Oncogene (2013) **32,** 1821–1830

www.nature.com/onc

ORIGINAL ARTICLE

KIBRA exhibits MST-independent functional regulation of the Hippo signaling pathway in mammals

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The Salvador/Warts/Hippo (Hippo) signaling pathway defines a novel signaling cascade regulating cell contact inhibition, organ size control, cell growth, proliferation, apoptosis and cancer development in mammals. The upstream regulation of this pathway has been less well defined than the core kinase cassette. KIBRA has been shown to function as an upstream member of the Hippo pathway by influencing the phosphorylation of LATS and YAP, but functional consequences of these biochemical changes have not been previously addressed. We show that in MCF10A cells, loss of KIBRA expression displays epithelial-to-mesenchymal transition (EMT) features, which are concomitant with decreased LATS and YAP phosphorylation, but not MST1/2. In addition, ectopic KIBRA expression antagonizes YAP via the serine 127 phosphorylation site and we show that KIBRA, Willin and Merlin differentially regulate genes controlled by YAP. Finally, reduced KIBRA expression in primary breast cancer specimens correlates with the recently described claudin-low subtype, an aggressive sub-group with EMT features and a poor prognosis.

Oncogene (2013) **32,** 1821–1830; doi:10.1038/onc.2012.196; published online 21 May 2012

Keywords: KIBRA; Hippo pathway; Merlin; Willin; breast cancer; claudin-low

INTRODUCTION

The Salvador/Warts/Hippo (Hippo) signaling pathway is a recently discovered biological pathway in *Drosophila melanogaster*, where it is implicated in the control of growth and organ size.[1] This pathway limits organ size by inhibiting cell proliferation and promoting apoptosis and is therefore of particular importance during development and tissue size control.[2–7] In addition, several lines of evidence suggest that the activities of multiple Hippo pathway components are deregulated in human cancer.[1,3,6,7]

The Hippo pathway consists of a series of kinases and adaptor proteins in which the Hippo kinase, in association with an adaptor protein Salvador, phosphorylates and activates Warts kinase, which is associated with Mats. This core kinase cassette phosphorylates and inactivates the transcriptional co-activator Yorkie, thereby suppressing expression of genes that promote cell survival, growth and proliferation.[5] Inactivation of Yorkie in *D. melanogaster* imaginal disc tissues results in cell cycle arrest and apoptosis.[3,6] Kibra, Expanded and Merlin function upstream of the core kinase cassette, activating the pathway via Hpo and Wts phosphorylation.[8–10] At least four upstream regulatory branches of the Hippo pathway exist in Drosophila: Fat/Dachsous, Kibra/Ex/Mer, Lethal Giant Larvae/atypical protein Kinase C, and Crumbs.[11] Studies suggest that loss-of-function mutant clones for these upstream regulators result in a comparatively milder overgrowth phenotype than Hpo core component mutants, whereas single mutants cause a mild overgrowth phenotype and double mutants display strong synergistic effects in some cases resembling those of Hpo mutants.[12] Studies have also shown that Ex and Mer may account for slightly different aspects of the phenotypes, implying that they may act not only redundantly but also in parallel with one another to control Hippo signaling.[8,13]

Less is known about the functioning of the Hippo signaling pathway in mammals, particularly the proteins that regulate the mammalian Hippo pathway upstream of the core kinase cassette. Components of the Hippo pathway are conserved in mammals and consist of MST1/2 (Hpo orthologs), WW45/Sav (Sav ortholog), LATS1/2 (Wts orthologs), MOBKL1A/B (Mats ortholog) and YAP (Yki ortholog),[3] but some upstream members of the mammalian Hippo pathway have remained enigmatic. For example, recent studies suggest that the Fat/Dachsous pathway does not control organ size and activity of the Yki orthologs, YAP and TAZ, in mice.[14,15] However recently, we identified that the predicted human ortholog of Ex, Willin, does activate the mammalian components of the Hippo signaling pathway and antagonises the activity of YAP.[16]

KIBRA (WWC1), the mammalian ortholog of Kibra, shows enriched expression in kidney and brain[17] and has been associated with memory performance and an age-dependent risk of Alzheimer disease.[18–21] KIBRA interacts with discoidin domain receptor 1 to modulate collagen-induced signaling.[22] KIBRA also interacts with and stimulates the phosphorylation of LATS1/2 and regulates the phosphorylation of YAP,[10,23] but the functional significance of these biochemical changes has not been addressed to date.

Here, we show that in MCF10A cells, loss of KIBRA expression displays EMT features, which are concomitant with decreased

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Received 18 November 2011; revised 29 March 2012; accepted 30 March 2012; published online 21 May 2012

LATS and YAP phosphorylation, but not MST1/2. In addition, ectopic KIBRA expression antagonizes YAP via the serine 127 phosphorylation site. However, we also show that KIBRA, Willin and Merlin differentially regulate genes controlled by YAP. Finally, we show that reduced KIBRA expression in primary breast cancer specimens correlates with the claudin-low subtype. Claudin-low tumors are a recently defined intrinsic breast cancer subtype characterized by a lack of luminal differentiation markers, a high enrichment for epithelial-to-mesenchymal transition (EMT) markers, immune response genes and cancer stem cell-like features.[24–27]

RESULTS

KIBRA knockdown induces features of EMT in mammary epithelial cells

Since deregulation of upstream Hippo pathway components such as Willin/FRMD6 induces EMT in MCF10A cells[16] as well as core components such as YAP,[28,29] we knocked down the expression of KIBRA in these cells using two independent lentiviral shRNA constructs to investigate whether this also occurred for KIBRA. While MCF10A cells expressing a non-targeting control (shScr) grew in epithelial-type islands on monolayer cultures, MCF10A cells expressing shRNA targeting KIBRA (MCF10A-shKIBRA) displayed abnormal features resembling YAP-induced EMT (Figure 1a). Interestingly, there was a concomitant decrease in the phosphorylation of the Hippo pathway kinases LATS1 and YAP, but not MST1/2 in these cells (Figure 1b; Supplementary Figure S1), treated with 50 ng/ml TNFα for 4 h before harvest to stimulate pathway activation. Phosphorylation of the MST1/2 substrate MOBKL1A/B[30] at T12 also did not decrease in shKIBRA cells, but rather phosphorylation increased at T12 (Figure 1b; Supplementary Figure S1). These findings were confirmed using another cell line (Supplementary Figure S4). MCF10A-shKIBRA cells produced 4-fold more colonies in soft-agar than MCF10A-shScr cells, after 21 days in culture (Figure 1c). This phenotype was enhanced by simultaneous knockdown of KIBRA and Merlin (Figure 1c; Supplementary Figure S1). MCF10A-shKIBRA cells also showed a 4-fold increased migration compared to MCF10A-shScr cells (Figure 1d). Consistent with the EMT-like phenotype, the mesenchymal markers N-cadherin and vimentin were up-regulated by YAP overexpression or by KIBRA knockdown, whereas the epithelial markers, E-cadherin and occludin, were down-regulated by both of these manipulations (Figure 1e). Efficient knockdown of KIBRA was assayed by immunoblotting (Figure 1e).

KIBRA knockdown phenotype is dependent on LATS and YAP but not MST1/2

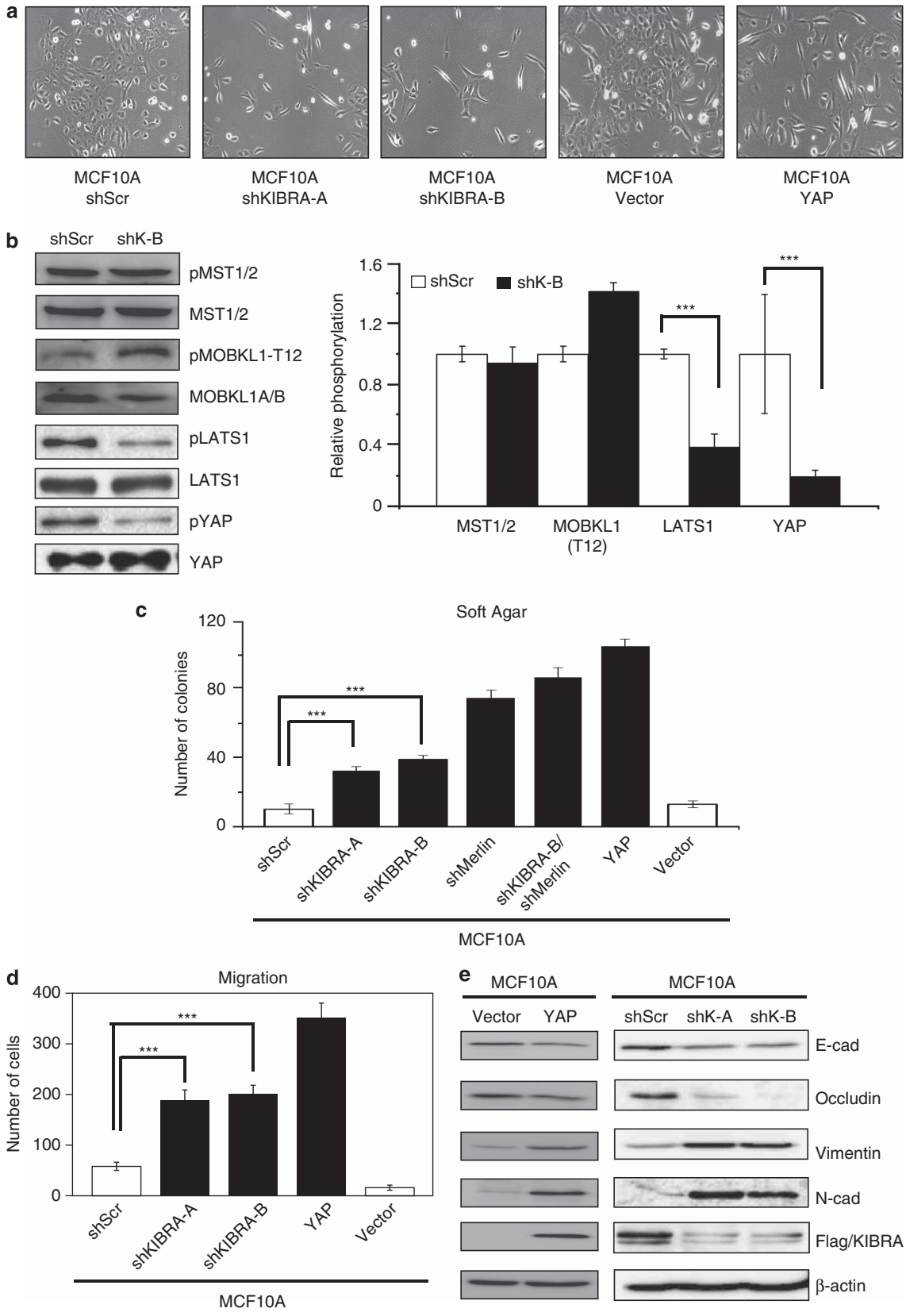
Next we sought to determine if the functional consequences of KIBRA knockdown, shown by increased migration, were dependent on Hippo pathway activity. The reduction of MST1/2 expression in MCF10A-shScr and MCF10A-shKIBRA cells by two independent siRNA duplexes did not show any abrogation of the enhanced migratory ability, but rather showed increased migration (Figure 2a). Surprisingly, reduction of MST1/2 expression in MCF10A-YAP(S127A) cells caused decreased migration (Figure 2a). An effective reduction of protein expression was confirmed by immunoblotting analysis (Supplementary Figure S2).

Similarly, expression of LATS1 and YAP was reduced by siRNA in MCF10A-shScr, MCF10A-shKIBRA and MCF10A-YAP(S127A) cells. LATS2 knockdown was not tested since LATS2 does not functionally antagonize YAP in MCF10A cells.[29] As expected, reduced LATS1 expression caused an increase in migration in MCF10A-shScr cells (Figure 2b). Surprisingly, reduced LATS1 expression caused a decrease in migration in MCF10A-shKIBRA and MCF10A-YAP(S127A) cells. Reduced YAP expression caused decreased migration in all 3 cellular backgrounds (Figure 2c). An effective reduction of protein expression was confirmed by immunoblotting analysis (Supplementary Figure S2). Interestingly, in the context of reduced migration due to the presence of siLATS in MCF10A-shKIBRA and MCF10A-YAP(S127A) cells, levels of phospho-YAP at S127 increased compared to siControl cells, but not in the context of increased migration due to the presence of siLATS in MCF10A-shScr cells (Supplementary Figure S2). Also, reduced migration due to the presence of siControl occurred in MCF10A-shKIBRA cells (compare Figures 1d and 2a–c) but not MCF10A-shScr or MCF10A-YAP(S127A) cells (Figures 2a–c). Taken together, these results suggest that the effects of KIBRA knockdown are MST-independent.

KIBRA expression antagonizes a YAP-induced EMT phenotype in MCF10A cells

Next, we investigated KIBRA expression in MCF10A cells over-expressing YAP (MCF10A-YAP). Upon infection of MCF10A-YAP cells with a retrovirus expressing KIBRA or empty vector, increases in LATS1 and YAP phosphorylation were observed when MCF10A-YAP cells over-expressed KIBRA. While over-expression of Willin in MCF10A-YAP cells increased levels of phosphorylated MST1/2 and MOBKL1A/B, KIBRA over-expression caused no significant changes in the levels observed for phosphorylated MST1/2 or MOBKL1A/B at T12 (Figure 3a; Supplementary Figure S3).[16] These findings were confirmed using another cell line (Supplementary Figure S4). Then, we explored whether KIBRA expression could antagonize a YAP-induced EMT phenotype. Notably, MCF10A-YAP cells expressing KIBRA produced approximately 70% fewer colonies in soft-agar than MCF10A-YAP cells containing empty vector, after 21 days in culture (Figure 3b; Supplementary Figure S3). Moreover, KIBRA was unable to antagonize a YAP(S127A) mutant under the same conditions (Figure 3b). MCF10A-YAP cells expressing KIBRA also showed attenuated migration by 50% as compared to MCF10A-YAP cells containing empty vector, while MCF10A-YAP(S127A) cells were refractory to the effects of KIBRA expression (Figure 3c; Supplementary Figure S3). Ectopic expression of YAP and KIBRA was confirmed by immunoblotting analysis (Figure 3d). Collectively, these results show that KIBRA antagonizes YAP via Ser127 phosphorylation.

Figure 1. Knockdown of KIBRA results in features of EMT. Drug-selected pools of infected cells were used in these analyses to avoid clonal selection effects. (**a**) KIBRA loss induces a morphology change in MCF10A cells that resembles YAP-induced EMT. Representative phase-contrast images of cells growing in monolayer cultures and transfected with either non-targeting shRNA (shScr) or shRNA targeting KIBRA (shKIBRA) or MCF10A cells expressing empty vector (MCF10A-vector) or YAP (MCF10A-YAP). (**b**) KIBRA knockdown in MCF10A cells results in a decrease of phosphorylated LATS1 and YAP but not of MST1/2 or MOBKL1A/B. Total MST1/2, MOBKL1A/B, LATS1 and YAP were used as loading controls. Relative phosphorylation was determined compared to background phosphorylation (shScr set to 1). The analysis was conducted in triplicate in three independent experiments. Error bars represent $\pm$ s.e. ($n = 3$). (**c**) KIBRA knockdown promotes anchorage-independent growth in soft agar. MCF10A-shScr, MCF10A-shKIBRA-A, MCF10A-shKIBRA-B, MCF10A-shMerlin, MCF10A-shKIBRA-B/shMerlin, MCF10A-YAP, MCF10A-vector, cells were cultured in soft agar for 21 days. Error bars represent $\pm$ s.e. ($n = 9$). shScr vs shKIBRA-A or shKIBRA-B: ***$P < 0.001$, Student's *t*-test. (**d**) KIBRA knockdown promotes migration. MCF10A-shScr, MCF10A-shKIBRA-A, MCF10A-shKIBRA-B, MCF10A-YAP or MCF10A-vector cells were cultured in Boyden chambers and migration assessed after 24 h. Error bars represent $\pm$ s.e. ($n = 9$). shScr vs shKIBRA-B: ***$P < 0.001$, Student's *t*-test. (**e**) Immunoblot analysis of E-cadherin and occludin (epithelial markers) and N-cadherin and vimentin (mesenchymal markers) shows loss of the epithelial markers and gain of mesenchymal markers in MCF10A-shKIBRA cells. β-actin was used as a loading control.



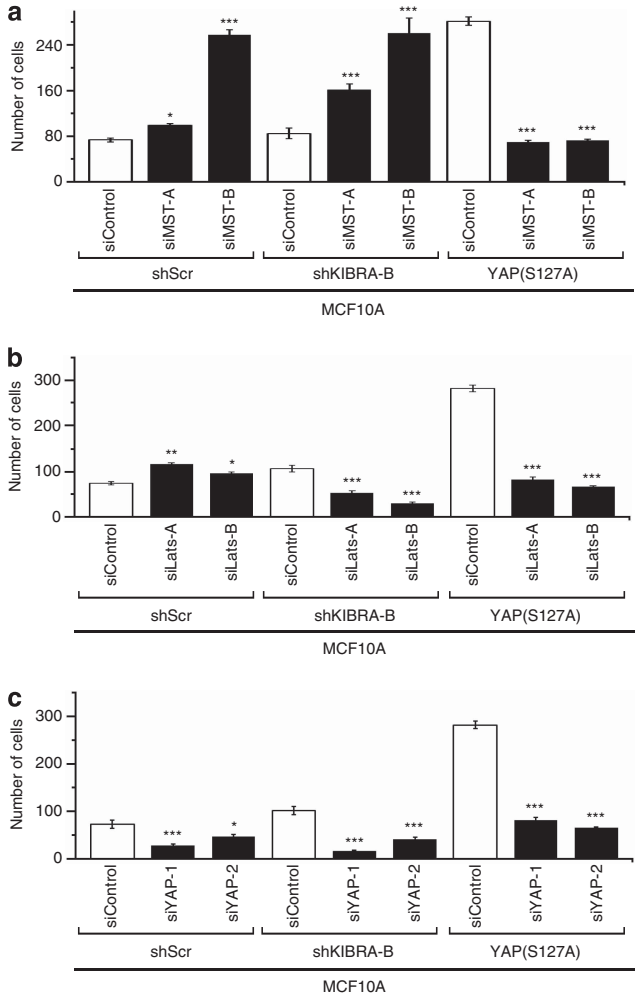


Figure 2. KIBRA knockdown phenotype is MST1/2 independent but requires LATS1 and YAP. (**a**) Effect of shKIBRA is MST1-independent. MCF10A-shScr, MCF10A-shKIBRA-B or MCF10A-YAP(S127A) cells with listed siRNA addition were cultured in Boyden chambers and migration assessed after 24 h. Error bars represent ± s.e. ($n = 9$). shKIBRA-Ctr vs shKIBRA-siMST-A or shKIBRA-siMST-B: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test. (**b**) Effect of shKIBRA is LATS1-dependent. MCF10A-shScr, MCF10A-shKIBRA-B or MCF10A-YAP(S127A) cells with listed siRNA addition were cultured in Boyden chambers and migration assessed after 24 h. Error bars represent ± s.e. ($n = 9$). shKIBRA-Ctr vs shKIBRA-siLATS-A or shKIBRA-siLATS-B: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test. (**c**) Effect of shKIBRA is YAP-dependent. MCF10A-shScr, MCF10A-shKIBRA-B or MCF10A-YAP(S127A) cells with listed siRNA addition were cultured in Boyden chambers and migration assessed after 24 h. Error bars represent ± s.e. ($n = 9$). shKIBRA-Ctr vs shKIBRA-siYAP-1 or shKIBRA-siYAP-2: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test.

The effect of Willin, Merlin and KIBRA antagonism on a YAP-induced EMT phenotype in MCF10A cells

To test whether genes regulated by YAP are altered by KIBRA expression, we investigated genes which were previously reported as altered upon Willin or LATS1 expression in MCF10A-YAP cells.[16,31] Quantitative PCR analysis showed that both KIBRA and Willin could antagonize the mRNA levels of genes known to be regulated by YAP: up-regulating BMP2 and RASSF4 and down-regulating CTGF, FGF1 and PDGFβ (Figure 4a). Interestingly, Merlin overexpression did not significantly influence the YAP-induced expression of the analysed genes (Figure 4a), even with an 8-fold overexpression of Merlin (Figure 4b). Ectopic expression of Merlin, KIBRA and Willin was confirmed by immunoblotting analysis (Figure 4b). Taken together, these results suggest that these three upstream components of the Hippo pathway differentially regulate genes controlled by YAP.

Decreased expression of KIBRA in breast cancer cell lines and primary breast tumors

In order to establish whether KIBRA expression is more generally associated with an EMT phenotype in breast cancer, we integrated published gene expression data for three breast cancer cell line datasets ($n = 173$ cell lines) and 17 primary human breast cancer datasets ($n = 2999$ tumours). In particular, we aimed to establish whether KIBRA expression was associated with EMT-related transcripts and the intrinsic breast cancer subtypes thought to be related to EMT, namely the Basal-B subgroup and the claudin-low subgroup.[24–27] KIBRA expression was significantly lower in invasive ductal carcinoma than normal breast ducts, in particular in ER-negative tumors ($P = 0.003$, Supplementary Figure S5A)[32] consistent with its role as a tumor suppressor gene. Claudin-low cell lines were identified by unsupervised clustering of the 500 most variable genes across the three integrated datasets (Figure 5). KIBRA expression was found to be lowest in claudin-low cell lines ($P = 2e-07$), which also expressed significantly lower levels of E-cadherin and higher levels of the YAP/TAZ target CTGF ($P = 0.005$) and the EMT-associated proteins TWIST ($P = 1e-11$), SNAI2 ($P = 2e-06$), and vimentin ($P = 1e-42$). Interestingly, this pattern of expression was most clearly demonstrated in the claudin-low subgroup, a recently described intrinsic subgroup characterised by enrichment of EMT and stem-cell markers. Therefore, we went on to assess whether the KIBRA/EMT signature was associated with the claudin-low and basal-like group in primary human cancers. Both basal-like and claudin-low tumors, in particular, showed significantly lower expression of KIBRA, CDH1 and CDH3 with reciprocal high expression of TWIST, SNAI2 and vimentin (Figure 6). This pattern was also clearly present in a second independent set of tumors (Supplementary Figure S5). The pattern of loss of expression of KIBRA is therefore associated with tumors that express markers of EMT both *in vitro* and in primary tumors *in vivo*. TAZ was not detectably expressed in most normal or breast cancer tissues using the Affymetrix GeneChip data that we analysed. There was no correlation of KIBRA expression with grade, although MOBKL1B ($P = 1e-11$), MST1 ($P = 4e-10$), MST2 ($P = 9e-7$) and LATS1 ($P = 0.009$) expression did show a correlation with higher histological grade (Supplementary Figure S6).

DISCUSSION

During the last decade, pivotal advances have been made in the understanding of the Hippo signaling pathway, but less is known about the proteins upstream of the core kinase cassette. We find that reducing endogenous KIBRA expression decreases LATS1 and YAP phosphorylation, but not MST1/2 or MOBKL1A/B phosphorylation. Moreover, KIBRA over-expression increases LATS1 and YAP phosphorylation, but not MST1/2 or MOBKL1A/B phosphorylation. These findings are in agreement with Xiao *et al.* (2011), supporting a model whereby LATS1 phosphorylation occurs in a MST1/2 independent manner or via Merlin in a WW45 dependent manner.[33] These observations contrast with Willin, that acts via MST1/2.[16] While the phosphorylation of YAP upon interaction of KIBRA with LATS has been reported,[23] here we show that decreased KIBRA increases anchorage-independent growth and migration, while the ectopic expression of KIBRA in MCF10A-YAP cells antagonizes the YAP-induced phenotypes of anchorage-independent growth and migration, but not when YAP is mutant at S127A. This phenocopies the action of Willin, which also functions through ser127 phosphorylation of YAP.[16] Interestingly,

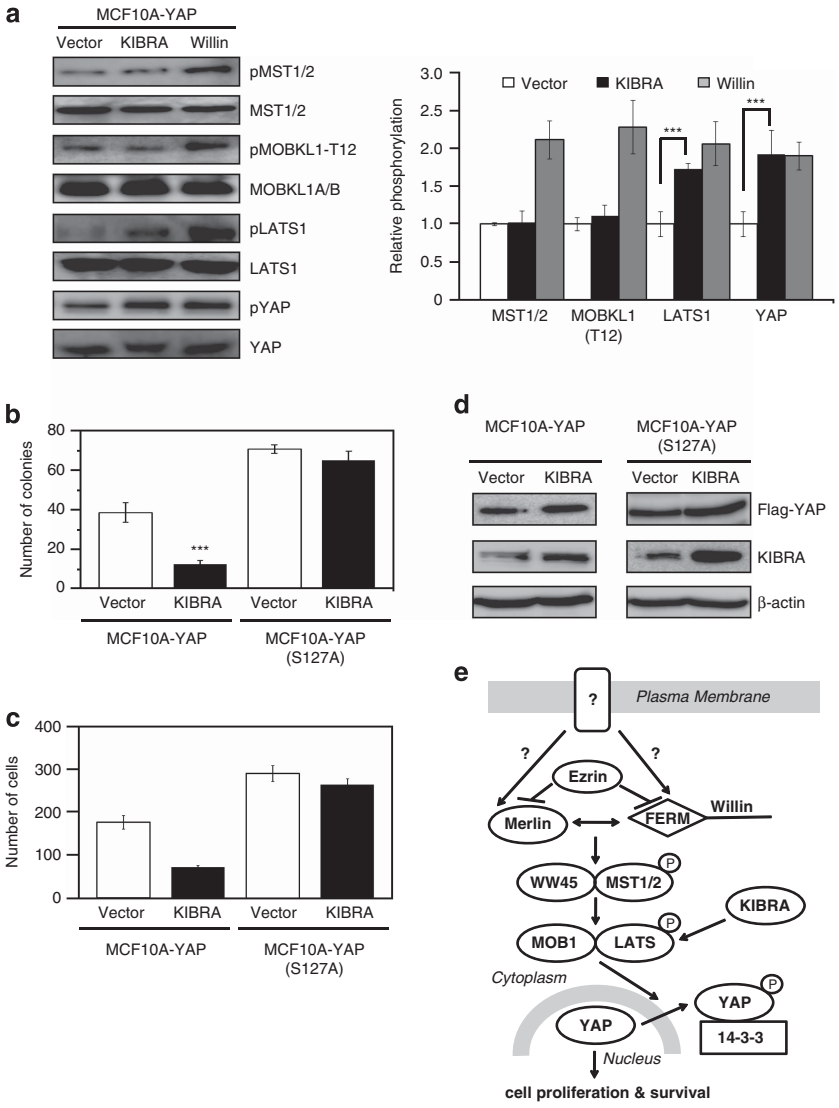


Figure 3. KIBRA expression antagonizes YAP-induced EMT in MCF10A cells. Drug-selected pools of infected cells were used in these analyses to avoid clonal selection effects. (**a**) Immunoblot analysis of retroviral infected MCF10A cells with either KIBRA or empty vector shows that KIBRA expression resulted in phosphorylation of LATS1 and YAP, but not MST1/2 or MOBKL1A/B. Relative phosphorylation to total protein levels (MST1/2, MOBKL1A/B, LATS1 or YAP) and background phosphorylation (in MCF10A-vector) is set to 1. Error bars represent ± s.d. ($n = 3$). (**b**) KIBRA expression in MCF10A-YAP cells suppresses anchorage-independent growth in soft agar. MCF10A-YAP-KIBRA, MCF10A-YAP-vector, MCF10A-YAP(S127A)-KIBRA or MCF10A-YAP(S127A)-vector cells were cultured in soft agar for 21 days. Error bars represent ± s.e. ($n = 9$). MCF10A-YAP-vector vs MCF10A-YAP-KIBRA: $^{***}P < 0.001$, Student's *t*-test. MCF10A-YAP(S127A)-vector vs MCF10A-YAP(S127A)-KIBRA: $P = 0.284$, Student's *t*-tests. (**c**) KIBRA expression in MCF10A-YAP cells suppresses migration. MCF10A-YAP-KIBRA, MCF10A-YAP-vector, MCF10A-YAP(S127A)-KIBRA or MCF10A-YAP(S127A)-vector cells were cultured in Boyden chambers and migration assessed after 24 h. Error bars represent ± s.e. ($n = 9$). MCF10A-YAP-vector vs MCF10A-YAP-KIBRA: $^{***}P < 0.001$, Student's *t*-test. MCF10A-YAP(S127A)-vector vs MCF10A-YAP(S127A)-KIBRA: $P = 0.281$, Student's *t*-test. (**d**) Immunoblot analysis of efficient FLAG-YAP and KIBRA expression. β-actin was used as a loading control. (**e**) Schematic representation of a model for the action of KIBRA on Hippo signaling.

mouse embryonic fibroblasts from mice lacking MST1/2 show phosphorylation of LATS1/2 and YAP,[34] further supporting the notion of MST-independent pathway activation.

Surprisingly, we observed a reduction in migration of shKIBRA/siLATS cells when compared to the shKIBRA/siControl cells. However, we find this reduction in migration of shKIBRA/siLATS cells correlates with an increase of anti-migratory p-YAP, suggesting compensatory feedback in these cells by other factors leading to maintenance of pathway function (and a return to migration levels seen in shScr cells). In addition, we treated shScr cells with siLATS and observed increased migration, agreeing with previous reports.[29] In this increased migration scenario, there was no increase in p-YAP levels compared to siControl cells. Overall, we believe that the migration results further support the notion that KIBRA is independent of MST, since migration in shKIBRA cells is not effectively changed by MST1/2 depletion, (it is increased compared to shKIBRA/siControl, but not compared to shKIBRA only), suggesting MST1/2 is upstream of KIBRA. The decreased migration of shKIBRA cells treated with siYAP reagents suggests YAP is downstream of KIBRA.

Next, we sought to investigate whether genes downstream of YAP are differentially regulated by KIBRA, Willin and Merlin. Surprisingly, we found that KIBRA and Willin regulate CTGF, FGF1, PDGFβ, RASSF4 and BMP2, but Merlin only regulates FGF1. PDGFβ

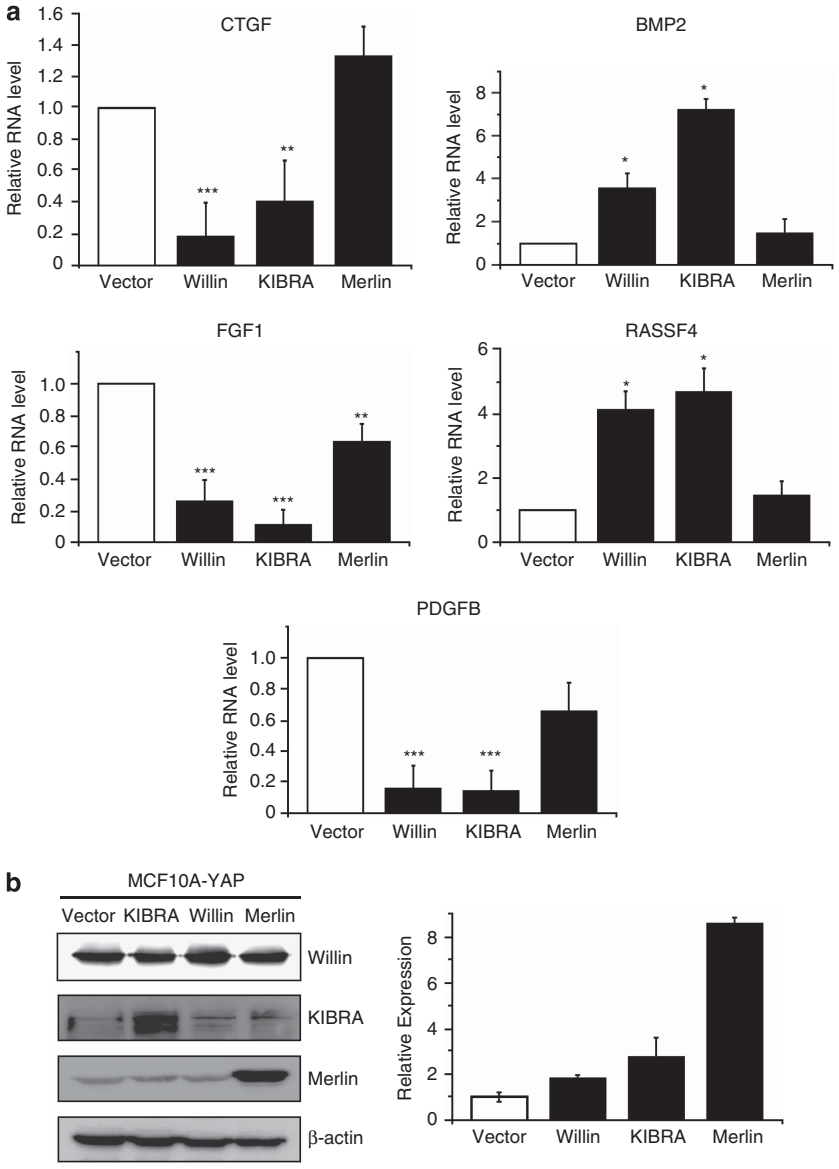


Figure 4. KIBRA expression antagonises YAP-induced transcriptional regulation. (**a**) mRNA expression of the YAP target genes BMP2, FGF1, RASSF4 and PDGFβ was assessed by quantitative real-time PCR in MCF10A-YAP cells overexpressing KIBRA, Willin or Merlin. mRNA levels were compared with the empty vector control MCF10A-YAP cells. Willin and KIBRA overexpression increased BMP2 and RASSF4 mRNA levels and decreased FGF1 and PDGFβ mRNA levels. Means were calculated from C_t values from two analyses conducted in triplicate. Error bars represent ± s.e. ($n = 6$). MCF10A-YAP-vector vs MCF10A-YAP-KIBRA or MCF10A-YAP-Willin for all the analysed genes: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ Student's *t*-test. MCF10A-YAP-vector vs MCF10A-YAP-Merlin: CTGF ($P = 0.129$), FGF1 ($P = 0.005$), BMP2 ($P = 0.585$), RASSF4 ($P = 0.307$), PDGFβ ($P = 0.06$), Student's *t*-test. (**b**) Immunoblot analysis of KIBRA, Willin or Merlin overexpression in MCF10A-YAP cells, showing 2.7, 1.8 and 8.6-fold increases compared to empty vector control. β-actin was used as a loading control.

and FGF1 were previously shown to be upregulated by YAP[31] and to be associated with transformed phenotypes, including EMT.[35,36] Furthermore, KIBRA and Willin inhibited YAP-induced transcriptional activation of RASSF4 and BMP2, which is consistent with the data presented by Angus *et al.*[16] and Hao *et al.*[31] These observations suggest that KIBRA and Willin regulate the Hippo pathway in MCF10A cells, but Merlin does not act in the same manner. As such, our findings support a model whereby KIBRA regulates LATS1 phosphorylation not by association with Merlin in a WW45-dependent manner, but through a MST1/2-independent mechanism (Figure 3e). These results highlight the upstream complexity and the ability of the pathway to fine-tune output. This is further supported by the fact that KIBRA and Willin, but not Merlin abrogation, resulted in the significant down-regulation of occludin (Figure 1e),[16] which together with claudins,[37] are components of the tight junctions.

In primary breast tumors, low KIBRA expression and high CTGF expression correlated with the claudin-low subtype, characterized by low expression of claudin-3, -4, -7; a lack of luminal differentiation markers; immune response gene enrichment; cancer stem cell-like features and high enrichment for EMT markers such as E-cadherin, vimentin and SNAI2. Consistent with our *in vitro* findings, down-regulation of E-cadherin and the up-regulation of the mesenchymal markers vimentin as well as N-cadherin, were described as occurring almost exclusively in this triple-negative subtype of tumor.[38] Importantly, claudin-low

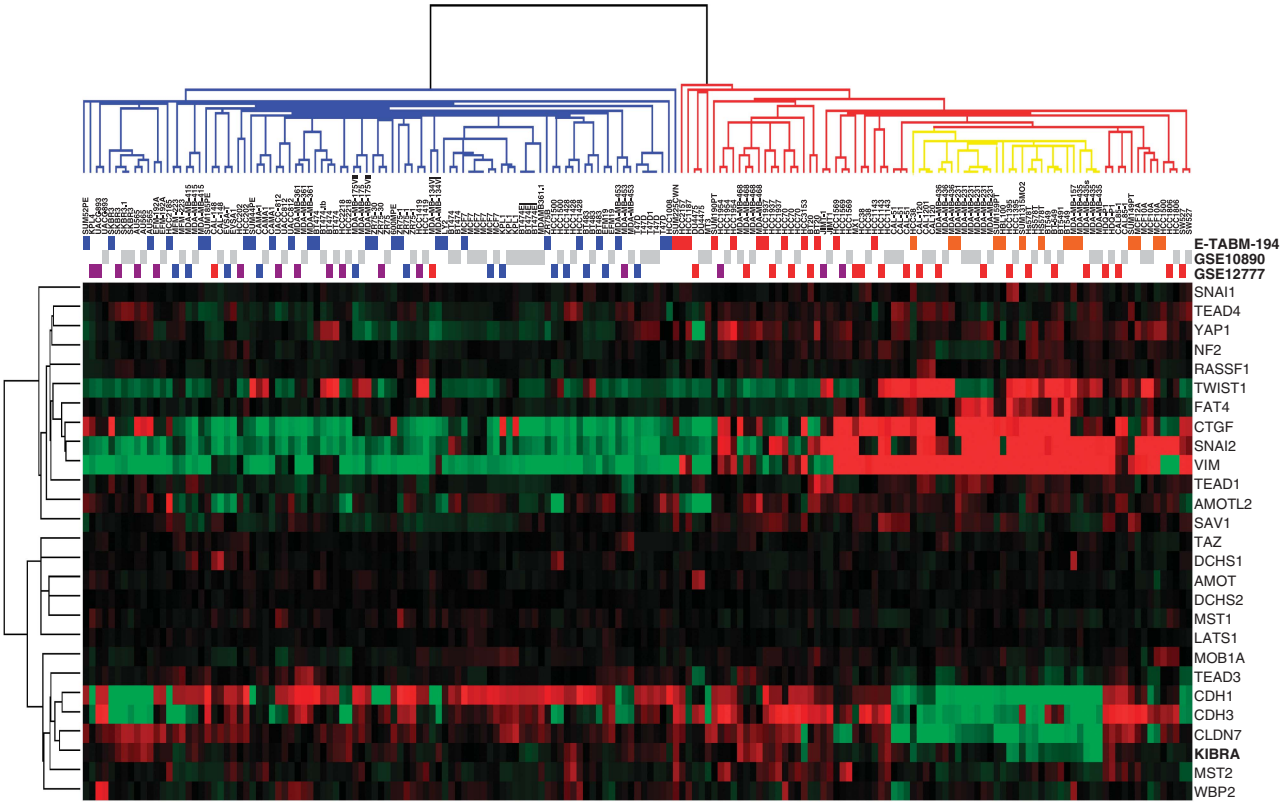


Figure 5. KIBRA expression is decreased in claudin-low breast cancer cell lines. Gene expression analysis data from 3 combined Affymetrix datasets using unsupervised clustering (average linkage, centred correlation) of the 500 most variable genes. The lines under the cell line names indicate the dataset from which each sample came and the colors indicate the type assigned in the original study (red = Basal/Basal A, orange = Basal B, purple = HER2 amplified, blue = luminal).

tumors have been linked to tumor initiating cells[24] and EMT development.[39] Interestingly, increasing evidence has shown the involvement of the Hippo pathway in the regulation of stem cell self-renewal and expansion,[40,41] however, the regulatory role of KIBRA in this scenario needs further investigation. Correlation of the Hippo pathway with histological grade supports recent findings of a 'gene signature for the Hippo signalling pathway' associated with grade.[42] The discrepancy for TAZ between our data and this study may be due to probe set issues arising in the Affymetrix GeneChip data and/or differences in the tumor samples analysed in the two studies. It was also previously reported that TAZ protein is highly expressed in some invasive ductal carcinomas.[43]

Previous data has described an interaction between KIBRA, DDR1 and aPKC, which occurs in a collagen-regulated manner,[22] suggesting a role for KIBRA in the collagen-stimulated activation of the MAPK cascade. Collagen in the extracellular environment inhibits the KIBRA-DDR1 interaction, but is required for KIBRA-induced ERK phosphorylation.[22] In normal mammary epithelium when context is essential, for example the organization of the mammary gland, then KIBRA expression would promote collagen sensing and Hippo pathway activation to maintain tissue architecture. Conversely, decreased KIBRA expression would allow cells to disconnect from sensing the extracellular environment and to turn on EMT/migration phenotypes.

To conclude, our studies have shown that Willin and KIBRA have similar effects on YAP activity. However, in our system, KIBRA activity is MST1/2-independent, while Willin is MST1/2-dependent. An understanding of how these Hippo pathway upstream components influence activation of the pathway in physiological settings represents important questions to be addressed.

MATERIALS AND METHODS

Cell culture and plasmids

MCF10A cells and derivatives were described previously[16] and grown according to Debnath *et al.*,[44] in standard cell culture flasks (Nunc Nunclon) in a humidified incubator at 37 °C with 5% CO_2. All experiments were carried out at 80–90% confluency. pBABE Nf2 (Johnson *et al.*[45]) was obtained from Addgene (plasmid 14116). Human KIBRA cDNA from the untagged expression plasmid pcDNA3.1(+) described in Hilton *et al.*[22] was excised and cloned into pBABEpuro. To establish the overexpressing cell lines, pBABE constructs were packaged in Phoenix A cells for viral production and retroviral infection of MCF10A cells was performed according to standard protocols. The transduced cells were selected, after 24 h, with 300 μg/ml hygromycin or with 2.5 μg/ml puromycin. The selections continued for 4 days (puromycin) or 8 days (hygromycin) after which cells were harvested for downstream experiments. Where indicated, TNFα was applied for 4 h at 50 ng/ml TNFα. To construct shRNA vectors, the following human oligonucleotides DNAs were synthesized and cloned into AgeI and EcoRI sites of pLKO.1 puro (Addgene; plasmid 8453) (19mer target sequences: KIBRA-A, 5′-GCTTCACTGACCTCTACTA-3′, KIBRA-B–5′-TG GTACAACCTTCTCAGCT-3′). Control shScramble was obtained from Addgene (plasmid 1864). Lentivirus packaging, MCF10A cell transduction and drug selection was performed according to standard protocols. siRNA duplexes targeting MST1 (ID # s8993 and s8994), LATS1 (ID # s17392 and s17393) and YAP (ID # s20366 and s20367) as well as a non-targeting siRNA control (Ambion, Warrington, UK) were transfected at a concentration of 20 nM, using Lipofectamine 2000 (Invitrogen, Paisley, UK), according to the manufacturer's instructions.

Immunoblot analysis

Cells were lysed in a lysis buffer (10 mM Tris at pH 8.0, 150 mM NaCl, 1% Na Deoxycholate, 1% nonidet P-40, 1% sodium dodecyl sulfate, 1 mM EDTA, 1:25 protease inhibitor cocktail and phosphatase inhibitors. Protein lysates (30 μg) were run on an SDS/10% polyacrylamide gel and transferred onto

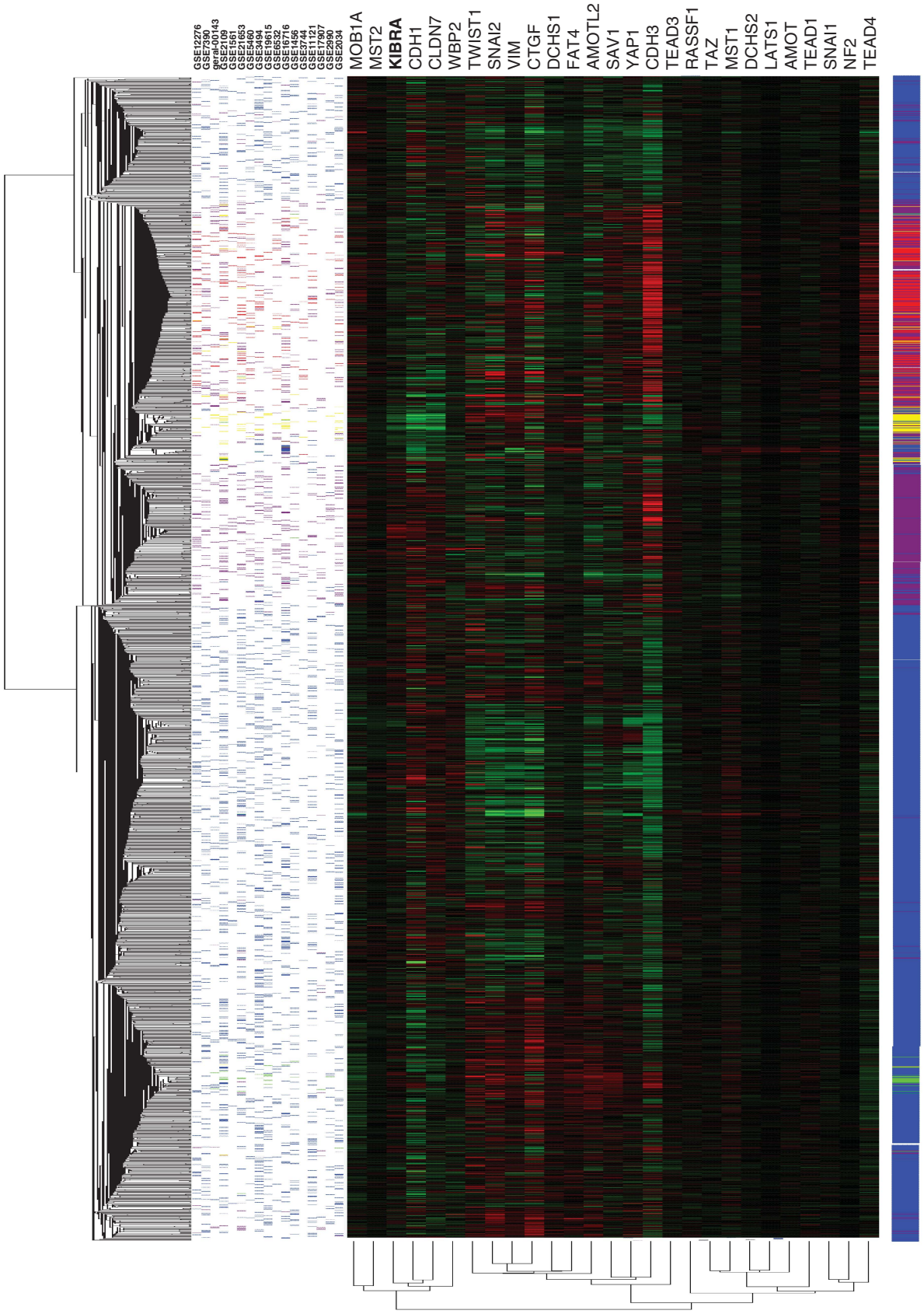


Figure 6. KIBRA expression is decreased in claudin-low primary breast cancers. Gene expression analysis data from 17 integrated Affymetrix datasets of primary breast tumors ($n = 2999$) using unsupervised clustering (average linkage, centred correlation) of the 500 most variable genes. The lines under the tree indicate the dataset (shown on right) from which each sample came and the colors indicate the intrinsic subtype assigned by highest correlation to the Prat *et al.*,[27] centroids (red = Basal, purple = HER2, blue = luminal, green = normal-like, yellow = Claudin-low).

PVDF transfer membrane (GE Healthcare, Amersham, UK). Protein expression was detected by specific antibodies. Primary antibodies: MST1/2, Phospho-MST1 (Thr183)/MST2 (Thr180), Phospho-MOBKL1A/B (Thr12), LATS1, Phospho-LATS1 (Ser909), YAP, Phospho-YAP (Ser127), (1:1000) (Cell Signaling Technology, Hitchin, UK), E-cadherin (1:500), N-cadherin (1:500), Vimentin (1:1000) (BD Biosciences, Oxford, UK), Occludin (1:1000) (Invitrogen), KIBRA (1:500) (Abcam, Cambridge, UK), Flag (M2) (1:1000), β-actin (1:10 000) (Sigma-Aldrich, Dorset, UK), MOBKL1A/B (Dr. Avruch). Secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit (1:10 000) (Stratech, Newmarket, UK). Protein expression was analyzed by Image J software and a student's unpaired *t*-test was performed to test significance.

Soft agar assay

Cells were seeded at 2×10^4 cells per well in 0.35% agarose/DMEM:F12 complete media onto a lower layer of 0.5% agarose/DMEM:F12 complete media. Once the agarose was set, a layer of DMEM:F12 complete media was added above the agarose layers. This media was replaced every other day and the number of colonies ($\geqslant 5$ cells represented a colony) in each well were counted after 21 days.

Migration assay

0.5 ml aliquots of serum-free cell suspension (5×10^5 cells) were added to the top chamber of 24-well chambers with 8.0 μm pores (BD Biosciences) and media supplemented with 20% serum was added to the lower chamber. After 24 h, the top of the insert membrane was scrubbed free of cells by using a cotton swab and PBS washes, and the bottom side was stained with 0.3% crystal violet (Sigma-Aldrich). The number of cells on the lower surface of each chamber was counted.

RNA extraction and quantitative real time PCR

Extraction of RNA from cell lysates was performed using peqGold MicroSpin Total RNA kit (PeqLab, Sarisbury Green, UK) followed by cDNA synthesis of 1 μg DNase-digested RNA, using First Strand cDNA Synthesis kit for RT–PCR (AMV) (Roche, Lewes, UK). The synthesized cDNA was analyzed by quantitative PCR using SYBR Green 2x Master Mix (Agilent, Wokingham, UK), Mx3005P machine (Agilent) and the following primers: PDGFβ, RASSF4, BMP2, FGF1. All measurements were performed in triplicate twice and standardized to the levels of β-actin. Sequence of the qPCR primers were as follows: CTGF: FW–GCAGAGCCGCCTGTGCATGG, RV–GGTATGTCTTCATGCTGGPDGFβ: FW–ACTTTGCGCGCACACACATACA-TAC, RV–TCACTCGCCGGCTACAGGCG; RASSF4: FW–GCACCCACAGGAAGCC-GACC, RV–GAAGCCGGGGACACTGTGGC; BMP2: FW–CGACCCTCGACCCCCG AGTC, RV–CACGCGGGGACACGTCCATT; FGF1: FW–AGCCCTCGGCCTACA AGCTCTTTA, RV– CACGCTTTCCGCACTGAGCTG.

Gene expression analysis

Raw.cel files from seventeen Affymetrix U133A/plus 2 primary breast tumour gene expression datasets were downloaded from NCBI GEO (GSE12276, GSE21653, GSE3744, GSE5460, GSE2109, GSE1561, GSE17907, GSE2990, GSE7390, GSE11121, GSE16716, GSE2034, GSE1456, GSE6532, GSE3494) or the caBIG (geral-00143) repositories, summarised with Ensembl alternative CDF[46] and normalised with RMA,[47] before integration using ComBat[48] to remove dataset-specific bias as previously described.[49] Similarly, expression data from three Affymetrix U133A/plus 2 breast cell line gene expression datasets were downloaded from ArrayExpress (E-TABM-194) and NCBI GEO (GSE10890, GSE12777) summarised with Ensembl alternative CDF[46] and normalised with RMA,[47] before integration using ComBat[48] to remove dataset-specific bias as previously described.[49] The intrinsic molecular subtypes were assigned based upon the highest correlation to the Prat *et al.*[27] centroids applied to each dataset separately. Centered average linkage clustering of the integrated tumor datasets was performed using the Cluster[50] and TreeView programs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We are very grateful to Dr Avruch for providing the MOBKL1A/B antibody and to Dr Takeichi for providing the Willin antibody. We thank V Fedele and E Campbell for technical assistance. We also thank the Scottish University Life Science Alliance for funding for SM and Breakthrough Breast Cancer for funding for AHS and DF. CJO was funded by The National Health and Medical Research Council of Australia, New South Wales Cancer Council, Cancer Institute New South Wales, Banque Nationale de Paris-Paribas Australia and New Zealand, RT Hall Trust, Australian Cancer Research Foundation and the National Breast Cancer Foundation.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)