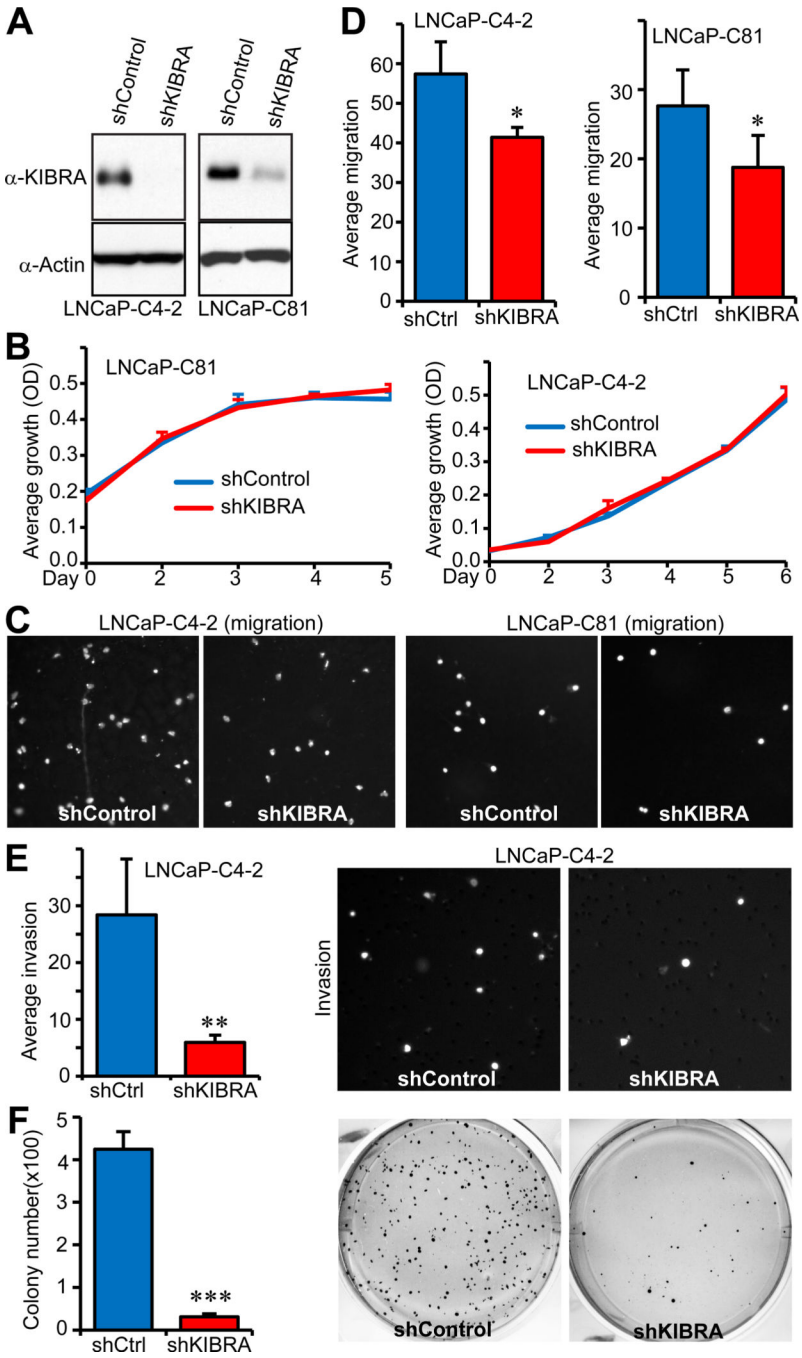

Fig.4. KIBRA knockdown impairs motility and anchorage-independent growth in prostate cancer cells

(A) Establishment of LNCaP-C4-2/C81 cells expressing control shRNA (shControl) or shRNA against KIBRA (shKIBRA).

(B) The proliferation curves (determined by MTT assays) of the cell lines established in (A).

(C, D) Cell migration effect was determined with the cell lines established in (A). Representative photos for migrating cells are shown in (C).

(E) Cell invasion effect was determined with the cell lines established in (A).

(F) Colony formation assays in LNCaP-C4-2 cells established in (A). Data were obtained from three (n=3) independent experiments (A–F) and expressed as mean ± s.e.m (B, D-F). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ (t-test).

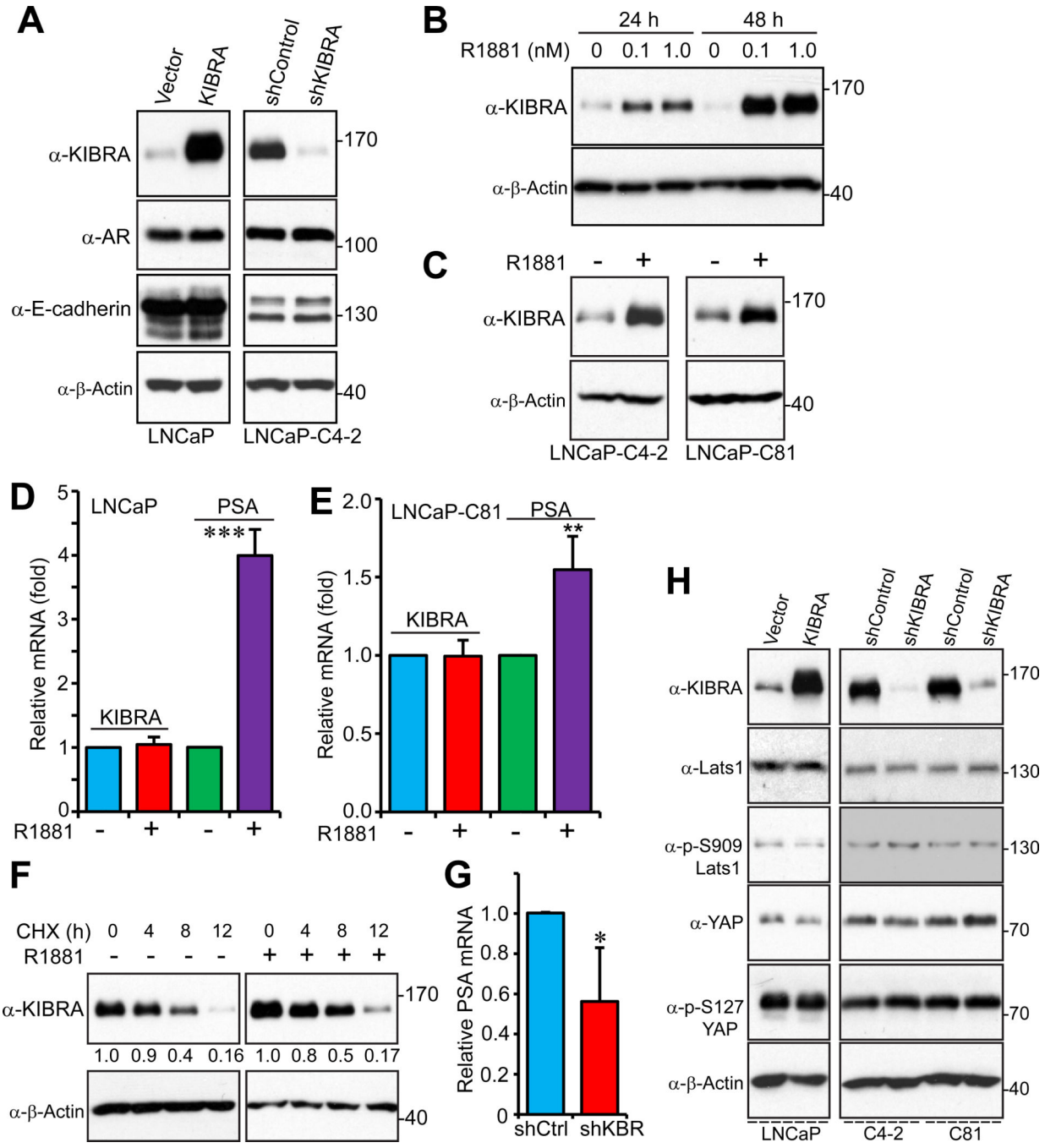

Fig. 5. KIBRA is induced by R1881 and is required for AR signaling activation

(A) Total cell lysates from various stable cell lines were probed with the indicated antibodies.

(B) LNCaP cells were treated with R1881 as indicated. Total protein lysates were subjected to Western blot analysis.

(C) LNCaP-C4-2 and LNCaP-C81 cells were treated with R1881 (1 nM) for 24 h and total protein lysates were subjected to Western blot analysis with the indicated antibodies.

(D, E) Quantitative RT-PCR in LNCaP or LNCaP-C81 cells treated or not treated with R1881 (1 nM) for 24 h.
(F) LNCaP-C81 cells were treated with cycloheximide (CHX, 50 μg/ml) at the indicated time points and the total cell lysates were analyzed with the indicated antibodies. The relative intensity was shown from the average of three blots (Image J).
(G) Quantitative RT-PCR for PSA in LNCaP-C4-2 cells with control or KIBRA knockdown.
(H) Total cell lysates from various stable cell lines were probed with the indicated antibodies. Data were obtained from three (n=3) independent experiments (A–H) and expressed as mean ± s.e.m (D–G). *: $p<0.05$; ***: $p<0.001$ (t-test).

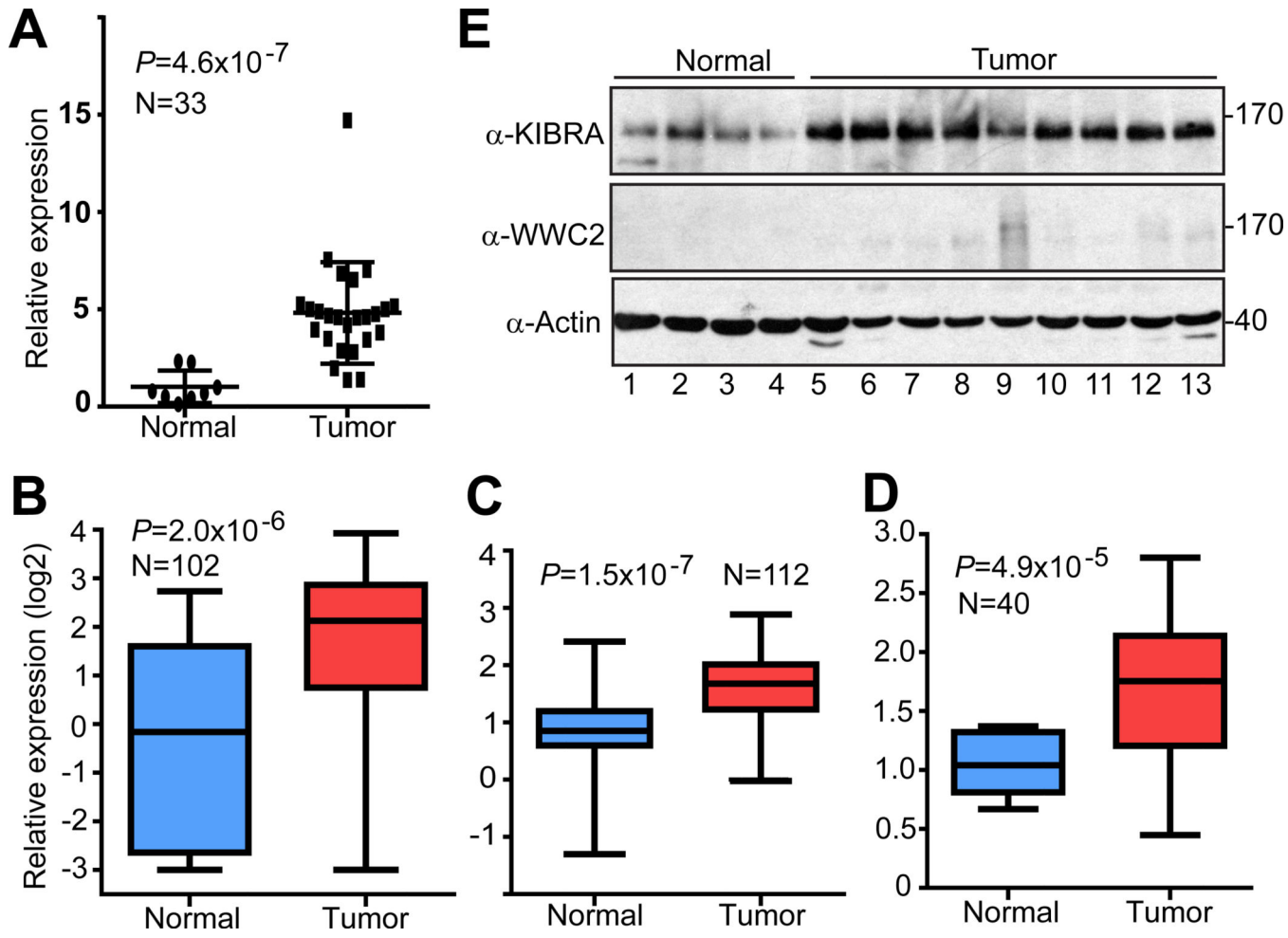

Fig. 6. KIBRA is overexpressed in prostate cancer

(A–D) KIBRA mRNA is increased in clinical samples. Data were mined from published studies through biogps.org (A) and oncomine.org (B–D). The original references are: [47] (A), [48] (B), [49] (C), and [50] (D). The box (B–D) extends from the 25th to 75th percentiles. The line is plotted at the median and the whiskers go to the smallest and the largest value for each group (B–D).

(E) Total protein lysates from prostate tumors and normal prostate tissue were subjected to Western blot analysis with the indicated antibodies.