The focal adhesion protein kindlin-2 controls mitotic spindle assembly by inhibiting histone deacetylase 6 and maintaining \(\alpha\)-tubulin acetylation

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ABSTRACT

Kindlins are focal adhesion proteins that regulate integrin activation and outside-in signaling. The kindlin family consists of three members, kindlin-1, -2, and -3. Kindlin-2 is widely expressed in multiple cell types, except those from the hematopoietic lineage. A previous study has reported that the Drosophila Fit1 protein (an ortholog of kindlin-2) prevents abnormal spindle assembly; however, the mechanism remains unknown. Here, we show that kindlin-2 maintains spindle integrity in mitotic human cells. The human neuroblastoma SH-SY5Y cell line expresses only kindlin-2, and we found that when SH-SY5Y cells are depleted of kindlin-2, they exhibit pronounced spindle abnormalities and delayed mitosis. Of note, acetylation of \(\alpha\)-tubulin, which maintains microtubule flexibility and stability, was diminished in the kindlin-2-depleted cells. Mechanistically, we found that kindlin-2 maintains \(\alpha\)-tubulin acetylation by inhibiting the microtubule-associated deacetylase histone deacetylase 6 (HDAC6) via a signaling pathway involving AKT Ser/Thr kinase (AKT)/glycogen synthase kinase 3 \(\beta\) (GSK3\(\beta\)) or paxillin. We also provide evidence that prolonged hypoxia down-regulates kindlin-2 expression, leading to spindle abnormalities not only in SH-SY5Y cell line but also cell lines derived from colon and breast tissues. The findings of our study highlight that kindlin-2 regulates mitotic spindle assembly and that this process is perturbed in cancer cells in a hypoxic environment.

Kindlins are essential 4.1-ezrin-radixin-moesin (FERM)-domain containing focal adhesion proteins in integrin-mediated cell adhesion (1). They promote integrin activation, clustering, and outside-in signaling (2-4). Although the kindlin family consists of three evolutionary-conserved members (kindlin-1, -2, -3) (5), they are functionally non-redundant. Loss of kindlin-1 gives rise to the skin blistering disease Kindler syndrome whereas loss of kindlin-3 leads to leukocyte adhesion deficiency III that is characterized by adhesion-defective immune cells and platelets. Kindlin-2 is expressed in multiple cell-types except hematopoietic cells (5). KINDLIN-2 gene ablation in mice and zebrafish is embryonic lethal (6,7). Loss of or diminished kindlin-2 expression leads to dysfunctional \(\beta\)1 and \(\beta\)3 integrins (6,8).

Kindlins contain F0, F1, F2, pleckstrin homology (PH), and F3 sub-domains (9). The F0 sub-domain promotes kindlin membrane localization (10,11). The F1 contains a lysine-rich
loop that binds negatively-charged lipids (12,13). The F2 is bisected by a PH domain, which binds to PIP2 and PIP3 (14-16). The F3 directly binds to the membrane distal highly conserved Nxx(Y/F) motif in the integrin β cytoplasmic tails (17,18). The F1, F2 and F3 sub-domains interact with each other in the clover-leaf-like conformation of kindlin-2 (19). Kindlin-2 regulates integrin avidity by removing integrin negative regulator and by clustering integrins. The former involves recruiting the LIM domain-containing protein migfilin by kindlin-2 to the integrin β cytoplasmic tail thereby displacing filamin A, a negative regulator of integrin activation because it prevents talin-integrin association (20-24). The latter involves kindlin-2 forming homodimers via its F2 sub-domain, which leads to integrin clustering (19).

The function of kindlin-2 is not limited to cell adhesion and migration. In endothelial cells, kindlin-2 interacts with clathrin heavy chain to regulate the expression of CD39 and CD73, which are involved in hemostasis (25). In breast cancer, kindlin-2 promotes metastasis by binding to and stabilizing EGFR (26), whereas in glioblastoma, kindlin-2 interacts with β-catenin and YB1 to enhance the expression of EGFR (27). Kindlin-2 also forms a ternary complex with β-catenin and TCF that enhances Wnt signaling in cancer cells (28).

In a genome-wide RNAi screen of Drosophila S2 cells, Fit1 (an ortholog of kindlin-2) was identified to play a role in preventing abnormal spindle assembly (29). The mechanism however remains unknown. We note that the other kindlin family member, kindlin-1, has been reported to interact directly with histone deacetylase 6 (HDAC6) to regulate α-tubulin acetylation affecting mitotic spindle assembly (30,31). However, kindlin-1 is expressed primarily in epithelial cells thus precluding its role as a regulator of mitotic spindle assembly in other cell types (5). Whether kindlin-2, which is widely expressed, regulates HDAC6 and α-tubulin acetylation thereby affecting mitotic spindle assembly warrants investigation.

Here, we show for the first time that kindlin-2 regulates mitotic spindle assembly by inhibiting HDAC6 activity via signaling pathways involving AKT, GSK3β and paxillin, thereby modulating α-tubulin acetylation in mitotic spindles. Further, this regulatory mechanism is perturbed in cancer cells under hypoxic stress as a consequence of kindlin-2 down-regulation.

**Results**

**Kindlin-2 depleted neuroblastoma SH-SY5Y cells exhibited delayed mitosis and spindle abnormalities**

To examine the role of kindlin-2 in maintaining mitotic spindle integrity, we used lentiviral-based shRNA transduction system to generate kindlin-2 depleted neuroblastoma SH-SY5Y stable cell line, which was verified by immunoblotting and qRT-PCR (Fig. 1A). We chose SH-SY5Y cell line because it only expressed kindlin-2. Depletion of kindlin-2 did not cause any up-regulation of kindlin-1 or kindlin-3 (Fig. 1B). Positive controls DLD-1 (colorectal adenocarcinoma cell line) and K562 (chronic myelogenous leukemia cell line) were included for kindlin-1 and kindlin-3, respectively. Hence, these data exclude functional compensation by other kindlins in kindlin-2-depleted SH-SY5Y cells in subsequent assays. In line with its role as a positive regulator of integrin function, cells depleted of kindlin-2 were defective in adhesion on fibronectin based on electric cell-substrate impedance sensing assay (Fig. 1C). We also transfected kindlin-2 depleted cells with a shRNA-resistant HA-tagged kindlin-2 WT plasmid to generate another stable cell line (henceforth referred to as HA-kindlin-2 (WT)-rescued cells). HA-kindlin-2 (WT)-rescued cells did not exhibit adhesion defect compared with WT and control shRNA cells (Fig. 1C). To determine cell proliferation, we performed tumor spheroid assays. Kindlin-2-depleted cells but not HA-kindlin-2 (WT)-rescued cells formed smaller tumor spheroids compared with wild-type (WT) or control shRNA cells (Fig. 1D), suggesting that kindlin-2 is important in cell proliferation.

We then performed cell cycle synchronization and determined the time taken for these cells to transit from G2/M phase to cytokinesis by live-cell imaging (Fig. 1E). Kindlin-2 depleted but not HA-kindlin-2 (WT)-rescued cells took a significantly longer time to transit from G2/M phase to cytokinesis compared with WT and control shRNA cells, suggesting a delay in mitosis upon kindlin-2 depletion. We ruled out shRNA off-target effects because the delay in mitosis was also
observed in another SH-SY5Y stable cell line expressing a different kindlin-2 targeting shRNA sequence (Supplementary Fig. 1A & B).

Mitotic spindles are microtubules (MT) formed by α- and β-tubulins. Hence we performed DAPI- and immunofluorescence (IF)-staining of α-tubulin to visualize the spindle assembly in all groups of mitotic cells. Kindlin-2 depleted but not HA-kindlin-2 (WT)-rescued cells exhibited multipolar spindles, abnormal bipolar spindles, and chromosome misalignment (collectively referred to as spindle abnormalities) when compared with WT and control shRNA cells (Fig. 1F & G), suggesting that kindlin-2 is important in mitotic spindle assembly. Defect in mitotic spindle assembly was also observed in another stable cell line expressing a different kindlin-2 targeting shRNA sequence (Supplementary Fig. 1C).

**Kindlin-2 depletion diminishes α-tubulin acetylation in mitotic spindles**

Tubulin acetylation provides mechanical stability to MT, which regulates intracellular trafficking, cell motility and cell cycle (32-34). In the early stages of mitosis, spindle MT contains a high level of acetylated Lys40 in α-tubulin (Ac-tubulin) (34-36). Acetylation of α-tubulin has been shown to increase the stability of bent microtubules (37,38). Ac-tubulin is a substrate of HDAC6, an enzyme that de-acetylates nuclear and cytoplasmic proteins (39). Tubacin is a selective inhibitor of HDAC6 (40). Tubacin has a hydroxamate group that chelates Zn$^{2+}$ ion of the catalytic centers in HDACs, and it has a 1,3-dioxane group that accounts for its selectivity for HDAC6 (41,42).

Hence we performed IF staining of Ac-tubulin and total α-tubulin in mitotic cells without or with tubacin treatment (Fig. 2A). Quantitative analysis of the fluorescence intensity ratio of Ac-tubulin to total α-tubulin of the mitotic spindles in each cell was determined (Fig. 2B). In the absence of tubacin treatment, a significantly low level of Ac-tubulin was detected in the mitotic spindles of kindlin-2-depleted but not HA-kindlin-2 (WT)-rescued cells compared with that of WT and shRNA control cells. The diminished level of Ac-tubulin in mitotic spindles was also detected in another stable cell line expressing a different kindlin-2-targeting shRNA sequence (Supplementary Fig. 1D), thus excluding shRNA off-target effect. In the presence of tubacin, there was an increase in the level of Ac-tubulin in mitotic spindles of all cells compared with untreated cells (Fig. 2B). Importantly, tubacin treatment restored the level of Ac-tubulin in mitotic spindles of kindlin-2-depleted cells to that comparable with other groups of treated-cells. Together, these data suggest that depleting kindlin-2 in cells leads to an increase in HDAC6 activity. Given that cells depleted of kindlin-2 exhibited delayed transit from G2/M to cytokinesis, we also examined the effect of tubacin on these cells. Tubacin treatment effectively recovered the G2/M to cytokinesis delay in kindlin-2-depleted cells (Fig. 2C). These data provide further evidence supporting a role of kindlin-2 in modulating acetylation of α-tubulin in mitotic spindles by inhibiting the activity of HDAC6.

Unattached kinetochore delays anaphase onset (43). The spindle assembly checkpoint (SAC) network is activated even when a single kinetochore is not attached to the spindle. Mitotic arrest deficiency protein 2 (MAD2), a key component of the SAC (44), is recruited to unattached kinetochores and it inhibits APC/C$^{Cdct20}$ activity that is required for entry into anaphase (45). We therefore performed IF staining of MAD2 together with an anti-centromere antibody (ACA) in all groups of mitotic cells. In line with spindle abnormalities observed in a high percentage of mitotic kindlin-2-depleted cells, MAD2 was readily detected at the centromere of non-aligned chromosomes in these cells but not HA-kindlin-2 (WT)-rescued cells (Fig. 2D).

**Kindlin-2 regulates HDAC6 activity via the PI3K/AKT (PKB)/GSK3β and paxillin signaling pathways**

Kindlin-1 localizes to centrioles in mitotic cells and it directly interacts with HDAC6 to regulate mitotic spindle MT acetylation (30). Hence we performed IF staining of kindlin-2 in both interphase and metaphase cells using two antibodies from different sources. Like focal adhesion protein paxillin, kindlin-2 was readily detected at focal adhesions in interphase cells on fibronectin (Supplementary Fig. 2A & B). However, its localization at centrosomes containing γ-tubulin in metaphase cells was not evident (Supplementary Fig. 2C & D). This suggests the possibility of a different mechanism by which kindlin-2 regulates mitotic spindle MT acetylation.
It has been shown that spindle abnormalities and mitotic arrest in kindlin-1-depleted cells is due to an increase in HDAC6 activity (30). The PI3K/AKT (PKB)/GSK3β signaling axis is important in mitotic spindle assembly in which AKT and GSK3β are detected at the centrosome (46). AKT inhibits GSK3β and prevents GSK3β from activating HDAC6 (47). Given that kindlin-2 is involved in integrin-mediated activation of AKT (48-50), we conjectured that HDAC6 activity will be elevated in kindlin-2-depleted cells. We first examined active AKT and GSK3β levels in G2/M synchronized cells on fibronectin without or treated with integrin-activating manganese by immunoblotting (Fig. 3A). Under both conditions, active AKT level was significantly lower in cells depleted of kindlin-2 compared with that in other groups of cells. By contrast, the opposite trend was observed for GSK3β. We then determined the activity of HDAC6 in these cells using a fluorometric enzyme assay (Fig. 3B). An increase in HDAC6 activity was detected in kindlin-2-depleted cells relative to other groups of cells. Consistent with these observations, Ac-tubulin level was also lower in kindlin-2-depleted cells (Fig. 3A). Kindlin-2 promotes integrin activation and outside-in signaling. Manganese directly activates integrins, bypassing the need for kindlin-2 in integrin activation. The similar activity profiles of AKT and GSK3β in both untreated and manganese-treated kindlin-2-depleted cells lend support to the regulation of HDAC6 by integrin outside-in signaling involving kindlin-2/AKT/GSK3β pathway. Because kindlin-2 mediates integrin outside-in signaling that regulates AKT activity, we asked whether inhibiting AKT activity can cause mitotic spindle defects. Indeed, WT SH-SY5Y cells treated with SH6, a phosphatidylinositol analog inhibitor of AKT (51), exhibited a significant increase in spindle abnormalities compared with untreated cells (Fig. 3A). Interestingly, paxillin has been reported to inhibit HDAC6 thereby regulating MT acetylation although direct interaction between them remains to be verified (52). Given that kindlin-2 recruits paxillin to nascent adhesion sites (50), we performed co-immunoprecipitation assay using 293T cells overexpressing HA-tagged kindlin-2 and mCherry-paxillin (Fig. 3D). Both mCherry-paxillin and endogenous HDAC6 were detected in the co-precipitate of HA-kindlin-2 (left panel). Both HDAC6 and HA-kindlin-2 were also detected in the co-precipitate of mCherry-paxillin (right panel). We extended the study to examine whether depletion of paxillin leads to spindle abnormalities in SH-SY5Y cells. We generated three stable cell lines expressing control shRNA and two paxillin-targeting shRNAs (different sequences) (Fig. 3E). Depletion of paxillin resulted in a significant increase in spindle abnormalities detected. Taken together, these data suggest that an alternative signaling axis that regulates MT acetylation can be one that involves integrin, kindlin-2, paxillin and HDAC6. Both signaling pathways need not be mutually exclusive.

To further verify the regulatory role of kindlin-2 in mitotic spindle MT acetylation, in addition to the HA-kindlin-2 (WT)-rescued cells, we generated stable cell lines using kindlin-2-depleted cells re-expressing shRNA-resistant HA-kindlin-2 integrin-binding defective (Q614W615/AA) mutant (17,53) or paxillin-binding defective PH-deleted mutant (50). Western blot analyses showed that the rescued expression levels of WT, Q614W615/AA and PH-deleted HA-kindlin-2 were comparable (Fig. 3F). Expression of HA-kindlin-2 (WT) significantly decreased the percentage of metaphase cells with spindle abnormalities detected but expression of HA-kindlin-2 mutants did not (Fig. 3G). To validate that the kindlin-2 expression is relatively even across the population of cells analyzed, we performed immunofluorescence staining of metaphase cells (Supplementary Fig. 3A). We examined the image of each cell using the image-J software to determine its kindlin-2 fluorescence intensity and its area. The ratio of kindlin-2 fluorescence intensity over cell area (in arbitrary units) was calculated and plotted. Expression of HA-kindlin-2 (WT) in the rescued cells was comparable, and the expression of HA-kindlin-2 (WT) but not its mutants significantly decreased the percentage of metaphase cells with spindle abnormalities (Supplementary Fig. 3B). Expression of HA-kindlin-2 (WT), but not its mutants, also rescued mitotic spindle MT acetylation (Fig. 3H), and fully recovered the delayed transit from G2/M to cytokinesis (Fig. 3I). Taken together, these data lend additional support to the regulatory role of
kindlin-2 in mitotic spindle MT acetylation and the maintenance of spindle integrity.

**Hypoxia down-regulates kindlin-2 expression which affects mitotic spindle assembly**

Our analyses thus far showed that kindlin-2 plays a role in mitotic spindle assembly by, in part, regulating mitotic spindle MT acetylation. Our assays were based on comparing cells with or without kindlin-2 depletion using the shRNA method. We then asked whether kindlin-2 expression is down-regulated under specific physiological conditions, leading to mitotic spindle anomalies. Hypoxia alters the cell cycle and perturbs normal mitotic spindle assembly (54-56). Hypoxia is a hallmark of solid tumors, and it is well documented to affect gene expression in cancer cells (57). Thus we examined the effect of hypoxia on SH-SY5Y cells in culture. Hypoxia reduced cell proliferation over time (Fig. 4A), and there was a significantly higher number of mitotic cells with spindle abnormalities compared with cells under normoxia (Fig. 4B). Mitotic spindle MT acetylation was also significantly reduced in cells under hypoxia compared with those under normoxia (Fig. 4C). Notably, the expression level of kindlin-2 transcript and protein decreased over time in cells under hypoxia (Fig. 4D & E) whereas the opposite trend was detected for HIF-1α and VEGFA, (Fig. 4F & 4G left panel), which are in line with hypoxia-mediated stabilization of HIF-1α and induction of VEGFA (57).

Next we determined the expression of microRNA (miR)-138 in these cells because miR-138 has been demonstrated to target the 3'-UTR of kindlin-2 transcript, leading to the down-regulation of kindlin-2 expression and β1 integrin signaling in prostate cancer cells (58). miR-138 expression was significantly up-regulated in cells under hypoxia over time (Fig. 4G right panel). This is concordant with changes in expression profile of miRNA, including miR-138, in cells under low oxygen tension (59). To further demonstrate that hypoxia induced miR-138 down-regulates kindlin-2 that leads to mitotic spindle abnormalities, we first generated a stable SH-SY5Y cell line expressing GFP-kindlin-2 that lacks the 3'UTR-targeting site of miR-138. These cells were then subjected to normoxia or hypoxia culture conditions. Over-expression of GFP-kindlin-2 effectively reduced the number of cells with mitotic spindle abnormalities under hypoxia (Fig. 4H), lending support to its role in maintaining normal mitotic spindle assembly. Taken together, these data suggest that hypoxia-induced miR-138 down-regulates kindlin-2, which could in part account for the spindle abnormalities observed in mitotic cells under hypoxia.

To rule out cell-type-specific effects, we also tested the effects of hypoxia on four additional cell lines: HCT116 (colorectal cancer), MDA-MB-231 (breast cancer), CCD841 CoN (colon epithelial, non-tumorigenic origin), and MCF10A (breast epithelial, non-tumorigenic origin). Similar to SH-SY5Y, kindlin-2 expression was reduced in these cell lines under hypoxia together with a concomitant increase in mitotic spindle abnormalities detected (Fig. 4I & Supplementary Fig. 4A). To further verify that kindlin-2 plays a role in regulating mitotic spindle assembly in these cells, kindlin-2 depletion was performed by using the lentiviral-based shRNA transduction system described in the earlier section. For HCT-116 and MDA-MB231, stable cell lines with kindlin-2 depletion were generated. For CCD841 CoN and MCF10A, transiently transduced cells were analyzed instead because of high rate of cell death under prolonged antibiotic selection for stable lines, and CCD841 CoN is very slow-growing. Nevertheless, in all four cell lines, an increase in mitotic spindle abnormalities was detected as a result of kindlin-2 depletion compared with control shRNA and WT cells (Fig. 4J & Supplementary Fig. 4B).

3D culture of cancer cells mimics better the tumor microenvironment compared with that of 2D culture (60-63). Thus we generated stable SH-SY5Y cells carrying the 5HRE-GFP plasmid in which GFP expression is regulated by five copies of human VEGF-hypoxia-responsive element (64). We used these cells for *in vitro* culture of tumor spheroids and isolated cells under hypoxic stress by flow cytometry cell-sorting based on GFP signal (Fig. 5A). Kindlin-2 expression was significantly lower in GFP<sup>hi</sup> cells compared with that of GFP<sup>lo</sup> cells based on immunoblotting (Fig. 5A) and qRT-PCR (Fig. 5B). By contrast, both miR-138 and VEGFA transcripts were up-regulated in GFP<sup>hi</sup> cells compared with that of GFP<sup>lo</sup> cells (Fig. 5B). To further validate the effect of hypoxia on kindlin-2 expression *in vivo*, we performed subcutaneous xeno-graft of the 5HRE-GFP cells in immune-
compromised Rag-/-/IL2R-/- mice. Tumors were excised and cryo-sectioned followed by IF staining and imaging (Fig. 5C). Hypoxic regions, as indicated by GFP expression, were detected in the tumor sections, and expression of kindlin-2 in these regions was lower than that in neighboring non-hypoxic regions based on fluorescence intensity analysis.

Discussion

Kindlins are well-established positive regulators of integrin-mediated cell adhesion and migration. Kindlin-2 ortholog Fit1 has previously been identified to maintain proper spindle assembly in Drosophila S2 cells but the mechanism is unknown (29). Here we show that kindlin-2 mediates integrin outside-in signaling that activates AKT (Fig. 5D). Active AKT then inhibits GSK3β, preventing GSK3β from activating HDAC6. In turn this prevents deacetylation of α-tubulin, leading to stable spindle MT assembly in mitotic cells. In parallel, kindlin-2 may recruit paxillin that directly inhibits HDAC6. Our findings also indicate that hypoxia up-regulates miR-138 that suppresses kindlin-2 expression, leading to mitotic spindle abnormalities.

Cell rounding is a hallmark of most cell types undergoing mitosis. Thus it appears intriguing that kindlin-2-mediated integrin signaling regulates spindle assembly in cells undergoing mitosis, which is known to disassemble focal adhesions. However, the contact between cells and ECM before entry into mitosis have been shown to be crucial for spindle positioning (65,66). Growth of MT from centrosome and bipolar spindle formation are also regulated by integrins (67). A recent detailed analysis on cell-substrate remodeling during mitosis revealed the presence of punctate active β1 integrin contacts that are maintained between the mitotic cell and its ECM substrate (68). Therefore the signaling axis involving integrin, kindlin-2, AKT, GSK3β, paxillin and HDAC6 remains relevant in cells undergoing mitosis.

Kindlin-2 is not the only kindlin that regulates mitotic spindle assembly. In an early study by Patel et al. (31), it was reported that mitotic spindle formation is regulated by kindlin-1, which is phosphorylated by Polo-like kinase 1 (Plk-1), a serine/threonine kinase that regulates mitotic checkpoints (69). Another key observation in their study is that kindlin-1 localizes to the centrosome. This may explain how kindlin-1 is phosphorylated by Plk-1, which is also found at the centrosome and mitotic spindle poles. In our analyses, we were unable to detect endogenous kindlin-2 at centrosomes of mitotic cells although kindlin-2 was readily detected at focal adhesions in interphase cells. Whether this is due to the level of sensitivity of detection remains to be explored. More studies are therefore needed to determine whether Plk-1 interacts and regulates kindlin-2 function. Recently, Patel et al., also reported that kindlin-1 directly interacts with histone deacetylase 6 (HDAC6) to regulate α-tubulin acetylation in mitotic spindles (30). In line with this, our data suggest that kindlin-2 inhibits HDAC6 activity, which affects mitotic spindle α-tubulin acetylation. However, this regulation occurs via signaling pathways involving AKT, GSK3β, and paxillin.

Finally, another important finding from this study is that kindlin-2 expression is down-regulated in cells under hypoxic stress as a result of elevated level of miR-138. Interestingly, cells under hypoxia exhibited pronounced mitotic spindle abnormalities that was effectively prevented by over-expressing kindlin-2 in 2D culture. The down-regulation of kindlin-2 expression was also observed in 3D tumor spheroid and in vivo tumor although we are technically limited in terms of specifically isolating G2/M phase cells from the tumors to examine mitotic spindle integrity and α-tubulin acetylation. Nevertheless, our data collectively provide mechanistic insights into the regulation of mitotic spindle assembly by kindlin-2, which could be perturbed in cancer cells in a hypoxic tumor microenvironment.

Experimental Procedures

Chemicals and reagents

All general chemicals and reagents were purchased from Bio-Rad Laboratories (Hercules, CA) and Merck (Sigma-Aldrich) (Darmstadt, Germany) unless stated otherwise.

Cells and culture conditions

The human neuroblastoma SH-SY5Y cell line was kindly provided by Dr. Esther Siew-Peng Wong (School of Biological Sciences, Nanyang Technological University, Singapore). The cell lines MDA-MB-231 (human breast cancer), HEK-293T (human embryonic kidney) and HCT 116
(human colorectal cancer) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). SH-SY5Y, HEK-293T and MDA-MB-231 cells were maintained in DMEM medium (Hyclone, Logan, UT) and HCT 116 cells were maintained in McCoy’s 5A (modified) medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HI-FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Nacalai Tesque, Kyoto, Japan). The cell line CCD841 CoN (human colon, non-tumorigenic origin) (ATCC) was kindly provided by Dr. Karen Crasta (Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore), and maintained in MEM medium (Gibco) containing supplements aforementioned. The cell line MCF10A (breast epithelial, non-tumorigenic origin) (ATCC) was kindly provided by Dr. Andrew Tan Nguan Soon (Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore), and maintained in DMEM F12 medium (Gibco) supplemented with 5% (v/v) heat-inactivated equine serum (Gibco), epidermal growth factor (0.02 μg/mL) (Peprotech, Israel), insulin (10 μg/mL) (Sigma), cholera toxin (0.1 μg/mL) (Sigma), penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Generation of stable cell lines

Stable expression of shRNA targeting kindlin-2 in SH-SY5Y cells was generated by using a lentiviral-based shRNA transduction system. Five GFP-C-shLenti plasmids were generated in which four plasmids contained different kindlin-2-targeting shRNA sequences (referred to as K2A, K2B, K2C and K2D) and one plasmid contained a scrambled negative control sequence (referred to as ctrl) (OriGene Technologies, Rockville, MD). Each of these plasmids was transfected together with third-generation lentiviral packaging plasmids (Applied Biological Materials) into HEK-293T cells and the culture supernatant that contained pseudo-virions was harvested. SH-SY5Y cells (2 x 10⁵) were cultured in 1 mL of fresh medium supplemented with 1 mL of virion-containing supernatant and 6 μg/mL Polybrene (Sigma-Aldrich, St.Louis, MO). 24 h post transduction, cells were sorted based on GFP signal on a FACS Aria flow cytometer (BD Biosciences, Mountain View, CA) and maintained in full medium containing 1.5 μg/mL puromycin (Thermo Fisher Scientific, Waltham, MA). The expression of kindlin-2 in these cells was determined by immunoblotting and qRT-PCR. Cells expressing the kindlin-2-targeting sequence (K2B) 5′-ACAACAGTGAAGAAATGGTGAAGTTGAT GAAGTT-3′ showed the highest efficacy in silencing the expression of kindlin-2. These cells were used in subsequent experiments. Another SH-SY5Y stable cell line expressing K2A shRNA with the targeting sequence 5′-GAAGAAGCTTATTTGG AATTGCATACAACAG-3′ was also used in verification studies (Supplementary Fig.1).

Prior to the rescue experiments, SH-SY5Y K2B cells were sub-cloned. The expression of kindlin-2 in these clones was screened and verified by immunoblotting. One clone (referred to as K2B clone 4) was selected and expanded in culture. To re-express kindlin-2 in K2B clone 4, transfection was performed by incubating 1.5 x 10⁵ cells in 250 μL serum-free DMEM medium containing 2.5 μg shRNA-resistant HA-kindlin-2 (WT; Q614W615/AA or PH-deleted) plasmid (zeocin resistance gene) and 7.5 μL TransIT-X2® reagent (Mirus Bio LLC, Madison, Wisconsin) according to manufacturer’s instructions. Cells with stable expression of kindlin-2 were selected in full culture medium containing 700 μg/mL Zeocin™ (Thermo Fisher Scientific).

The stable SH-SY5Y cell line that expressed GFP under hypoxia was generated by transfecting 2 x 10⁵ wild-type SH-SY5Y cells with 3 μg 5HRE-GFP plasmid (neomycin resistance gene) (a gift from Dr. Martin Brown and Dr. Thomas Foster, Stanford University School of Medicine, Stanford, CA (64), Addgene plasmid #46926) in 250 μL serum-free DMEM medium containing 3 μL of Lipofectamine 2000® according to manufacturer’s instructions. Cells were maintained in full culture medium containing 900 μg/mL G418-Sulphate (PAA Laboratories).

To generate stable paxillin-depleted cells, psi-H1 vector set (eGFP reporter, puromycin selection marker) containing paxillin-targeting shRNA (4 different targeting sequences) or control sequence was purchased from Genecopoeia, Rockville, MD. Cells were transfected with 2.5 μg of plasmid each using Lipofectamine 2000® aforementioned followed by selection in medium containing 1.5 μg/mL puromycin. Efficient silencing of paxillin expression in cells expressing paxillin- shRNA (targeting sequence A) (5′-
GGAGAGTCTCTTGGATGAACT-3') or (targeting sequence B) (5'-GCAACCTTTCTGAA CTGACC-3') compared with cells expressing control shRNA, and they were used in subsequent experiments.

Stable kindlin-2-depleted HCT 116 and MDA-MB-231 cells were generated by using the GFP-lentiviral-based shRNA transduction system (as described above for SH-SY5Y cells) followed by selection in full medium containing 1.5 μg/mL puromycin. The same lentiviral system was used to transduce CCD841 CoN and MCF-10A but stable cell lines were not generated due to high % of cell death after prolonged exposure to puromycin selection. Instead, these cells were used immediately for subsequent experiments 2 days after transduction.

Expression plasmids
HA-tagged full length human kindlin-2 (isoform 2) in pcDNA3.1(zeo) (3,70) was used to generate shRNA-resistant HA-kindlin-2 expression plasmid. Silent mutations were made at the third base of each codon found in the kindlin-2 cDNA sequence that is the target of kindlin-2 shRNA K2B using the QuikChange™ site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and relevant primers. This K2B shRNA resistant HA-kindlin-2 expression plasmid was then used to generate the two mutants: the integrin-binding defective Q614W615/AA HA-kindlin-2 mutant (17,53) (in this case Q621W622/AA in the kindlin-2 isoform 2) by performing additional site-directed mutagenesis using relevant primers, and the paxillin-binding defective PH-deleted HA-kindlin-2 mutant using overlapping PCR with relevant primers to remove the region Pro350-Gln480.

Quantitative RT-PCR
TRIzol (Invitrogen) was used to extract total RNA from cells. Reverse transcription was performed using the Power SYBR® Green Cells-to-Ct™ kit (Thermo Fisher Scientific). Quantitative RT-PCR was performed using the KAPA SYBR Fast qPCR Master Mix Universal Kit (KAPA Biosysyms, Wobum, MA) with relevant forward and reverse primers on a CFX96TM Real-time PCR detection system (Bio-RAD, Hercules, CA) at 95 °C for 10 min, 95 °C for 30 sec, 60 °C for 30 sec and repeated from step 2 for 40 cycles. Primers used are as follows: VEGFA (forward) 5’-GAAGTGCTGAA GTTCATGGATCCTCA-3’, (reverse) 5’-TGGAA GATGTCCACCAGGGT-3’; kindlin-2 (forward) 5’-GAACAGCATATAACGGAGA-3’, (reverse) 5’-TGGAACTCTGCAAATGAGTG 3’; miR-138-5p (forward) 5’-GGCAGCTGTGTT GTGAATC-3’, (reverse) 5’-CTAAGTTGTTGG TCGACCGC-3’; 18s RNA (forward) 5’-GTAACCCGTTGAAACCCATT-3’, (reverse) 5’- CACCTCAATCGGTAGTAGCC-3’; U6 (forward) 5’-CTCGCTTGCGACGACATA-3’, (reverse) 5’-AACGCTTCAGAATTTGGC-3’.

Tumor spheroid culture
Cells (8 x 10^3) in 100 μL full culture medium were seeded into each well of a 96-well culture plate that was pre-coated with 1 % (w/v) autoclaved agarose (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) followed by centrifugation at 500 x g for 5 min. Cells were maintained under standard culture conditions for 7 days. To determine the size of the spheroids, they were visualized under a microscope with a 4X objective and phase contrast (Olympus IX83 microscope, Japan). Images were taken and analyzed using the CellSens Dimension software.

Hypoxia treatment
For comparison between the effects of normoxia versus hypoxia on cells after 24 h or 48 h, the seeding density for cells was 2 x 10^6 cells per 100 mm cell culture dish. To make comparison after 96 h, the seeding density for cells was reduced to 4 x 10^5 cells per 100 mm cell culture dish to prevent overgrowth of cells. For each time point, cells were seeded into two cell culture dishes. The medium was replaced with fresh medium after 24 h. One dish of cells was maintained under standard culture conditions (normoxia) whereas another dish of cells was placed in a custom-built hypoxia chamber. The chamber was connected to a flow meter (Dwyer Instruments Inc., Michigan city, IN) and filled with a gas mixture of 95% N2 and 5% CO2, and the level of O2 measured using a gas detector (Industrial Scientific, Pittsburgh, PA). The chamber was then transferred to a 37°C incubator. The chamber was flushed twice with the gas mixture on the same day.

Fluorescence-activated cell sorting (FACS)
SH-SY5Y 5HRE-GFP tumor spheroids were cultured as described in the previous section. The spheroids were washed in PBS, trypsinized, and resuspended in PBS containing 20% (v/v) HI-FBS. Cells were passed through an appropriate size filter to remove clumps and sorted by FACS based on GFP signal on a FACSAria flow cytometer (BD
Biosciences, Mountain View, CA). Cells sorted into GFP<sup>lo</sup> and GFP<sup>hi</sup> groups were used for further analyses. Flow cytometry data are presented using the FlowJo software. (Tree Star Inc., Ashland, OR).

**Cell proliferation assay**

Cell proliferation and viability was determined using the ADAM-MCT™ Automatic Cell Counter and viability kit (NanoEnTek, Inc., Korea).

**Electric cell-substrate impedance sensing (ECIS) measurement**

ECIS measurement was performed as described previously (3). In brief, 8 x 10<sup>4</sup> cells were seeded into each well of a 16-well E-plate device (ACEA Biosciences, San Diego, CA) that was pre-coated with 2.5 μg/cm<sup>2</sup> human fibronectin (Merck-Sigma-Aldrich). AC impedance measurements were taken at 5 min intervals on a Real-Time Cell Electronic System (ACEA Biosciences) placed in a cell culture incubator. AC impedance is expressed as arbitrary units termed cell index. This value correlates with the magnitude of cell adhesion and spreading. Each datum point represents mean (± S.D.) of technical triplicates.

**Immunoblotting**

Cells were lysed in lysis buffer (150 mM NaCl, 1% (v/v) Igepal and 10 mM Tris, pH 7.4) containing inhibitors of proteases (Roche, Basel, Switzerland) and phosphatases (Nacalai Tesque, Nakagyo-ku, Kyoto). Protein concentration was measured using the bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA). Proteins were resolved by SDS-PAGE under reducing conditions and electro-transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). PVDF membrane was incubated in TBST blocking buffer (150 mM NaCl, 0.1% (v/v) Tween-20, 25 mM Tris, pH 8.0) containing 5% (w/v) non-fat milk at room temperature (RT) for 30 min. For phosphorylated protein detection, TBST containing 5% (w/v) BSA was used instead. The membrane was then incubated in blocking buffer containing the primary antibody at 4 °C overnight. The following primary antibodies from Cell Signaling Technology (Danvers, MA) were used: rabbit-anti-kindlin-2 (D1K4C) (1:2000 dilution), mouse anti-HA tag (1:1000 dilution), rabbit anti-phosphorylated AKT (Ser473) (1:1000 dilution), rabbit anti-AKT (1:1000 dilution), rabbit anti-phosphorylated GSK3β(Ser9)(5B3) (1:1000 dilution), rabbit anti-GSK3β (27C10)(1:1000 dilution), and rabbit anti-HIF-1α (D1S7W) (1:1000 dilution). Other primary antibody used are as follows: rabbit-anti-kindlin-2 (1:1000 dilution, Sigma-Aldrich), rat anti-human/mouse kindlin-3 clone 9 mAb (3) (1:1000 dilution), mouse anti-actin Ab-5 (1:1000 dilution, BD Transduction Laboratories, San Jose, CA), mouse anti-acetylated tubulin (1:1000 dilution, Sigma-Aldrich), rabbit anti-α tubulin (1:1000 dilution, Thermo Fisher Scientific), mouse anti-β tubulin (1:2500 dilution, Sigma-Aldrich), mouse anti-paxillin (clone 5H11) (1 μg/mL, Merck, Darmstadt, Germany), and rabbit anti-GAPDH (1:2000 dilution, Cell Signaling Technology).

After incubation with the primary antibody, the membrane was washed three times in TBST buffer and incubated with appropriate HRP-conjugated secondary antibody at RT for 1 h. The following secondary antibodies were used: HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG antibodies were from Advansta (Menlo Park, CA), HRP-conjugated goat anti-rat IgG antibody (GE Healthcare, UK). The membrane was washed three times in TBST buffer followed by ECL detection using the WesternBright ECL kit (Advansta). Images were captured on a ChemiDoc™ Touch imaging system (Bio-Rad, Hercules, CA).

**Immunoprecipitation assay**

Immunoprecipitation assay was performed as previously described with modifications (70). 3 x 10<sup>6</sup> 293T cells were seeded into a 10 cm culture dish. The following day, cells were co-transfected with expression plasmids HA-kindlin-2 (WT) pcDNA3.1(zeo) (30 µg) and pmCherry-paxillin (a gift from Kenneth Yamada; Addgene plasmid #50526; http://n2t.net/addgene:50526; RRID: Addgene_50526) (10 µg) using polyethylenimine (PEI) (30 µg) (Sigma-Aldrich)-based method. Cells were harvested by trypsinization 24 h post-transfection, re-suspended in full culture medium containing 5 mM MnCl<sub>2</sub>, and seeded on fibronectin-coated 10 cm culture dish followed by 3 h incubation under culture conditions. Cells were harvested and lysed in lysis buffer (150 mM NaCl, 1% (v/v) Igepal and 10 mM Tris, pH 7.4) containing protease inhibitor cocktail (Roche, Basel, Switzerland) on ice for 30 min. The lysate was pre-cleared with 3 µg mouse serum IgG (Sigma-Aldrich) and protein A-Sepharose beads (GE Healthcare, Piscataway, NJ) for 30 min at 4°C on a roller. The pre-cleared lysate was separated
into two equal portions. For HA-kindlin-2 immunoprecipitation experiment, 3 μg mouse serum IgG was added to one portion and 3 μg mouse anti-HA antibody (Merck, Darmstadt, Germany). For paxillin immunoprecipitation experiment, 4 μg mouse serum IgG and 4 μg of mouse anti-paxillin antibody (clone 5H11) were added to the two portions of lysate, respectively. Protein A-Sepharose beads were added to each portion and the samples were incubated for 3 h at 4°C on a roller. Beads were recovered by centrifugation followed by washing three times in lysis buffer. Precipitated proteins were eluted by boiling beads in Laemmli buffer containing DTT followed by SDS-PAGE and immunoblotting.

Synchronization of cells

Cells were seeded in a culture dish pre-coated with fibronectin (2.5 μg/cm²). Thymidine (Sigma-Aldrich) was added to a final concentration of 2 mM followed by incubation for 14 h under standard culture conditions. The medium was discarded and cells were washed in PBS. Deoxycytidine (Sigma-Aldrich) was added to the culture at a final concentration of 24 μM followed by incubation for 9 h under standard culture conditions. Thymidine was then added to a final concentration of 2 mM followed by another 14 h of incubation. Cells were washed twice in PBS and the medium replaced with fresh medium containing 24 μM deoxycytidine followed by 2 h of incubation. Cells were then washed twice in PBS before adding fresh medium containing 10 μM RO3306 (Sigma Aldrich) followed by 19 h of incubation. Cells were released from the effect of RO3306 by washing twice in PBS and adding fresh medium to the cells. Cells were immediately used for live-cell imaging or incubated for another 90 min under standard culture conditions before immunoblotting assay or immunofluorescence staining. In experiments involving tubacin treatment, immediately after RO3306 release, cells were either treated with 2 μM tubacin (Sigma-Aldrich) and subjected to live-cell imaging or treated with 2 μM tubacin for 90 min followed by fixing, immunostaining and confocal laser scanning microscopy analysis.

For AKT inhibition experiment, cells were first treated with thymidine, deoxycytidine and RO3306 as described above. After 6 h of RO3306 treatment, AKT inhibitor SH6 (Abcam, UK) was added to the cell culture to a final concentration of 10 μM followed by overnight incubation. Cells were released from RO3306 treatment by washing and fresh medium containing 10 μM SH6 was added back to the cells. After 90 min incubation, cells were washed and fixed for IF microscopy.

Live-cell imaging to determine the time taken to transit from G2/M to cytokinesis

Aforementioned, cells were released from the effect of RO3306 by washing in PBS and adding fresh medium to the cells. The dish containing the cells was placed immediately on an inverted microscope (Olympus IX83) equipped with a motorized temperature controlled (37 °C) and 5 % CO₂ incubator stage, and a cooled monochrome digital camera. The capturing field was selected randomly within 5 min after the dish was placed on the stage. Live-cell images under 10x phase contrast objective (UPLFLN-PH, Olympus) were captured every 2 min for 6 h using CellSens Dimension software (Olympus). Duration of mitosis was calculated based on the time taken for a round cell to divide into two daughter cells.

Immunofluorescence (I.F.) microscopy

Cells (3 x 10⁶) were seeded into a glass bottom 14 mm microwell dish (MatTek Corporation, Ashland, MA) that was pre-coated with 2.5 μg/cm² fibronectin followed by synchronization. Cells were cultured for 90 min after release from RO3306. Cells were fixed in PBS containing 4 % (w/v) paraformaldehyde at RT for 10 min. Cells were permeabilized in cytoskeleton stabilization buffer (CSK) (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 10 mM PIPES, pH 6.8) containing 0.3 % (v/v) Triton X-100 at RT for 1 min. Cells were washed three times in PBS and non-specific binding sites were blocked by incubating cells in PBS containing 5% (w/v) BSA at RT for 30 min. Cells were then stained with relevant primary antibody in PBS containing 5% (w/v) BSA at RT for 1 h. The following primary antibodies were used: rabbit anti-α tubulin (1:300 dilution, Thermo Fisher Scientific), mouse anti-acetylated tubulin (1:100 dilution, Sigma-Aldrich), rabbit anti-MAD2 (1:300 dilution, Thermofisher Scientific), human anticentromere antibody (1:300 dilution, Antibodies, Inc. Davies, CA), which was kindly provided by Dr. Soak-Kuan Lai and Dr. Hoi-Yeung Li, Nanyang Technological University, Singapore. Cells were washed three times before staining with secondary antibody in PBS. The following secondary antibodies were used: Alexa Fluor-647 conjugated goat anti-mouse IgG (1:600 dilution, Invitrogen),
Alexa Fluor-594 conjugated goat anti-rabbit IgG (1:600 dilution, Invitrogen), Rhodamine Red-X-conjugated donkey anti-human IgG (1:10000 dilution, Jackson Immunoresearch Laboratories Inc., West grove, PA.), which was kindly provided by Dr. Soak-Kuan Lai and Dr. Hoi-Yeung Li, School of Biological Sciences, Nanyang Technological University, Singapore. DNA was stained with DAPI (0.1 mg/mL). Images of cells were acquired on a confocal laser scanning microscope (Zeiss LSM710, Carl Zeiss, Oberkochen, Germany) with a 63X oil objective lens. Data were analyzed using the Zen 2011 software (Carl Zeiss). To improve clarity in the figure presentations, the overall brightness and contrast of entire images were adjusted.

**HDAC6 activity assay**

HDAC6 activity in G2/M synchronized cells was measured using a fluorimetric-based detection kit according (BioVison, Inc., Milpitas, CA) to manufacturer’s instructions.

**Xenograft tumor formation and cryosectioning**

Animal studies were approved by the Institutional Animal Care and Use Committee at the Nanyang Technological University, Singapore. SH-SY5Y 5HRE-GFP cells (5 x 10⁶) in 100 μL of Matrigel® (Growth factor reduced and phenol red-free)(Corning®, Corning, NY) and PBS mixture were subcutaneously injected into the right flank of immune-compromised Balb/c-Rag-IL2Rγ null mice (kindly provided by Dr. Klaus Erik Karjalainen and Dr. Christiane Ruedl, School of Biological Sciences, Nanyang Technological University, Singapore). After 24 days, mice were sacrificed and the tumors were excised. Each tumor was rinsed in PBS before immersing into PBS containing 4% (w/v) paraformaldehyde and incubated at 4 °C overnight. The tumor was then immersed in PBS containing 15% (w/v) sucrose and incubated at 4 °C until the tumor settled at the bottom of the container. The step was repeated in PBS containing 30% (w/v) sucrose. The tumor was embedded in OCT (Baxter, Deerfield, IL) and frozen in liquid nitrogen. The tumor was sectioned at 8 μm per slide on a CM1950 cryostat (Leica, Wetzlar, Germany). Tumor tissue slide was then fixed in PBS containing 4% (w/v) paraformaldehyde at RT for 10 min followed by permeabilization in CSK buffer containing 0.3 % (v/v) Triton X-100 at RT for 1 min. Tumor tissue slide was washed three times in PBS followed by incubation in PBS containing 1% (w/v) BSA at RT for 30 min. Tumor tissue was then stained with rabbit anti-kindlin-2 (1:100 dilution, Sigma-Aldrich) primary antibody in PBS containing 1% (w/v) BSA at RT for 1 h. Tumor tissue slide was washed three times in PBS before staining with Alexa Fluor-635 conjugated goat anti-rabbit IgG (1:600 dilution, Invitrogen), Alexa Fluor-594-phalloidin (0.27 ng/mL) and DAPI (0.1 mg/mL) in PBS. Images were acquired on a confocal laser scanning microscope (Zeiss LSM710) (Carl Zeiss) with 10X and 63X (oil) objective lens. Data were analyzed using the Zen 2011 software (Carl Zeiss).

**Statistical calculations**

The GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used to perform statistical calculations. p < 0.05 is considered significant. The statistical test used in each analysis is described in the figure legends.

**Data Availability**

All data are contained within the manuscript.
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Conflict of interest

The authors declare that they have no conflict of interest

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Fig. 1 Depletion of kindlin-2 in SH-SY5Y cells caused adhesion defects, delayed mitosis, and mitotic spindle abnormalities. (A) (left panel) Immunoblotting of cell lysate from cells expressing control (ctrl) shRNA or kindlin-2 (K2) targeting shRNA with antibody against kindlin-2. GAPDH serves as loading control. Protein bands were quantified by densitometry. Values below protein bands represent the mean fold differences in protein expression levels relative to wild-type (normalized to 1.0) from three independent experiments. (right panel) qRT-PCR analysis of kindlin-2 transcript level in cells. The expression of kindlin-2 transcript in wild-type cells is normalized to 1.0. Three independent experiments were performed. Statistical calculation was performed using a two-tailed paired Student’s t test. p < 0.05 is considered significant. n.s.: not significant. (B) Immunoblotting of cell lysate with antibodies against kindlin-1 or kindlin-3. GAPDH serves as loading control. (C) ECIS measurements of cells on fibronectin. Each datum point is the mean (± SD, gray bar) of technical triplicates. (D) 3D culture of cells was performed and the spheroid image area was measured. Each datum point in the graph represents one cell. Two-tailed unpaired t test was performed. Representative images of spheroids are shown. Scale bar: 400 μm. (E) Time taken (min) for cells to transit from G2/M to cytokinesis. Statistical calculation was performed using a two-tailed unpaired Student’s t test. (F) Immunofluorescence staining of α-tubulin and chromosomes in metaphase WT, ctrl shRNA, K2 shRNA and HA-K2 (WT) rescued cells. Scale bar: 5 μm. (G) The percentage of metaphase cells containing normal versus abnormal spindles was determined. Number of cells analyzed: 17 (wild-type), 19 (ctrl shRNA), 20 (K2 shRNA), 42 (HA-K2 (WT) rescued) collated from three independent experiments. Statistical calculation was performed using a two-tailed unpaired Student’s t test. p < 0.05 is considered significant. n.s.: not significant.
### Fig. 2

#### A

|                      | Without tubacin treatment | With tubacin (2 μM) treatment |
|----------------------|---------------------------|-------------------------------|
|                      | WT | ctrl shRNA | K2 shRNA | HA-K2 (WT) rescued | WT | ctrl shRNA | K2 shRNA | HA-K2 (WT) rescued |
| **Ac-tubulin**       | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| **α-tubulin**        | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |
| **DAPI**             | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) | ![Image](image21) | ![Image](image22) | ![Image](image23) | ![Image](image24) |
| **Merge**            | ![Image](image25) | ![Image](image26) | ![Image](image27) | ![Image](image28) | ![Image](image29) | ![Image](image30) | ![Image](image31) | ![Image](image32) |

#### B

|                      | n.s. | *p < 0.0001* | *p = 0.0156* | *p = 0.0006* | *p < 0.0001* | n.s. | n.s. | n.s. |
|----------------------|------|--------------|--------------|--------------|--------------|------|------|------|
|                      |      | Ac. + n. p. tubulin |      | Ac. + n. p. tubulin |      | Ac. + n. p. tubulin |      | Ac. + n. p. tubulin |      |
|                      | WT   | ctrl shRNA  | K2 shRNA   | HA-K2 (WT) rescued | WT | ctrl shRNA | K2 shRNA | HA-K2 (WT) rescued |

#### C

|                      | n.s. | *p < 0.0001* | *p = 0.0469* | n.s. | *p < 0.0001* | n.s. | n.s. | n.s. |
|----------------------|------|--------------|--------------|------|--------------|------|------|------|
|                      |      | time (min) from G2/M to G0/G1 |      | time (min) from G2/M to G0/G1 |      | time (min) from G2/M to G0/G1 |      | time (min) from G2/M to G0/G1 |      |
|                      | WT   | ctrl shRNA  | K2 shRNA   | HA-K2 (WT) rescued | WT | ctrl shRNA | K2 shRNA | HA-K2 (WT) rescued |

#### D

|                      | WT   | ctrl shRNA  | K2 shRNA   | HA-K2 (WT) rescued | K2 shRNA |
|----------------------|------|--------------|------------|-------------------|---------|
| **MAD2**             | ![Image](image33) | ![Image](image34) | ![Image](image35) | ![Image](image36) | ![Image](image37) |
| **ACA**              | ![Image](image38) | ![Image](image39) | ![Image](image40) | ![Image](image41) | ![Image](image42) |
| **DAPI**             | ![Image](image43) | ![Image](image44) | ![Image](image45) | ![Image](image46) | ![Image](image47) |

- Presence of MAD2 at centromere
- Absence of MAD2 at centromere

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Fig. 2  The level of acetylated α-tubulin in mitotic spindles was reduced in kindlin-2 depleted SH-SY5Y cells. (A) Immunofluorescence staining of acetylated α-tubulin, total α-tubulin and chromosomes in metaphase cells with or without tubacin (2 μM) treatment. Representative images are shown. Scale bar: 5 μm. (B) Fluorescence intensity ratio of acetylated α-tubulin to total α-tubulin of the mitotic spindle was determined. Each datum point represents one cell analyzed. (C) Time taken (min) for cells to transit from G2/M to cytokinesis. Each datum point represents one cell. Data were collated from at least three independent experiments. (D) Immunofluorescence staining of MAD2, ACA and chromosomes in all four groups of cells. Representative images are shown. Scale bar: 5 μm. The percentage of metaphase cells with MAD2 localization at centromere was determined. Number of cells analyzed: 32 (WT), 39 (ctrl shRNA), 41 (K2 shRNA); 36 (HA-K2 (WT) rescued) collated from three independent experiments. In (B) and (C), statistical calculations were performed using a two-tailed unpaired Student’s t test. p < 0.05 is considered significant. n.s.: not significant.
Fig. 3  Kindlin-2 regulates the acetylation of α-tubulin via HDAC6. (A) Akt and GSK3β activities in G2/M synchronized cells were determined by immunoblotting using antibodies against phosphor-Akt at S473 and phosphor-GSK3β at Ser9. Protein bands were quantified by densitometry. Values below protein bands represent the mean fold differences of phosphor-protein/total protein relative to wild-type cells (normalized to 1.0) from at least three independent experiments. The level of acetylated α-tubulin and total α-tubulin in cells was also determined by immunoblotting. Statistical calculation was performed using a two-tailed unpaired Student’s t test. (B) HDAC6 activity in G2/M synchronized cells was determined using a fluorometric enzyme assay kit. Eight independent experiments were performed. Statistical calculation was performed using a two-tailed paired Student’s t test. (C) Treatment of G2/M synchronized WT SH-SY5Y cells with 10 µM Akt inhibitor (SH6) induced a significant increase in spindle abnormalities compared with untreated cells. 43 cells per group from three independent experiments were analyzed. Statistical calculation was performed using a two-tailed unpaired Student’s t test. (D) Immunoprecipitation assay using 293T cells transfected with HA-kindlin-2 and mCherry-paxillin. (Left panel) immunoprecipitation using anti-HA antibody. (Right panel) immunoprecipitation with anti-paxillin antibody. (E) Stable SH-SY5Y cells expressing control shRNA, paxillin-targeting shRNA (A) or (B) were generated. (Top panel) Paxillin expression in these cells was determined by immunoblotting. GAPDH serves as loading control. (Bottom panel) Depletion of paxillin in SH-SY5Y cells resulted in an increase number of cells with spindle abnormalities. Number of cells analyzed: 38 (ctrl shRNA), 42 (pax-shRNA A), 49 (pax-shRNA B) from three independent experiments were analyzed. Statistical calculation was performed using a two-tailed unpaired Student’s t test. (F) Immunoblot analysis of stable HA-kindlin-2 (WT)-rescued cells, HA-kindlin-2 (Q614W615/AA)-rescued cells and HA-kindlin-2 (PH-deleted)-rescued cells. Kindlin-2 depleted cells were transfected with shRNA-resistant HA-tagged WT, integrin-binding defective (QW/AA), or paxillin-binding defective (PHA) kindlin-2 expression plasmid. Re-expression of kindlin-2 was determined by immunoblotting with anti-kindlin-2 antibody. Actin serves as loading control. (G) The percentage of metaphase cells containing normal spindles versus abnormal spindles was determined. Number of cells analyzed: 47 (ctrl shRNA), 45 (K2 shRNA), 42 (K2 WT rescued), 30 (K2 QW/AA rescued) and 36 (K2 PHΔ rescued) from at least three independent experiments. Statistical calculation was performed using a two-tailed unpaired Student’s t test. (H) Fluorescence intensity ratio of acetylated α-tubulin to total α-tubulin in mitotic spindles was determined. (I) Time taken for cells to transit from G2/M to cytokinesis. In (F) and (G), each datum point represents one cell analyzed. Data were collated from at least three independent experiments. Statistical calculation was performed using a two-tailed unpaired Student’s t test.
Fig. 4 Kindlin-2 expression was reduced in SH-SY5Y cells under hypoxia. (A) Proliferation of cells under normoxia (N) or hypoxia (H) at different time points was determined. Cell proliferation under N at each time point was normalized to 1.0. Data were obtained from three independent experiments. (B) The percentage of metaphase cells containing normal spindles versus abnormal spindles under N or H was determined. Total number of metaphase cells analyzed: N (47), H (50). (C) Fluorescence intensity ratio of acetylated α-tubulin to total α-tubulin in mitotic spindles of metaphase cells under N or H was determined. Each datum point represents one cell analyzed. Data are collated from three independent experiments. (D) qRT-PCR analysis of kindlin-2 expression in cells under N or H. Values represent mean ± S.D. from three independent experiments. Kindlin-2 expression in cells under N at each time point was normalized to 1.0. 18S rRNA was used as internal control. (E) Immunoblotting of kindlin-2. Protein bands were quantified by densitometry. Values below protein bands represent the mean fold differences of kindlin-2 expression between cells under N (normalized to 1.0) and H from four independent experiments. β tubulin serves as loading control. (F) Immunoblotting of HIF-1α. β tubulin serves as loading control. (G) qRT-PCR analysis of VEGFA and miR138 expression levels in cells under N and H at different time points. Values represent mean ± S.D. from three independent experiments. VEGFA or miR138 expression in cells under N at each time point was normalized to 1.0. 18S rRNA and U6 snRNA were used as internal controls, respectively. (H) SH-SY5Y cells over-expressing GFP-kindlin-2 were subjected to N or H for 96 h followed by immunoblotting (left panel) to verify the maintained expression of GFP-kindlin-2. The % of spindle abnormalities in these cells during metaphase was also determined. (I) Immunoblotting to detect kindlin-2 in HCT-116 and MDA-MB231 cells under N or H for 96 h. β-tubulin serves as loading control. Values below protein bands represent the mean fold differences of kindlin-2 expression between cells under N (normalized to 1.0) and H from three independent experiments (top panel). The % of spindle abnormalities in these cells during metaphase was also determined (bottom panel). (J) Spindle abnormalities in stable HCT-116 and MDA-MB231 cell lines expressing kindlin-2 targeting shRNA. Expression of kindlin-2 was verified by immunoblotting. Values below protein bands represent the mean fold differences of kindlin-2 expression from three independent experiments (top panel). The % of spindle abnormalities in these cells during metaphase (bottom panel). All statistical calculations were performed using a two-tailed unpaired Student’s t test.
Fig. 5 Hypoxia down-regulates kindlin-2 expression in tumor spheroids and in vivo tumor. (A) 3D spheroid culture of 5HRE-GFP SH-SY5Y cells was performed. A representative image of a spheroid is shown. Scale bar: 100 μm. GFP signal in dissociated cells was determined by flow cytometry analysis. Cells cultured under 2D conditions were included as control. High (hi) GFP and low (lo) GFP expressing cells from 3D spheroid were sorted by flow cytometry cell sorting and the expression levels of kindlin-2 determined by immunoblotting. (B) Expression levels of VEGFA and miR138 in GFP(hi) and GFP(lo) cells were determined by qRT-PCR. For (A & B) four independent experiments were performed. Statistical calculation was performed using a two-tailed unpaired Student’s t test. (C) Subcutaneous xenotransplantation of 5HRE-GFP SH-SY5Y cells into immune-compromised mice. Tumor was excised and subjected to cryo-sectioning followed by immunofluorescence staining. Scale bar (top panel tissue section): 200 μm. Scale bar (bottom panel expanded images): 20 μm. LUT presentations are shown in expanded images. (D) An illustration of kindlin-2 signaling in the regulation of α-tubulin acetylation. The interaction between kindlin-2 and integrin promotes phosphorylation and activation of Akt. Activated Akt inhibits GSK3β activity, leading to a decrease in HADC6 activity, which in turn allows the acetylation of α-tubulin at mitotic spindles. Acetylation of α-tubulin has been shown to increase the stability of bent microtubules (37,38). The activity of HDAC6 can be inhibited by paxillin (52). In solid tumors, hypoxia could lead to a decrease in kindlin-2 expression as a consequence of an increased expression of miR138. The result of a diminished level of kindlin-2 expression includes the formation of abnormal spindles in mitotic cells.
The focal adhesion protein kindlin-2 controls mitotic spindle assembly by inhibiting histone deacetylase 6 and maintaining α-tubulin acetylation

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