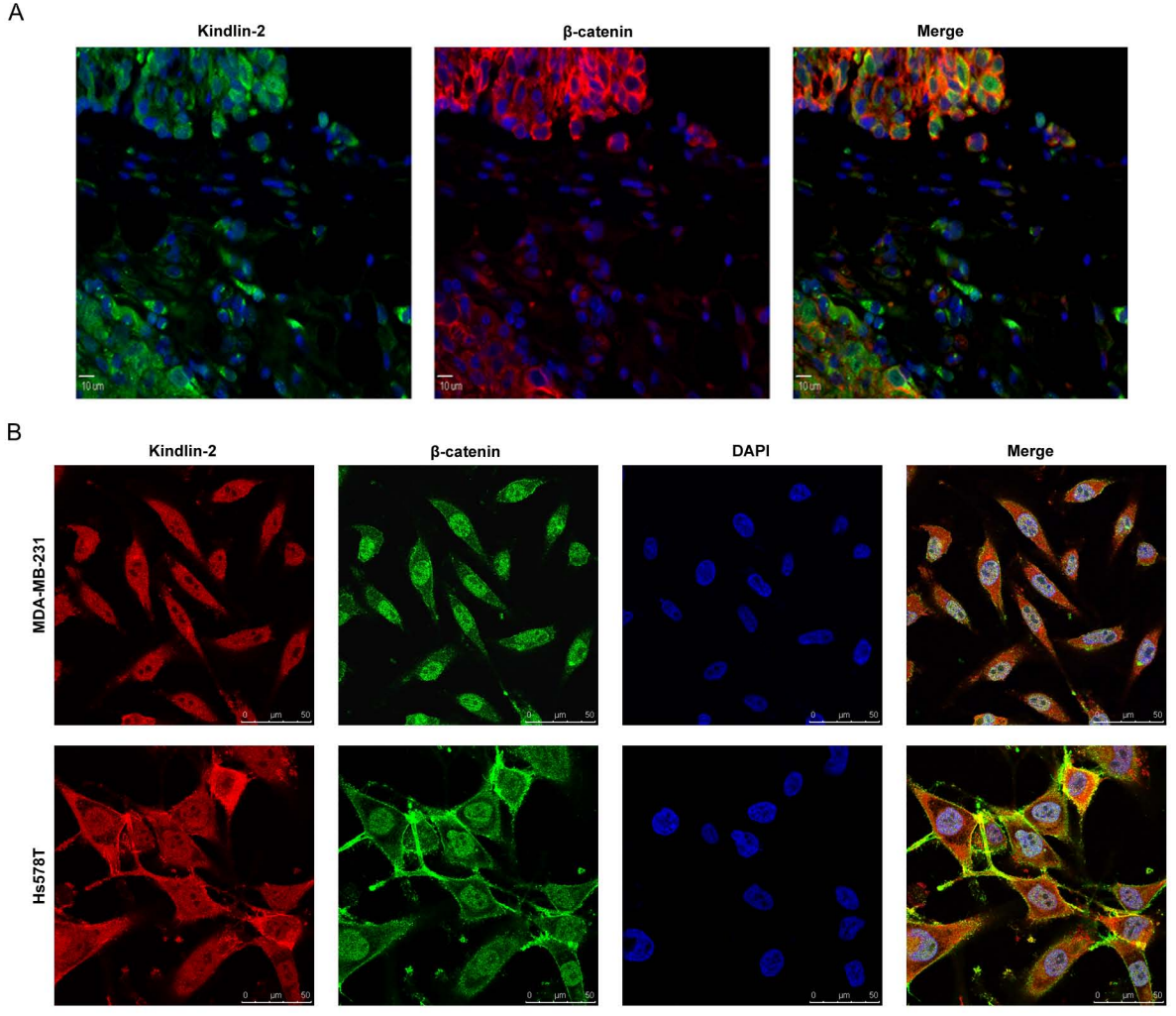
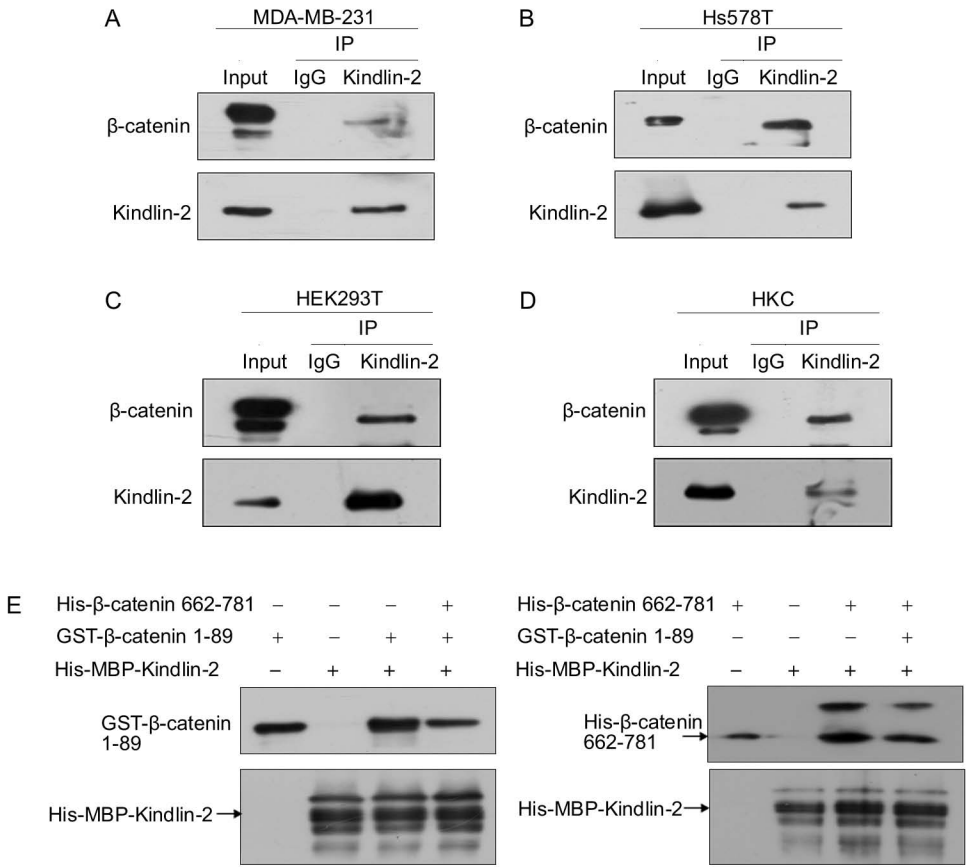
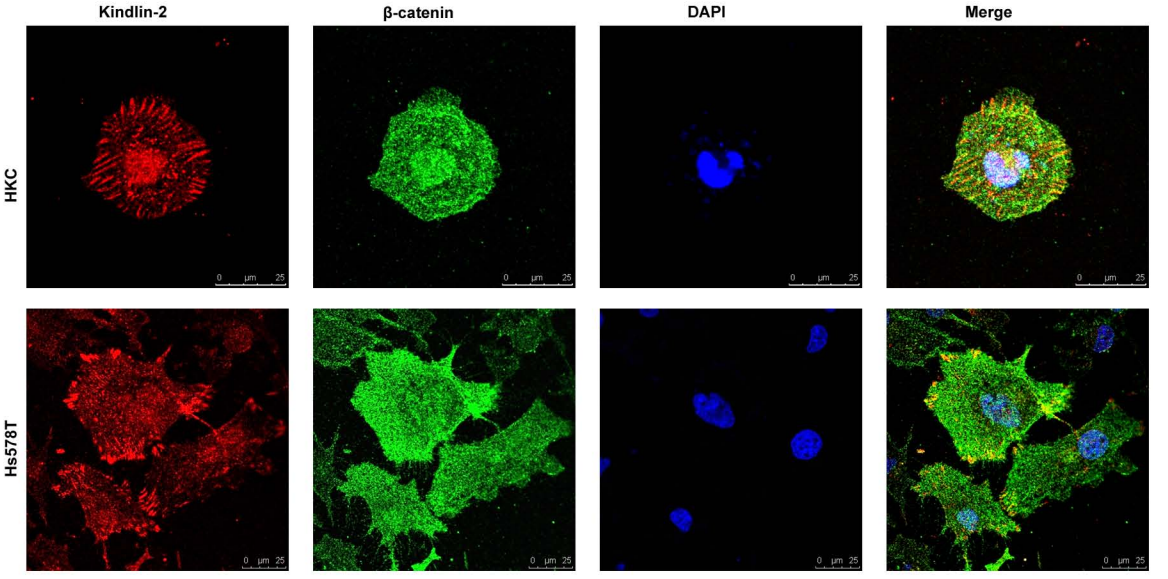
Supplementary Figure 1



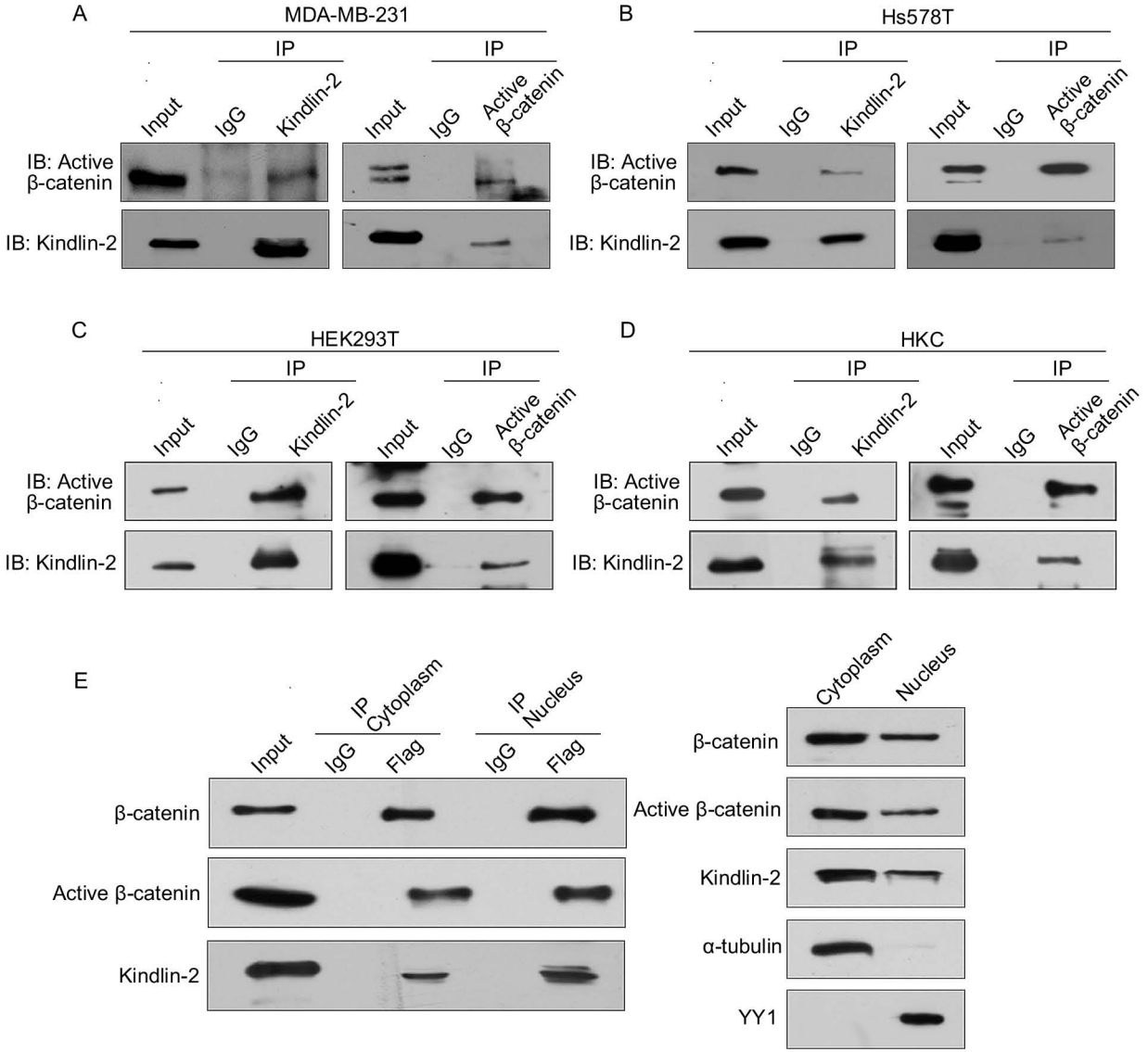
Supplementary Figure 2



Supplementary Figure 3

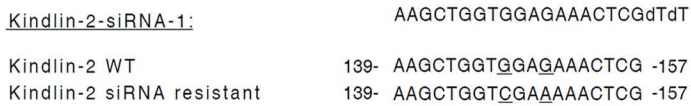


Supplementary Figure 4



Supplementary Figure 5

A



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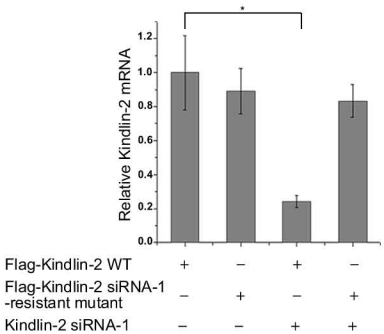

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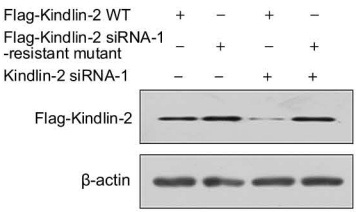

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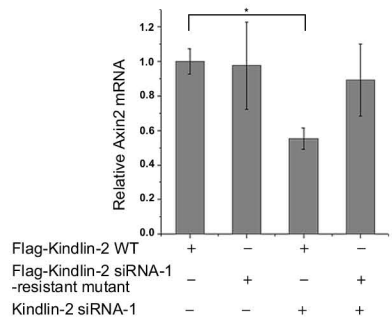

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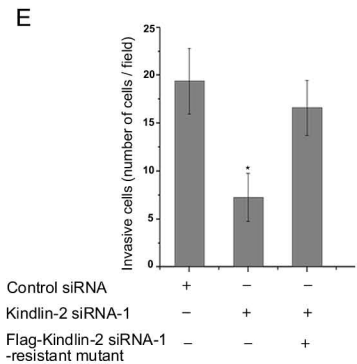
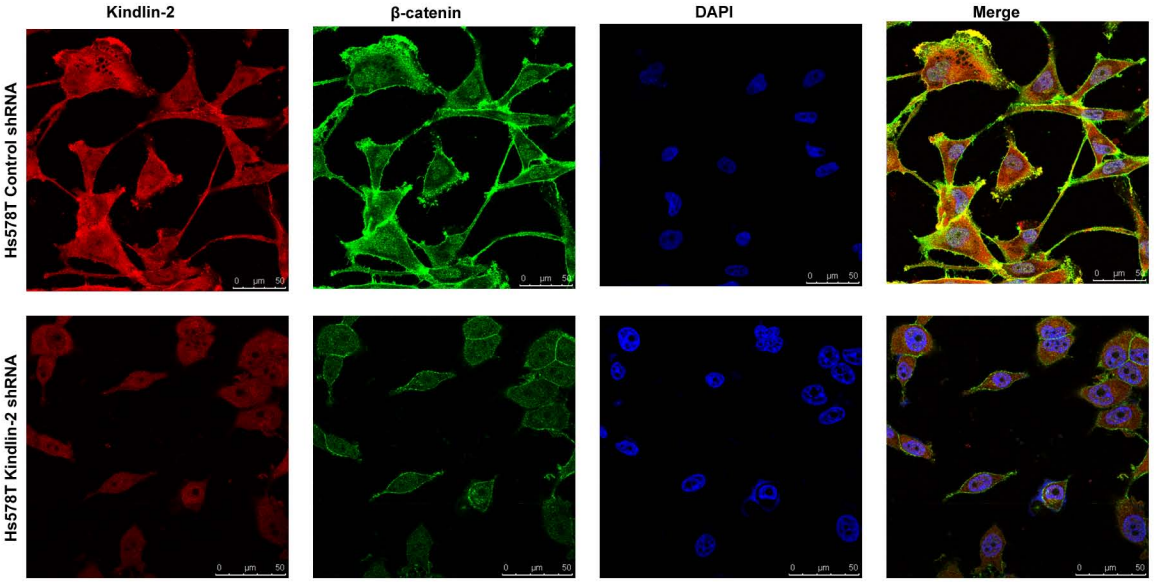
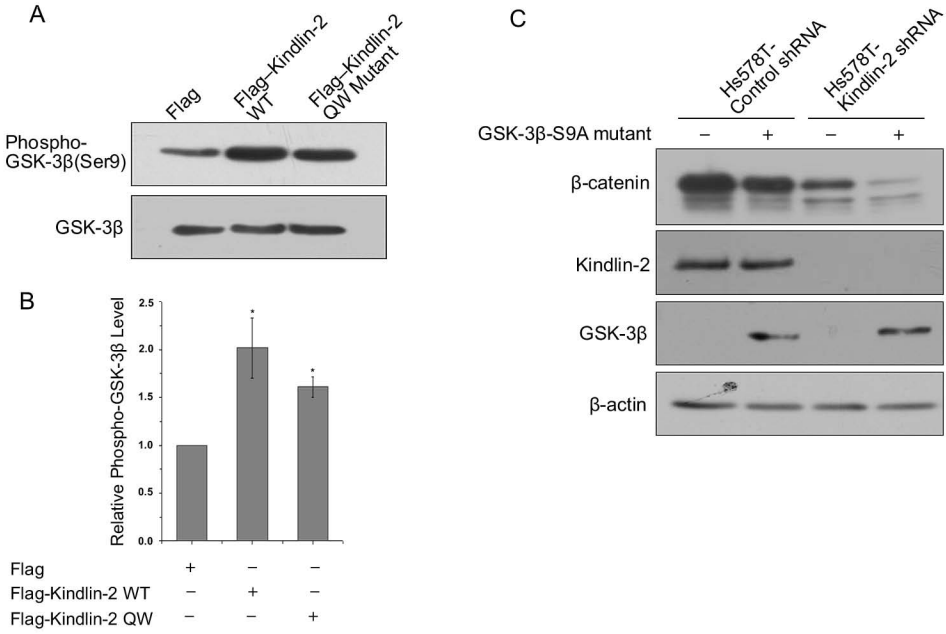


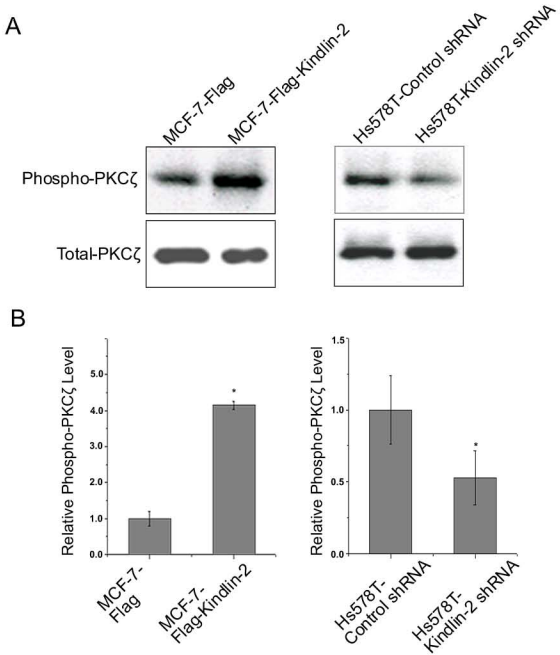
Supplementary Figure 6



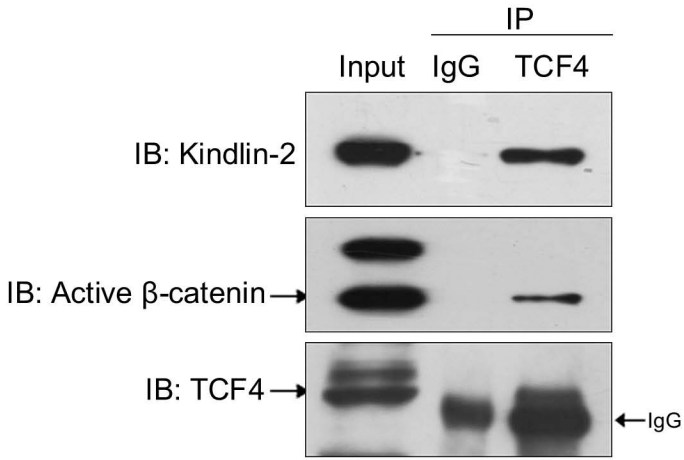
Supplementary Figure 7



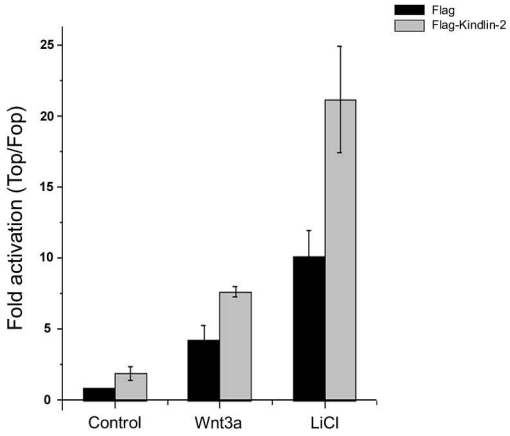
Supplementary Figure 8



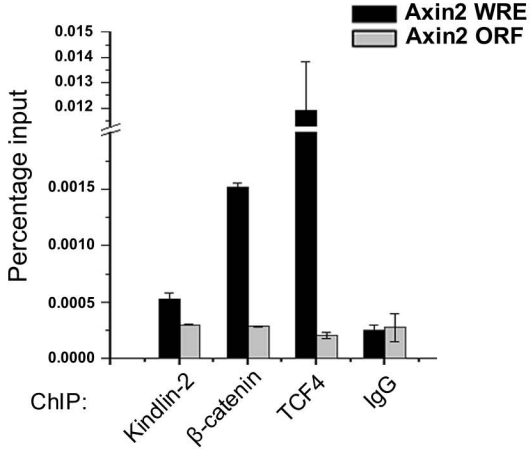
Supplementary Figure 9



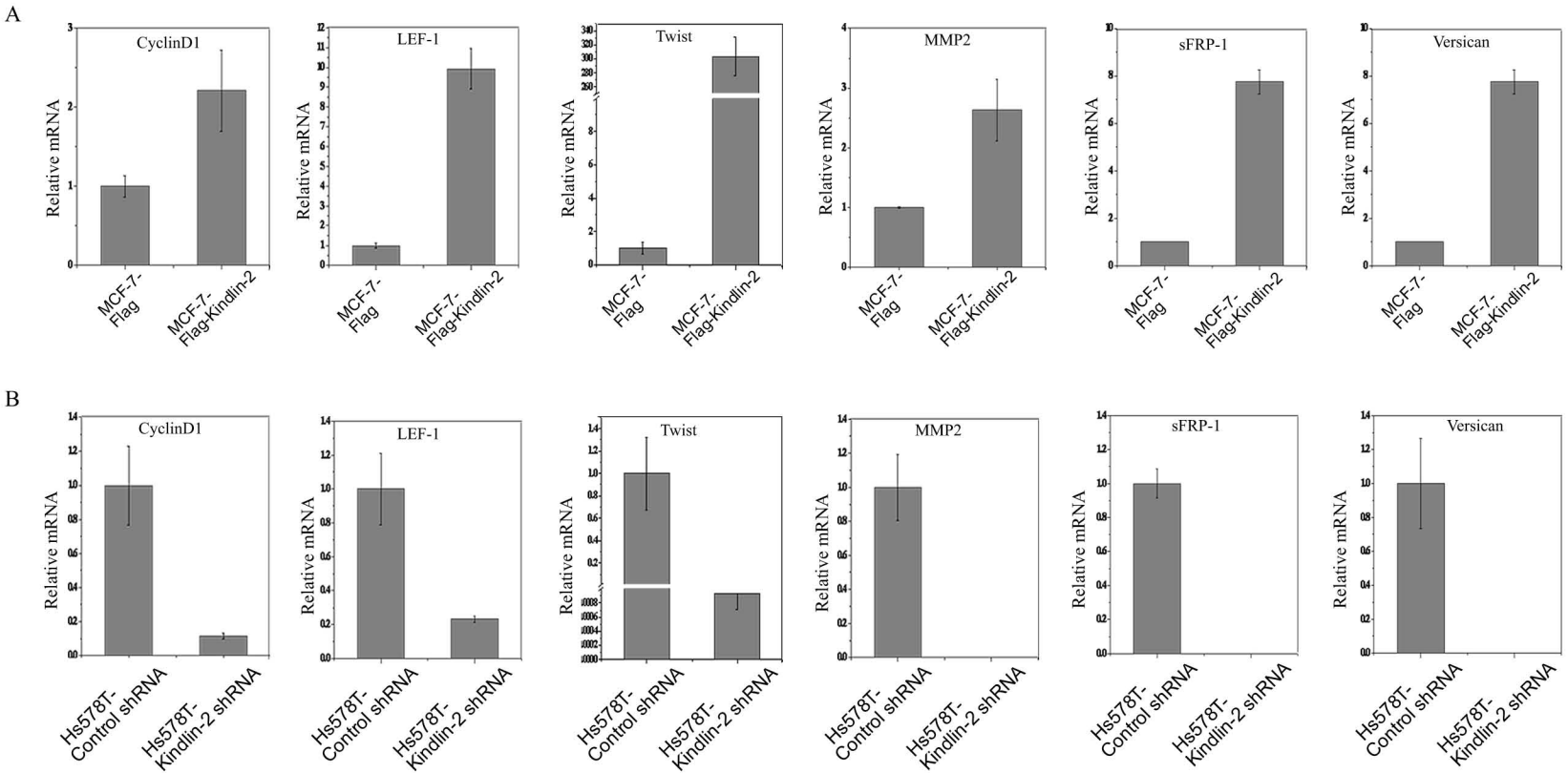
Supplementary Figure 10



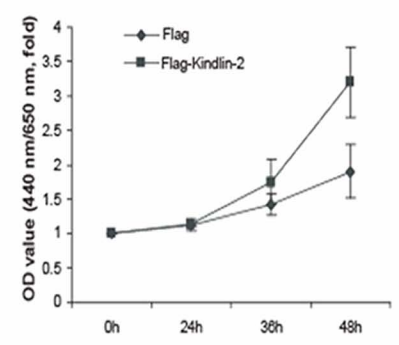
Supplementary Figure 11



Supplementary Figure 12



Supplementary Figure 13



Supplementary Figure Legends

SFig 1 | The localization of Kindlin-2 and β-catenin. (A) The localization of Kindlin-2 and β-catenin in the invasive front of human tumor. Immunofluorescence (IF) staining was performed using anti-Kindlin-2 (Alexa Flour 488, green), anti-β-catenin (Alexa Flour 568, red) and nuclei (DAPI, blue). (B) IF staining for Kindlin-2 (Alexa Flour 568, red), β-catenin (Alexa Flour 488, green) and nuclei (DAPI, blue) was performed in MDA-MB-231 and Hs578T cells. Images were captured with a confocal microscopy.

SFig 2 | Kindlin-2 directly interacts with β-catenin. (A–D) Cell lysates from different cell lines were prepared and Kindlin-2 antibodies were then used in Co-IP, followed by Western blot (WB) analysis using indicated antibodies. (E) His-MBP-Kindlin-2 was incubated with GST-β-catenin N-terminal (1-89) or/and His-β-catenin C-terminal (662-781) *in vitro* for a competition assay.

SFig 3 | The localization of Kindlin-2 and β-catenin at focal adhesion structures. HKC or Hs578T cells were plated on collagen-coated coverslips for 24 h, fixed, and then stained with anti-Kindlin-2 (Alexa Flour 568, red) and anti-β-catenin (Alexa Flour 488, green). Images were captured with a confocal microscopy.

SFig 4 | Kindlin-2 specifically associates with active β-catenin. (A–D) Lysates from different cell lines were extracted, and then Kindlin-2, or active β-catenin antibodies were used for Co-IP, followed by WB. (E) Both cytoplasmic and nuclear lysates were extracted from MCF-7–Flag-Kindlin-2 stable cells for performing Co-IP. Both lysates were evaluated by WB. The absence of α-tubulin demonstrates that the fraction was from the nucleus. The absence of YY1 indicates that the fraction was from the cytoplasm.

SFig 5 | The identification of the specificity of Kindlin-2 siRNA. (A) Kindlin-2 G147C/G150A mutant was generated, which is resistant to Kindlin-2 siRNA-1 or shRNA. (B-D) Flag-Kindlin-2 WT or Flag-Kindlin-2 siRNA-resistant mutant was transfected into control or siRNA-1 treated 293T cells. Total RNA was extracted to detect the levels of Kindlin-2 (B) and Axin2 (D) by qPCR. Total protein was extracted

to detect the level of Flag-Kindlin-2 by Western blot (C). (E) Flag-Kindlin-2 siRNA-resistant mutant was cotransfected with control or Kindlin-2 siRNA-1 into Hs578T cells, and then invasion assay was performed. Error bars indicate s.d. values, n=3; * indicates $p<0.05$ by Student's *t*-test.

SFig 6 | The depletion of Kindlin-2 leads to the weaken of fluorescent intensity of β-catenin. IF staining for Kindlin-2 (Alexa Flour 568, red) and β-catenin (Alexa Flour 488, green) was performed in control or Hs578T-Kindlin-2-shRNA stable cells. Images were captured with a confocal microscopy.

SFig 7 | The effect of Kindlin-2 on GSK-3β activity. (A) A shortly exposed blot from Fig 2G was shown. (B) The quantified values of phosphor-GSK-3β (Ser9) were showed in Flag or Flag-Kindlin-2-treated 293T cells, Error bars indicate s.d. values, n=3; * indicates $p<0.05$ by Student's *t*-test. (C) GSK-3β-S9A mutant was transfected into the indicated stable cells, and then protein was extracted for WB.

SFig 8 | The regulation of Kindlin-2 in PKCζ activation. (A) Both phospho-PKCζ and total-PKCζ were examined by Western blot in indicated stable cells. (B) The quantified values of phospho-PKCζ were showed in indicated stable cells. Error bars indicate s.d. values, n=3; * indicates $p<0.05$ by Student's *t*-test. (C) 293T cells were treated with Flag, Flag-Kindlin-2, PKCζ siRNA, PKCζ inhibitor Gö6983 (1 μM, 24 h, Sigma) or PKCζ pseudosubstrate (20μM, 24 h, BIOSOURCE), later protein was extracted for WB.

SFig 9 | Co-IP assay was performed in LiCl-treated 293T cells (20 mM, 6 h) using endogenous anti-TCF4 antibody, followed by Western blot using anti-Kindlin-2, anti-active β-catenin and anti-TCF4 antibodies.

SFig 10 | 293T cells were transfected with Flag or Flag-Kindlin-2 plus SuperTop/Fopflash for 24 h and then treated with Wnt3a (200 ng, 6 h), LiCl (20 mM, 6 h). Error bars indicate s.d. values, n=3; * indicates $p<0.05$ by Student's *t*-test.

SFig 11 | ChIP assay was performed in LiCl-treated 293T cells (20mM, 6 h) using anti-Kindlin-2 (sigma), anti-β-catenin (BD), and anti-TCF4 (Upstate) antibodies.

SFig 12 | mRNA levels of Wnt target genes were examined by qPCR in indicated stable cells.

SFig 13 | Kindlin-2 promotes cell proliferation. Cell growth curve was made in MCF-7-Flag and MCF-7-Flag-Kindlin-2 stable cells.

Supplementary Methods

Cell culture and establishment of stable clines

Human breast cancer cell lines MCF-7, Hs578T and MDA-MB-231 was grown in DMEM supplemented with 10% fetal bovine serum (Gibico) and an appropriate amount of penicillin/streptomycin in a 37 °C, 5% CO_2, humidified incubator. Medium was changed twice per week. For the generation of stable cell lines, MCF-7 cells were transfected with pCMV-3xFLAG or pCMV-3xFLAG-Kindlin-2 plasmid using Lipofectamine 2000. pSuper-control shRNA or pSuper-Kindlin-2 shRNA was transfected into Hs578T cells. Clones were selected by 800 μg/ml of G418 (Sigma) for 2 week. Mix clones (6-10 independent clones) were maintained in medium containing 200μg/ml of G418.

Western blotting

Briefly, the cells were harvested with a scraper and then washed once with cold phosphate-buffered saline. The cells were then lysed in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1% NP40, and 1% Protease Inhibitor Cocktail). Equal amounts of proteins were size-fractionated by 6–12% SDS-PAGE. The antibodies used were anti-β-catenin (Santa Cruz Biotechnology), anti-Kindlin-2 (Millipore), anti-active β-catenin (Millipore 8E7), anti-phospho- β-cateninS33/37/T41 (Cell Signaling Technology), anti-GSK3β (Cell Signaling Technology), anti-TCF4 (Upstate), anti-E-cadherin(Santa Cruz Biotechnology), anti-vimentin (Epitomics), anti-N-cadherin (Epitomics), anti-fibronectin (Epitomics), anti-snail (Cell Signaling Technology), anti-Axin2 (Epitomics), anti-HA (sigma), anti-Flag (sigma), anti-GST (sigma), anti-His (Cell Signaling Technology).

Co-immunoprecipitation (Co-IP)

A Co-IP assay was performed as described previously (Yu, Cai et al. 2009). Briefly,

total cell lysates were incubated with antibodies at 4 °C for 4 h followed by incubating with protein A/G-Sepharose (Santa Cruz Biotechnology) overnight. After the beads were washed three times with NP40 buffer, the bound proteins were eluted with 2× SDS loading buffer at 100 °C for 5 min. The immunoprecipitates were analyzed by Western blotting with different antibodies.

Purification of Fusion Proteins and GST/His/MBP Pull-down Assay

GST, GST-β-catenin or His-MBP-Kindlin-2 was expressed in *E. coli* BL21 (Tiangen Biotechnology, beijing, china), and purified with Glutathione Sepharose 4B beads (Pharmacia Biotech), HIS-Select HF Nickel Affinity Gel (Sigma) or MBP-Affinity Matrix (Amylose Resin, New England Biolabs). In a GST pull-down assay, GST or GST-β-catenin fusion protein was incubated with Glutathione Sepharose 4B beads by rocking at 4°C for 1hr, and the beads were then washed three times with TEN buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and 100 mM NaCl). His-MBP-Kindlin-2 were then added to the beads and incubated by rocking at 4 °C overnight. The beads were washed three times with TENT buffer (0.5% NP40, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and 300 mM NaCl), and then dissolved into 2× SDS loading buffer after centrifugation, and boiled 5 min at 100 °C. After centrifuging, the supernatant was extracted and analyzed by Western Blot. In a MBP or HIS pull-down assay, His-MBP-Kindlin-2 fusion protein was incubated with MBP-Affinity Matrix or HIS Affinity Gel by rocking at 4°C for 1hr, and then GST or GST-β-catenin fusion protein was added and incubated at 4 °C overnight. The affinity matrix or gel was washed with TENT buffer, and then dissolved into 2× SDS loading buffer.

Immunofluorescence and Confocal Microscopy

The cellular protein fractionation was performed as described previously (Yu, Cai et al. 2009) with modifications. After the cells were fixed with 4% paraformaldehyde

solution at RT for 15 min, they were treated with 0.5% Triton X-100 at 37 °C for 5 min and blocked with 5% BSA at room temperature for 1hr. The cells were then incubated with 1:100 dilution of Kindlin-2 antibody or β-catenin antibody overnight at 4 °C, and then with a 1:100 dilution of Alexa Fluor 488 or 568-conjugated IgG (Invitrogen) for 1hr at room temperature. The images were captured with a TCS SP5 confocal microscope (Leica, Germany).

RNA interference (RNAi)

Sequences of RNA interference (RNAi) oligonucleotides were as follows: Control siRNA, UUCUCCGAACGUGU CACGU; Kindlin-2 siRNA-1, AAGCUG GUGGAGAAACUCG; Kindlin-2 siRNA-2, CAGCGAGAAUCUUGGAGGCTT; β-catenin siRNA, TGTGAGGCCTCTTGTCAGAAA; Axin2 siRNA, CACCACC ACCACCACCATT; Snail siRNA, UGCACATCCGAAGCCACACTT; PKCzeta siRNA: GATGGAGGAAGCTGTACCG. All RNAi oligonucleotides were purchased from Shanghai GenePharma Company (Shanghai). Kindlin-2 shRNA was designed according to the sequence of siRNA-1. The forward primer is 5'-GATCCCCAAGCTGGTGGAGAAACTCGTTCAAGAGACGAGTTT CTCCACCAGCTTTTTTTGGAAA-3'. These RNAi oligonucleotides and shRNA were transfected into cells by using a Lipofectamine 2000 kit (Invitrogen) for 48 hrs according to the manufacturer's instructions.

Real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using the SuperScript kit (Invitrogen). The primer sequences were as followed: Axin2, forward primer 5'-CTGGGGGCAGCGAGTATTAC-3' and reverse primer 5'-GCCT TTCCCATTGCGTTTGG-3'; Kindlin-2, forward primer 5'-TGTCCCCGCTATCTA AAAAAGT-3' and reverse primer 5'-TGATGGGCCTCCAAGATTCT-3'; GAPDH,

forward primer 5’-CTGAGAACGGGAAGCTTGT-3’ and reverse primer 5’-GGGT GCTAAGCAGTTGGT-3’; LEF-1, forward primer 5’-AATGAGAGCGAATGTCG TTGC-3’ and reverse primer 5’-GCTGTCTTTCTTTCCGTGCTA-3’; CyclinD1, forward primer 5’-GTGCTGCGAAGTGGAAACC-3’ and reverse primer 5’-ATCCA GGTGGCGACGATCT-3’; MMP2, forward primer 5’-CCGTCGCCCATCATCAAG TT-3’ and reverse primer 5’-CTGTCTGGGGCAGTCCAAAG-3’; sFRP-1, forward primer 5’-AACGTGGGCTACAAGAAGATG-3’ and reverse primer 5’-CAGCGAC ACGGGTAGATGG-3’; Versican, forward primer 5’-GTAACCCATGCGCTACAT AAAGT-3’ and reverse primer 5’-GGCAAAGTAGGCATCGTTGAAA-3’.

Chromatin Immunoprecipitation (ChIP) Assay

A chromatin immunoprecipitation (ChIP) assay was performed as described previously (Wu, Wang et al. 2008). Briefly, cells were fixed with 1% formaldehyde at 37°C for 10 min. Cells were lysed on ice for 15 min and then sonicated to yield DNA fragments (500-700 bp in size). After centrifugation, the soluble chromatin was subjected to immunoprecipitation with FLAG-M2 beads (Sigma) or with other antibodies (anti-β-catenin, BD; anti-TCF4, Upstate; anti-Kindlin-2, Sigma) followed by incubating with protein A/G-Sepharose (Santa Cruz Biotechnology). Then, the protein A/G-Sepharose was washed sequentially with low-salt, high-salt, LiCl, and TE buffers and finally eluted with freshly prepared 1% SDS–0.1 M NaHCO3. Then the sample was heated at 65°C overnight to reverse the DNA-protein cross-links. DNA was purified with a Qiagen DNA extraction kit. The primers for quantitative ChIP (q-ChIP) PCR were as follows (Li and Wang 2008): Axin2-WRE-F, ctggagccggctgcgctttgataa; Axin2-WRE-R, cggccccgaaatccatcgctc tga; Axin2-ORF-F, ctggctttggtgaactgttg; Axin2-ORF-R, agttgctcacagccaagaca.

Subcellular Fraction

Cells were rinsed twice in cold PBS, then incubated with buffer A (50 mM Tris-HCl pH7.8, 420 mM NaCl, 1mM EDTA, 0.5% NP40, 0.34 M sucrose, 10% glycerol, 1 mM Na_3VO4, and protease inhibitor mixture) for 5 min on ice, and the cells were then scraped and centrifuged. The supernatant was the cytoplasmic fraction, and the pellet was then lysed in buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, protease inhibitor mixture). After centrifuging, the supernatant was the nuclear fraction.

Cell Invasion Assay

5×10^4 cells were plated onto the upper wells of the modified Boydean Chamber (BD) coated with Matrigel. After 2 days, the top Matrigel was removed and the cells passed through the membrane were stained with crystal violet and counted.

Reference

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