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Hakai, an E3-ligase for E-cadherin, stabilizes δ-catenin through Src kinase

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ABSTRACT

Hakai ubiquitinates and induces endocytosis of the E-cadherin complex; thus, modulating cell adhesion and regulating development of the epithelial-mesenchymal transition of metastasis. Our previous published data show that δ-catenin promotes E-cadherin processing and thereby activates β-catenin-mediated oncogenic signals. Although several published data show the interactions between δ-catenin and E-cadherin and between Hakai and E-cadherin separately, we found no published report on the relationship between δ-catenin and Hakai. In this report, we show Hakai stabilizes δ-catenin regardless of its E3 ligase activity. We show that Hakai and Src increase the stability of δ-catenin synergistically. Hakai stabilizes Src and Src, which in turn, inhibits binding between glycogen synthase kinase-3β and δ-catenin, resulting in less proteosomal degradation of δ-catenin. These results suggest that stabilization of δ-catenin by Hakai is dependent on Src.

1. Introduction

δ-Catenin or NPRAP is a member of p120ctn subfamily of armadillo proteins and was first identified through its interactions with presenilin-1 [1]. δ-Catenin is abundantly expressed in the brain and has been implicated in the regulation of dendrogenesis and cognitive function. It also has 10 Arm repeats, indicating its potential participation in various protein-protein interactions. Overexpression of δ-catenin has been reported in a variety of cancer tissues, including prostate [2–4], lung [5–8], ovarian [9], brain [10], and colorectal tumors [11]. Burger et al. showed that δ-catenin mRNA is overexpressed in prostate cancer compared to that in benign prostatic hyperplasia [2]. Lu et al. demonstrated that the δ-catenin protein is upregulated in over 80% of prostatic adenocarcinomas, and its expression is correlated with an increase in Gleason score [3]. Our previous published data show that δ-catenin promotes E-cadherin processing and thereby activates β-catenin-mediated oncogenic signaling [12].

E-cadherin is one of the major classical cadherins involved in epithelial cell-cell adhesion. It is frequently absent or downregulated in various human cancers. E-cadherin contains an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The E-cadherin cytoplasmic domain is associated with catenins, which link the actin cytoskeleton network [13,14]. E-cadherin is required for maintenance of stable junctions [15]. However, reduction of E-cadherin expression is heavily involved in the epithelial-mesenchymal transition and is correlated with malignant cancer behavior. The juxtamembrane regions of E-cadherin are bound by p120ctn and δ-catenin. p120 catenin promotes cell surface trafficking of cadherins to regulate cadherin stability and turnover at the plasma membrane [16–19], and to facilitate recycling of cadherin back to the cell surface [16,20]. Hakai was discovered by Fujita et al. as a novel ubiquitin E3 ligase protein that targets pTyr sites on E-cadherin [21].

Hakai is a RING-finger type E3 ubiquitin-ligase [22,23]. According to Fujita et al., Hakai possesses a RING domain, a short pTyr recognition sequence, and a proline-rich domain. Mukherjee et al. established that molecular structure of E3 ubiquitin-ligase Hakai contains a novel domain, HYB (Hakai pTyr-binding). HYB domain consists of a pair of monomers arranged in an anti-parallel configuration. Each monomer consists of two zinc-finger domains: a RING finger domain and a short pTyr-binding domain that incorporates a novel, atypical Zn-finger coordination motif. Both domains are important for dimerization. The minimum E-cadherin phosphotyrosine-binding sequence in Hakai is contained within amino acids 148–206. The N-terminal RING domain (residues 106–148) is followed by the C-terminal atypical zinc-binding domain contained within the pTyr-binding domain [24].

As Hakai mediates ubiquitination and endocytosis of the E-cadherin complex, expression of Hakai in epithelial cells enhances endocytosis of E-cadherin, causing a perturbation in cell-cell adhesion [21]. In addition

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to cell-cell contact, Hakai is involved in the reduction of cell-substratum adhesion and increases epithelial cell invasion [25]. Figueroa et al. reported that Hakai regulates cell proliferation in an E-cadherin down-regulation-independent manner. They demonstrated that Hakai expression is upregulated in colon and gastric cancer [26]. Gong et al. characterized Hakai as a transcriptional coregulator in breast cancer cell, suggesting that it may play an important role in the development of breast cancer [27]. These findings suggest roles for Hakai in E-cadherin-dependent and -independent tumorigenesis. Cooper et al. has also discussed the probability of ubiquitin independent functions of Hakai in cellular signaling [28]. It is important to study the different functional roles of Hakai in different physiological or pathological conditions to investigate its clinical potential usefulness as therapeutic target for cancer [29].

In this report, we show that Hakai stabilizes δ-catenin regardless of its E3 ligase activity. Furthermore, we show that Src kinase activity is essential for Hakai to stabilize δ-catenin. Hakai stabilizes Src and Src, in turn, inhibits binding between glycogen synthase kinase-3β (GSK-3β) and δ-catenin, resulting in less ubiquitination and proteosomal degradation of δ-catenin.

2. Materials and methods

2.1. Plasmids and antibodies

Preparation of δ-catenin wild type (WT) and T1078A δ-catenin constructs in pEGFP-C1 has been described previously [30]. The deletion constructs of 1–690, 690–1040, 1–1070, and 1–1200 were generated by PCR amplification and cloned into pEGFP-C1 vector. The constructs of pCMV5 RF-Src [dominant-negative (K295R, Y527F)] and c-Src/GFP constructs were kindly provided by Joan Brugge (Harvard Medical School). The Hakai-Flag and Hakai-GFP plasmids were provided by Professor Keesook Lee (Chonnam National University). ΔN-terminal Hakai (amino acids 1–148 deleted) was constructed by ligating the digested products of pCDNA3-Flag-Hakai with ApaII/XhoI and EcoRV/XhoI after forming blunt ends with Klenow. ΔPEST Hakai (amino acids 195–220 deleted) was constructed from pCDNA3-Flag-Hakai by digestion with NdeI.

The antibodies for immunoblotting were purchased commercially as follows: anti-Hakai (#50993; Abcam), anti-δ-catenin (#611537; BD Bioscience), anti-E-cadherin (#sc-7870; Santa Cruz Biotechnology); anti-GFP (#G1544; Sigma), anti-β-actin (#A5441, Sigma), anti-py20 (SC-508; Santa Cruz Biotechnology) and (SC-7080; Santa Cruz Biotechnology) anti-Src (#05-184; Sigma), anti-α-tubulin (#T9026, Cell Signaling), anti-lamin B (SC-6216, Santa Cruz Biotechnology), GSK 3α/β (#5676, Cell Signaling) and phospho-GSK3α/β (9331S; Cell Signaling). HA epitope was detected using media from 12CA5 hybridoma. Antibodies for immunofluorescence were also obtained commercially: anti-E-cadherin (#sc-7870; Santa Cruz Biotechnology), anti-Hakai (21179-1-AP; Proteintech) and anti-Flag (#F7425; Sigma).

Hakai si-RNA (#SC-89853), Src si-RNA (#SC-29228) and control si-RNA (SC-37007) were purchased from Santa Cruz Biotechnology. Src inhibitor, SU6656 (#S9692), was purchased from Sigma Aldrich.

2.2. Methods

2.2.1. Cell culture and transfection

The Bosc23 cells, which are human kidney cells derived from the 293 cell line, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2. CWR22Rv-1 and PC3 cells (human prostate cancer cell lines) were grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. MCF7 cells (human breast cancer cell line) were cultured in DMEM/high glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2.

Bosc23 cells were transfected using calcium phosphate and the other cells were transfected using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.2.2. Western blot and immunoprecipitation

Cells were harvested with MLB lysis buffer. The cell lysates were quantified with a protein assay to measure protein concentration of each sample with the BCA kit (Pierce, Rockford, IL, USA). Next, 20 μg of each sample was loaded onto a Tris-Glycine gel and electrophoresed. The proteins were transferred to a polyvinylidenedifluoride membrane (Millipore, Billerica, MA, USA). The membrane was cut according to the target protein's molecular weight and was activated with methanol. The cut membranes were blocked with 5% skim milk buffer and were probed with primary antibodies. Bound antibodies were visualized with horse-radish peroxidase-conjugated secondary antibodies followed by visualization with enhanced chemiluminescence Western Blotting Detection reagents (Millipore).

Immunoprecipitation was performed using lysates from transfected cells. The cells were lysed in MLB lysis buffer as described above and incubated with primary antibodies according to the manufacturer's protocol for 16 h at 4 °C and then removed with Protein G Sepharose (GE Healthcare, Uppsala, Sweden). The immunoprecipitated proteins were eluted at 95 °C for 2 min with 15 μl of 2× sample buffer (0.1 M Tris-HCl, pH 6.8, 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 1.43 M β-mercaptoethanol) and analyzed by Western blot as described above.

2.2.3. Protein stability assay

The cells were incubated in fresh growth medium 24 h after transfection and treated with 50 μM cycloheximide (CHX) for different times.

2.2.4. Immnunofluorescense staining and image acquisition

PC3 and CWR22Rv-1 cells transfected with GFP-Hakai and RFP-δ-catenin were grown on glass coverslips. After 24 h of transfection, the cells were fixed with 4% PFA in PBS. Next, the fixed cells on glass coverslips were subjected to confocal analysis. PC3 cells transfected with GFP-tagged δ-catenin constructs with or without Flag-tagged Hakai were fixed and subjected to immunofluorescence staining using the anti-Flag antibody and Alexa Fluor 568 Goat anti-rabbit IgG. GFP-tagged Hakai transfected CWR22Rv-1 and MCF7 cells were fixed and subjected to immunofluorescence staining using anti-E-cadherin antibody and Alexa Fluor 568 Goat anti-rabbit IgG. Images were visualized and acquired using a TCS SP5 AOBS/Tandem microscope (Leica Microscope Systems GmBh, Wetzlar, Germany) at Korea Basic Science Institute, Gwangju.

2.2.5. Cell fractionation

The Subcellular Protein Fractionation Kit for Cultured Cells (78840; Thermo Scientific, Rockford, IL, USA) was used to separate the cytoplasmic, membrane, and nuclear protein extracts. Briefly, the cells were harvested and centrifuged at 500 × *g* for 5 min. The supernatant was removed, and the cell pellets were washed with PBS followed by centrifugation at 500 × *g* for 2–3 min. The supernatant was discarded, and the cell pellet was made as dry as possible. Next, specific buffers in the Kit were added to the cell pellets according to the protocol.

The protein extracts from the different cell compartments were subjected to Western blot. Lamin B antibody was used as the nuclear protein marker and α-tubulin and EGFR antibodies were used as the cytoplasmic and membrane protein markers respectively.

3. Results

3.1. Hakai increases the stability of δ-catenin

To investigate the effects of Hakai on the protein expression of δ-catenin, Bosc23 cells were transiently co-transfected with different Hakai gene dosage ratios and same dose of δ-catenin-GFP plasmids, as indicated in the Fig. 1A. Total cell lysates were immunoblotted using anti-δ-catenin, anti-Hakai, and anti-actin antibodies. Interestingly, overexpressing Hakai increased δ-catenin protein level in Bosc23 cells. Such effect of Hakai on δ-catenin protein level was further confirmed in CWR22Rv-1 cells which endogenously express δ-catenin. Some other cell lines which endogenously express δ-catenin are shown with Western blot data in Supplementary Fig. 1 along with its mRNA level in Supplementary Fig. 2. In CWR22Rv-1 cells, overexpressed Hakai similarly increased endogenous δ-catenin (Fig. 1B), despite δ-catenin mRNA levels remained unchanged (Supplementary Fig. 3). Increased δ-catenin level in presence of overexpressed Hakai prompted us to investigate if knocking down Hakai decreases δ-catenin level. We used Hakai si-RNA in δ-catenin stable CWR22Rv-1and found out that lower level of δ-catenin was expressed in Hakai si-RNA transfected cells than in control si-RNA transfected cells (Fig. 1C).

In addition, further study using the protein biosynthesis inhibitor, cycloheximide, showed that δ-catenin half-life was increased in Hakai overexpressed Bosc23 cells compared with Hakai non-transfected cells (Fig. 1D–F). The protein half-life of δ-catenin was about 16 h, whereas it was >20 h in the presence of Hakai, indicating that overexpressing Hakai increases δ-catenin stability.

To investigate which δ-catenin region is mainly responsible for the stabilizing effect of Hakai, we tested effect of Hakai on different δ-catenin deletion mutants, 1–690, 690–1040, 1–1070 and 1–1200. We transiently transfected Bosc23 cells with different Hakai-Flag gene dosage ratios and the same doses of respective δ-catenin deletion mutants (Fig. 2A). We observed that overexpressing Hakai did not increase δ-catenin 1–690, 690–1040, and 1–1070, protein levels in a dose-dependent manner (Fig. 2A–C). Whereas, we found stabilizing effect of overexpressing Hakai on δ-catenin 1–1200 level was same as on δ-catenin WT (Fig. 2D). The effect of Hakai overexpression on different δ-catenin constructs is summarized in Fig. 2E. As Hakai did not stabilize δ-catenin 1–1070, we concluded that δ-catenin amino acids 1070–1200 are essential for Hakai mediated stabilization of δ-catenin.

After finding out the region of δ-catenin that is essential for the stabilizing effect of Hakai on δ-catenin, we investigated whether Hakai ubiquitin-ligase activity is necessary for the Hakai-mediated stabilizing effect on δ-catenin. The RING finger domain is important for ubiquitin-ligase activity, whereas the PEST sequence motif is important for degrading target proteins [27]. We used both the Hakai RING domain and PEST-domain deletion mutants. Both the Hakai RING domain and PEST-domain deletion mutants increased δ-catenin levels distinctly (Fig. 3A–C). These findings suggest that neither the E3-ligase activity nor PEST motif of Hakai is involved in the stabilization of δ-catenin by Hakai.

3.2. Hakai and δ-catenin do not associate physically

To demonstrate whether there is a physical association between Hakai and δ-catenin, we performed immunoprecipitaion experiments using anti-δ-catenin antibody. Bosc23 cells were transiently transfected with δ-catenin-GFP and Hakai-Flag. GFP and Presenilin 1 CTF were used as negative and positive controls respectively for immunoprecipitation with δ-catenin antibody. As a result, we observed that there is no physical association between Hakai and δ-catenin. We subsequently confirmed this result by immunoprecipitation experiment in reverse direction using Hakai antibody and Western blot with δ-catenin (Fig. 4B). We concluded that the stabilizing effect of Hakai on δ-catenin does not occur from a physical interaction between these two proteins as no direct binding was detected between these two proteins (Fig. 4A and B).

Next, we investigated the cellular localization of Hakai and δ-catenin proteins by transfecting Hakai-GFP and δ-catenin-RFP expression

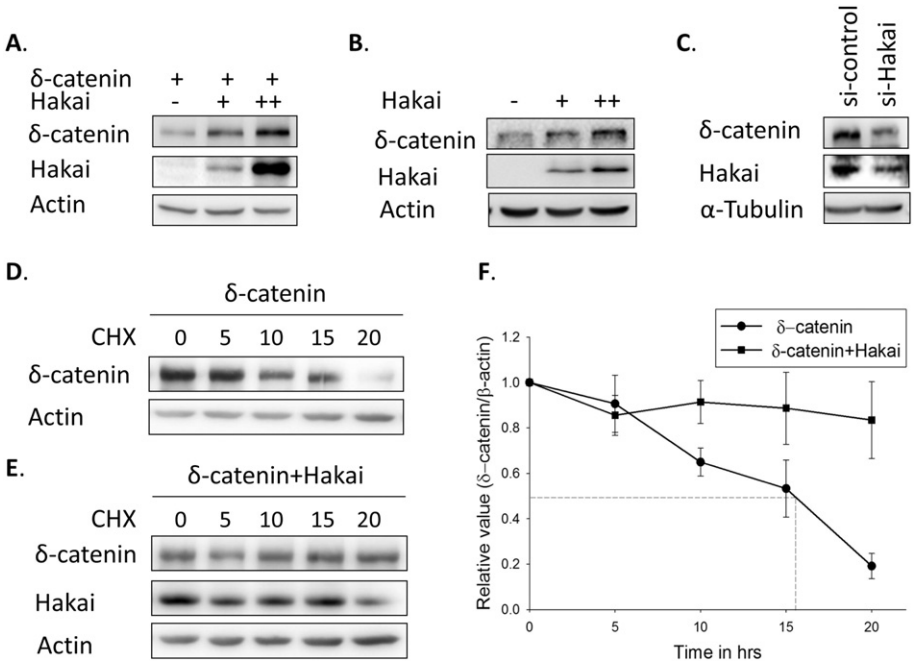

Fig. 1. Hakai increases δ-catenin stability. (A) Overexpressing Hakai in Bosc23 cell increased δ-catenin protein level. (B) Increased endogenous δ-catenin was observed with Hakai overexpression in CWR22Rv-1 cells. (C) Hakai si-RNA in δ-catenin stable CWR22Rv-l cells decreased the level of δ-catenin compared to the cells transfected with control si-RNA. (D, E) Bosc23 cells were transfected with δ-catenin alone or δ-catenin along with Hakai-Fl and the cells were treated with 50 μM cycloheximide for different times after transfection for 24 h. The cell lysates were collected and subjected to Western blot. (F) Relative δ-catenin protein levels in Hakai-expressing and non-expressing cells. Protein densities in Western blots in D and E were measured, and the δ-catenin protein level was normalized to the β-actin protein level. The experiment was repeated three times and the average and standard deviation are shown.

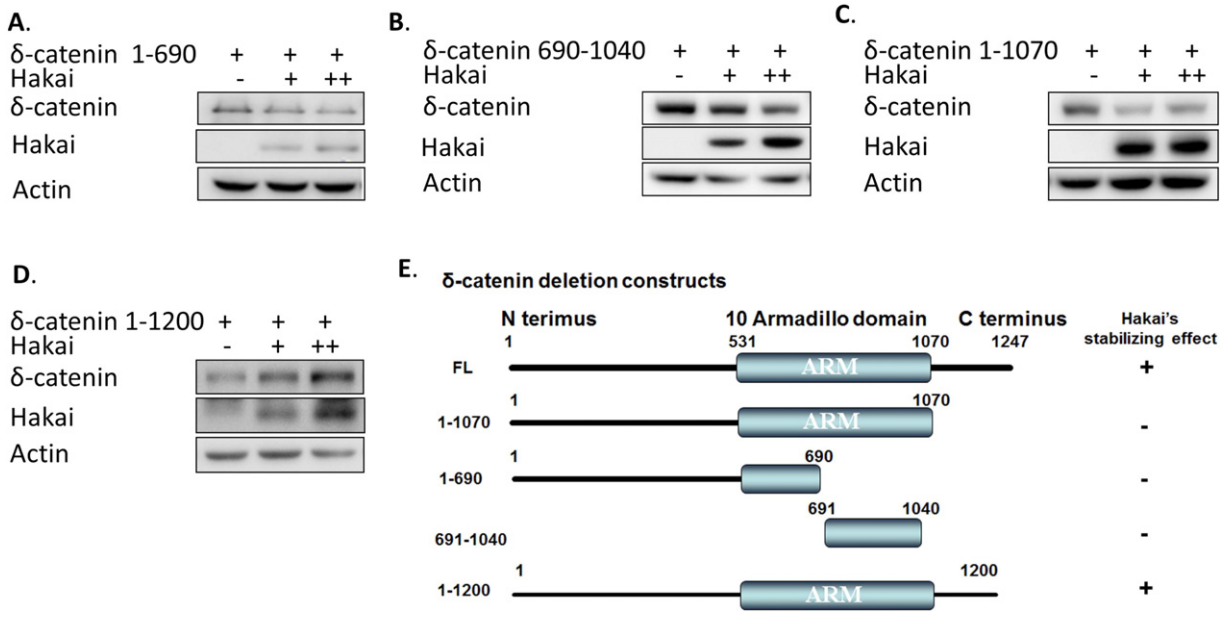

Fig. 2. Effect of Hakai overexpression on different δ-catenin deletion mutants. (A–C) No change in δ-catenin 1–690, 690–1040, and 1–1070 mutants expression was observed when transiently co-transfected with Hakai in Bosc 23 cells. (D) However, increased expression of the δ-catenin mutants was observed when δ-catenin 1–1200 was transiently transfected with Hakai in Bosc 23 cells, indicating that δ-catenin amino acids 1070–1200 are essential for the stabilizing effect of Hakai on δ-catenin. (E) Pictorial summary of the effects of overexpressing Hakai on different δ-catenin deletion mutants showing that δ-catenin amino acids 1070–1200 are essential for the stabilizing effect of Hakai on δ-catenin.

plasmids into CWR22Rv-1 and PC3 cells. We used confocal microscopy to localize Hakai and δ-catenin (Fig. 4C). We found out that Hakai was mainly localized in the nucleus, some in the cytoplasm and rarely in plasma membrane. In contrast, most of the δ-catenin was localized in the plasma membrane. The different subcellular localization of Hakai and δ-catenin further supports the suggestion that Hakai-mediated stabilization of δ-catenin is not through their physical association.

3.3. Hakai stabilizes δ-catenin through Src kinase

Our previously published data show that Src kinase phosphorylates δ-catenin on its tyrosine residues and Y1073, Y1112, and Y1176 are the predominant sites responsible for tyrosine phosphorylation mediated by c-Src [31]. As Hakai is a known E3 ubiquitin ligase that interacts with E-cadherin in a tyrosine phosphorylation-dependent manner, our previously published data on tyrosine phosphorylation of δ-catenin by Src suggests that Hakai possibly act as an E3 ligase for δ-catenin. To investigate the possible E3 ligase activity of Hakai for δ-catenin, we transfected δ-catenin-GFP, Hakai-Flag, Src kinase, and HA-ubiquitin expression plasmids into Bosc23 cells (Fig. 5A). The cells were treated with ALLN, a specific inhibitor of proteosome-dependent proteolysis to inhibit ubiquitinated δ-catenin proteosome-dependent proteolysis. Ubiquitinated δ-catenin was immunoprecipitated with HA antibody. Interestingly, Hakai decreased the level of ubiquitinated δ-catenin and

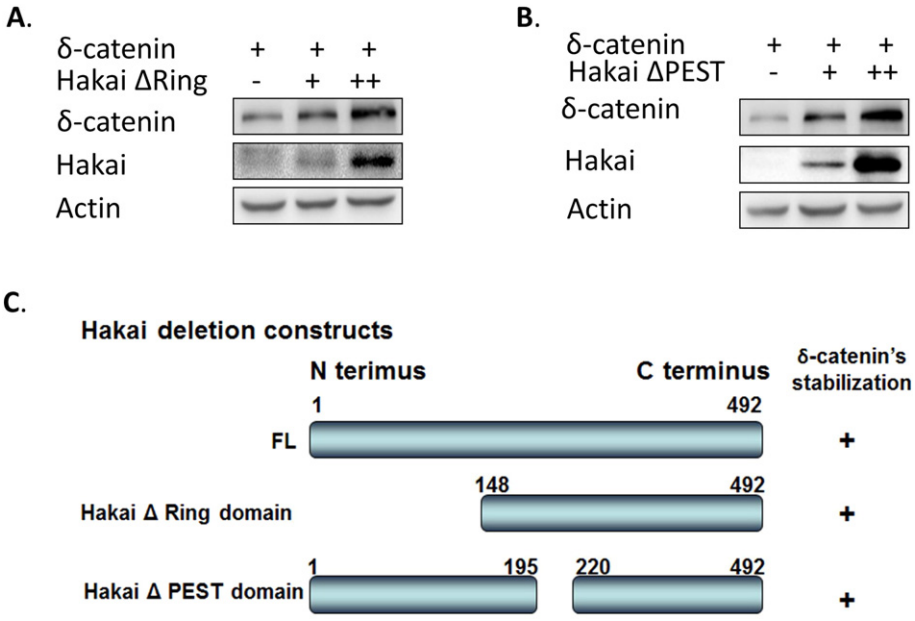

Fig. 3. Effects of Hakai mutants on δ-catenin. (A) Effect of the Hakai Ring domain deletion mutant on the protein stability of δ-catenin. (B) Effect of Hakai deletion mutant of the PEST motif on the stability of δ-catenin. (C) Pictorial summary of effects of overexpressing Hakai constructs on δ-catenin, showing neither the E3-ligase activity nor PEST motif of Hakai is necessary for Hakai mediated stabilization of δ-catenin.

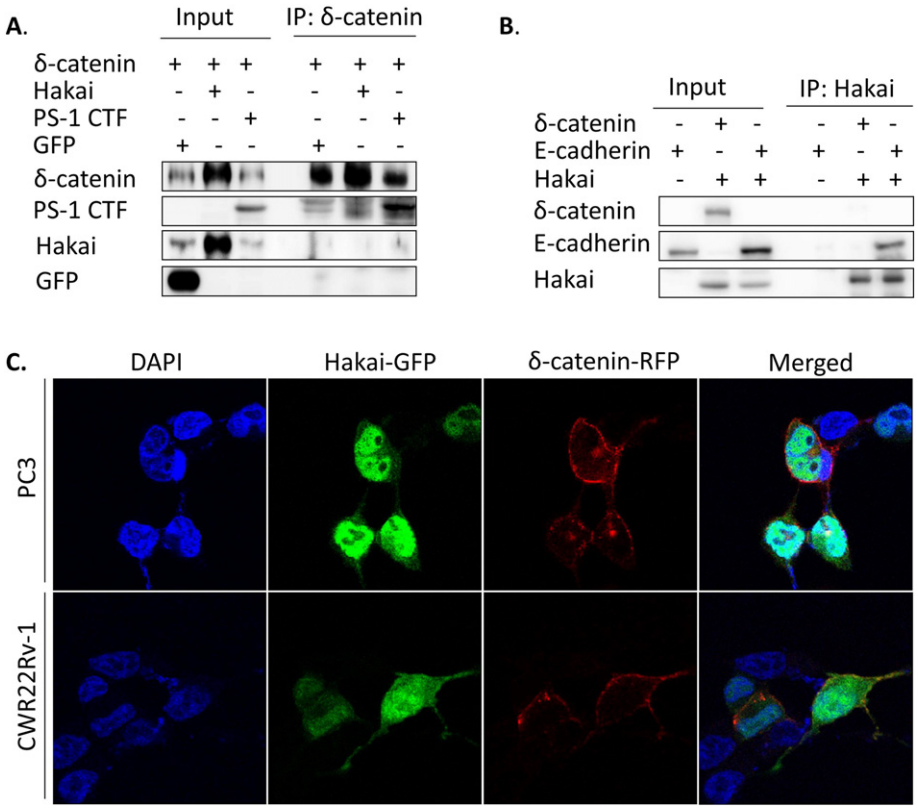

Fig. 4. Hakai and δ-catenin do not associate physically. (A) Immunoprecipitation with anti-δ-catenin antibody was performed using whole-cell lysates of Bosc23 cells, which revealed that there is no direct binding between δ-catenin and Hakai. GFP was used as the negative control and Presenilin 1-CTF was used as the positive control for immunoprecipitation experiment. (B) Immunoprecipitation with anti-Hakai antibody was performed using whole-cell lysates of Bosc23 cells. Consistent with previous finding, immunoprecipation with anti-Hakai antibody shows that there is no direct binding between δ-catenin and Hakai. E-cadherin was used as the positive control for immunoprecipitation with Hakai antibody. (C) Confocal microscopy shows localization of Hakai-GFP (Green) and δ-catenin-RFP (Red) in the cell. Hakai was localized mainly in the nucleus, some in the cytoplasm and rarely in plasma membrane, whereas δ-catenin was localized mostly in the plasma membrane in PC3 and CWR22Rv-1 cells.

increased total δ-catenin level. This effect was more profound in the presence of Src (Fig. 5A). Taken together, we concluded that Hakai decreases ubiquitination of δ-catenin resulting in up-regulation of δ-catenin stability and this stabilizing effect was enhanced by overexpressed Src kinase.

As stabilizing effect of δ-catenin by Hakai was greater in the presence of Src, we performed Src knockdown experiments in Bosc23 cells. We observed that Hakai did not increase δ-catenin level in cells transfected with Src si-RNA in contrast to that in cells transfected with control si-RNA (Fig. 5B). Taken together, these results suggest that c-Src-mediated Tyr-phosphorylation of δ-catenin is the possible reason of the stabilizing effect of Hakai on δ-catenin.

Our previously published data show that GSK-3β interacts with and phosphorylates δ-catenin and thereby enables its ubiquitination/proteosome-mediated proteolysis [32]. On the other side, c-Src-mediated Tyr-phosphorylation of δ-catenin increases its stability by decreasing its affinity to GSK-3β [31], suggesting a possible mechanism for the Hakai mediated stabilization of δ-catenin. To confirm that Hakai decreases the affinity of δ-catenin to GSK-3β by enhancing the Src level, we checked the effect of Hakai on δ-catenin and GSK3β binding by immunoprecipitation with anti-δ-catenin antibody. Protein density was measured and it was found out that the ratio of GSK3β bound to δ-catenin was lower in presence of Hakai (Fig. 5C). We confirmed less binding between δ-catenin and GSK3β in presence of Hakai by performing immunoprecipitation in reverse direction using anti-HA antibody (Fig. 5D). The presence of Hakai decreased the binding between GSK3β and δ-catenin whereas no change in the levels of GSK3β or phospho-GSK3β were detected (Fig. 5E). Moreover, we checked the effect of Hakai on δ-catenin T1078A construct in Bosc23 cells and found out that Hakai does not stabilize this mutant (Fig. 5F). As Thr 1078 phosphorylation of δ-catenin is essential for GSK-3β mediated ubiquitination and degradation of δ-catenin [32], all of these findings indicate that Hakai stabilizes δ-catenin through Src kinase by inhibiting affinity of δ-catenin to GSK-3β. To confirm that kinase activity of Src is essential for Hakai's stabilizing effect on δ-catenin, we transiently co-transfected Bosc23 cells with different Hakai gene dosage ratios and same dose of δ-catenin. We treated the transfected cells with SU6656, a specific Src family kinase inhibitor. Overexpressed Hakai did not increase protein level of δ-catenin when the cells were treated with SU6656 (Fig. 5G). Furthermore, we also overexpressed Hakai to see its effect on δ-catenin in presence of overexpressed RF-Src which is kinase inactive form of c-Src. Consistent with Src inhibitor experiment; Hakai did not increase level of δ-catenin when RF-Src was dominant (Fig. 5H). Taken together, these results suggest that c-Src-mediated Tyr-phosphorylation of δ-catenin is necessary for Hakai mediated δ-catenin's stabilization.

Moreover, we investigated the effect of Hakai on Src. Bosc23 cells were transiently co-transfected with different Hakai gene dosage ratios and same dose of Src plasmids. Total cell lysates were immunoblotted using anti-δ-catenin, anti-Hakai, and anti-actin antibodies. We found out that overexpressed Hakai increased the level of Src (Fig. 6A). The increased level of Src by Hakai was further confirmed with endogenously expressed Src (Fig. 6B). Furthermore, we transfected si-Hakai in CWR22Rv-1 cells and found out that lower level of endogenous Src was expressed in si-Hakai transfected cells than that in control si-RNA transfected cells (Fig. 6C).

In addition, Hakai overexpressed cells exhibited an increased Src half-life compared with that of the Hakai non-transfected cells, as

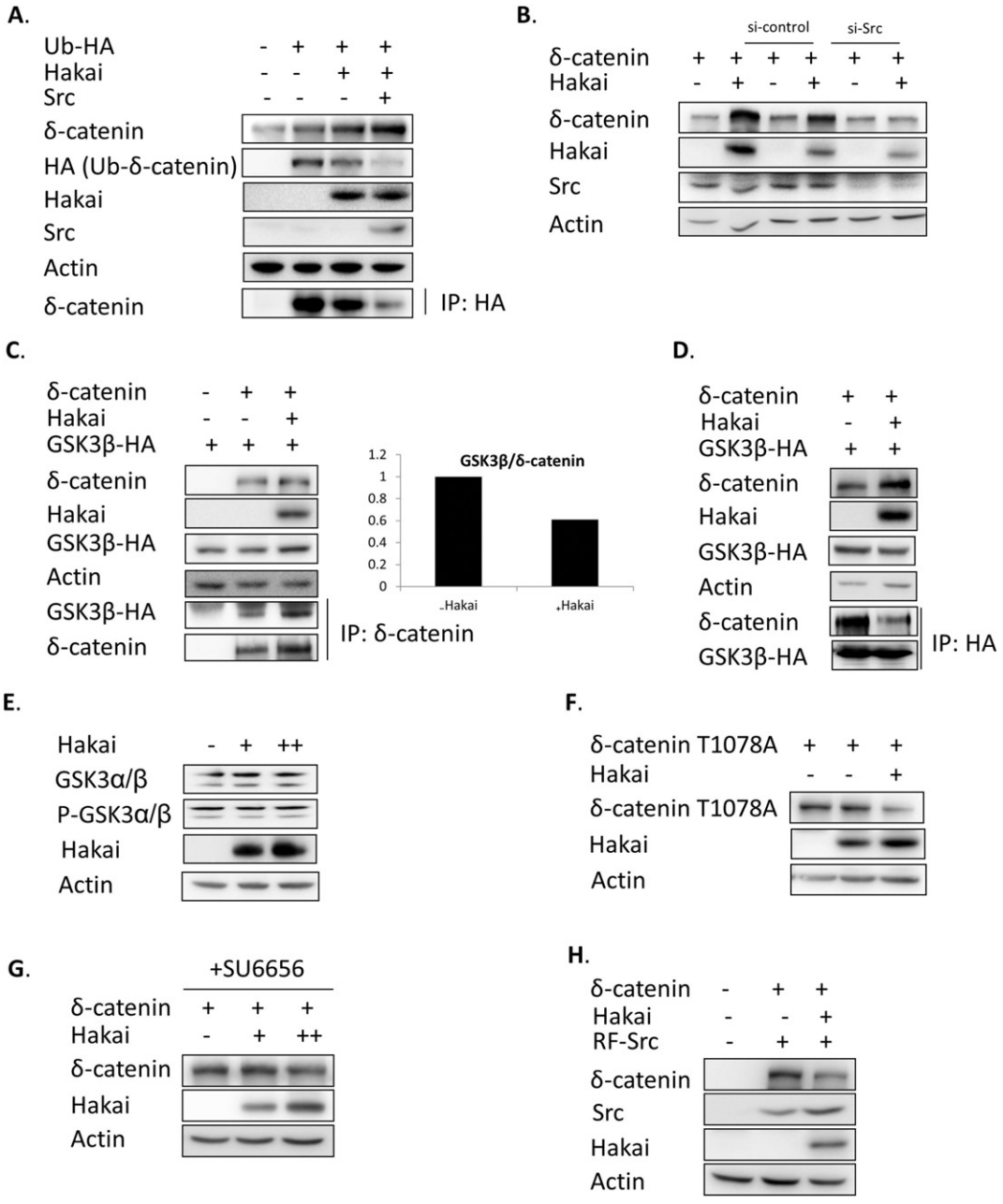

Fig. 5. Hakai stabilizes δ-catenin through Src kinase. (A) Hakai decreases δ-catenin ubiquitination. Bosc23 cells were transiently transfected with δ-Catenin-GFP, Hakai-Flag, Src kinase, and HA-ubiquitin expression plasmids. Ubiquitinated δ-catenin was immunoprecipitated with anti-HA antibody. Hakai increased total level of δ-catenin but decreased ubiquitinated δ-catenin. (B) Hakai did not increase δ-catenin level in cells transfected with Src si-RNA in contrast to that in cells transfected with control si-RNA. (C, D) Less binding of GSK-3β to δ-catenin was observed in the presence of Hakai, suggesting inhibited GSK-3β-dependent proteosomal degradation of δ-catenin. (E) No change in GSK-3β or phospho-GSK-3β levels was detected when Hakai was overexpressed. (F) Hakai did not stabilize δ-catenin T1078A. (G) δ-Catenin and two different dosage ratios of Hakai was co-transfected into Bosc23 cells. After transfection for 24 h, a specific Src kinase inhibitor, SU6656, was added into the culture media. The cells were maintained in this media containing 10 nM SU6656 for 24 h before being harvested. Western blot analysis of the cell lysates showed overexpressed Hakai does not increase protein level of δ-catenin when the cells were treated with SU6656. (H) Hakai did not increase δ-catenin level in cells when kinase inactive RF-Src was overexpressed.

demonstrated by treating the cells with protein biosynthesis inhibitor cycloheximide. The protein half-life of Src was about 12 h, whereas it was >18 h in the presence of Hakai (Fig. 6D–E). We also checked the physical interaction of Hakai and δ-catenin in Bosc23 cells and found out that Hakai and Src interacts physically (Fig. 6F). The physical association between Hakai and Src-HA was further confirmed by performing immunoprecipitation in reverse direction using anti-HA antibody and immunoblotted with anti-Hakai antibody (Fig. 6G). All of these findings indicate that Hakai stabilizes Src kinase which, in turn, stabilizes δ-catenin by inhibiting GSK-3β mediated ubiquitination and degradation.

3.4. δ-Catenin mainly localizes in the plasma membrane regardless of E-cadherin endocytosis after Hakai overexpression

δ-Catenin has been found to be colocalized with E-cadherin at the plasma membrane as it binds to the juxtamembrane domain of E-cadherin. As Hakai is known to endocytose E-cadherin, we were curious to know the change in localization of δ-catenin after Hakai overexpression. Firstly, we could not observe much change in total E-cadherin level when Hakai was overexpressed. However, we observed total E-cadherin was slightly downregulated when Hakai and Src both were

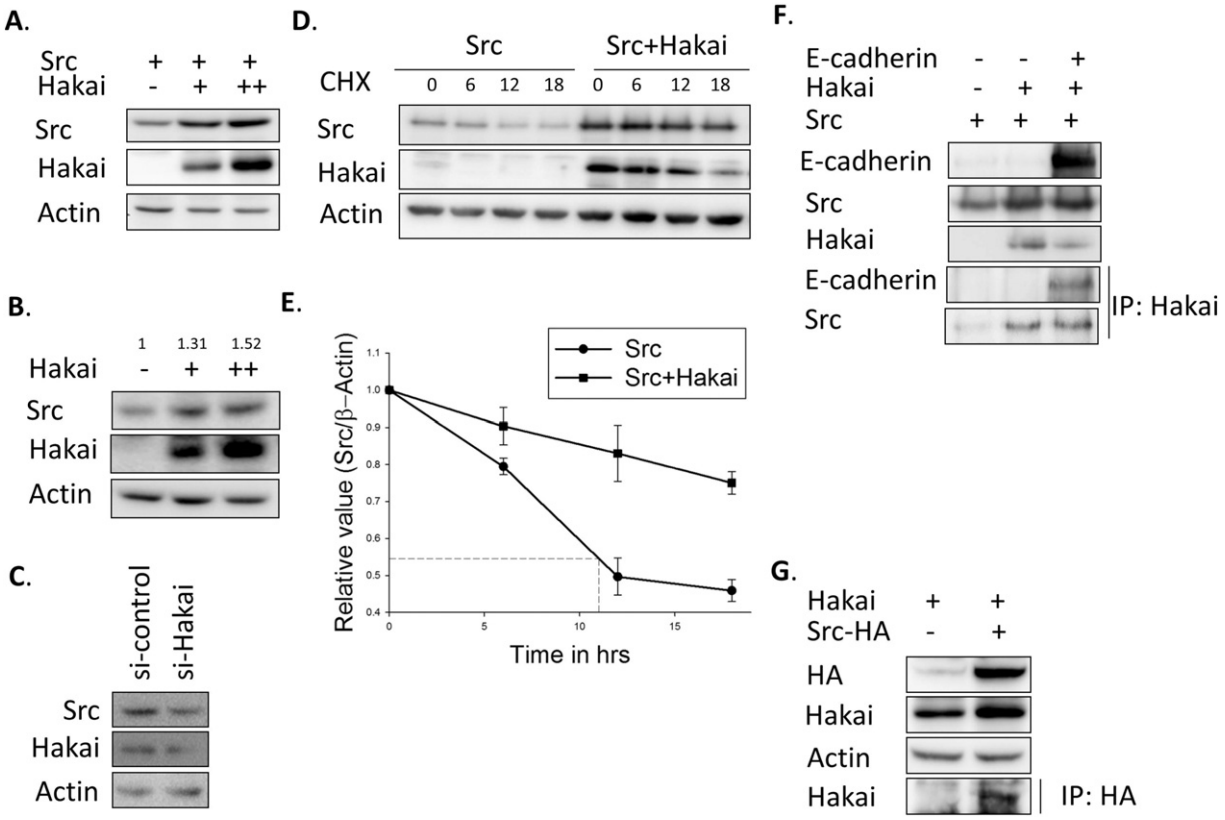

Fig. 6. Hakai stabilizes δ-catenin through Src kinase. (A) Increased Src was observed when Src was co-transfected with Hakai into Bosc23 cells. (B) Overexpressing Hakai in CWR22Rv-1 cells increased endogenous Src protein expression. The numbers at the top represent relative values of the protein density of Src to protein density of β-Actin. (C) Hakai did not stabilize δ-catenin in Src si-RNA transfected cells. (D) Bosc23 cells were transfected either with Src only or with both Src and Hakai. The cells were treated with 50 μM cycloheximide for different times, as indicated, after transfection for 24 h. The cell lysates were collected and subjected to Western blot analysis. (E) Src protein density was measured in the Western blots and normalized by protein level of β-actin. Src protein half-life was about 12 h, whereas it was >18 h in the presence of Hakai. The experiment was repeated three times and average and standard deviations are shown. (F) Immunoprecipitation with anti-Hakai antibody was performed to investigate physical interaction between Hakai and Src using whole-cell lysates of Bosc23 cells, which revealed that Hakai and Src interact physically. E-cadherin was used as the positive control for immunoprecipitation experiment with anti-Hakai antibody. (G) The physical interaction between Hakai and Src-HA was further confirmed by performing immunoprecipitation in reverse direction using anti-HA antibody for Src-HA and immunoblotted with anti-Hakai antibody.

transiently transfected in CWR22Rv-1 cells (Fig. 7A and B). Decreased membranous E-cadherin level was observed when Hakai was overexpressed and the decrease in membranous E-cadherin was higher when Hakai and Src both were overexpressed (Fig. 7C and D). The immunostaining of E-cadherin before and after Hakai overexpression also did not show remarkable change in membranous E-cadherin level in CWR22Rv-1 and MCF7 cells (Fig. 7E). This implies that endocytosis of E-cadherin seems to have not much significant effect on change in localization of membranous δ-catenin. We did immunostaining experiments to check the effect of Hakai overexpression on the localization of δ-catenin and found out that δ-catenin remains at plasma membrane even after Hakai overexpression (Fig. 8).

In terms of the localization of δ-catenin in plasma membrane after Hakai overexpression, possible explanation is that δ-catenin associates with other membranous proteins that Hakai does not downregulates. It has also been reported that palmitoylation increases δ-catenin's hydrophobicity and traffics it to the plasma membrane [33]. The localization of δ-catenin in plasma membrane even in PC3 cells, which has very low level of expression of E-cadherin (Fig. S1) justifies our explanation on localization of δ-catenin in plasma membrane regardless of E-cadherin endocytosis after Hakai overexpression. All the δ-catenin constructs were found to be mainly localized in the plasma membrane except δ-catenin 690–1040 which was mainly localized in cytoplasm. Even though Hakai overexpression increases levels of δ-catenin WT and 1–1200 but decreases δ-catenin 1–690, 690–1040 and 1–1070 (Fig. 2), all the plasma membrane-localized δ-catenin mutants remained in the plasma membrane even after Hakai overexpression. This strongly justifies our explanation on unaltered localization of δ-catenin from plasma membrane after Hakai overexpression regardless of E-cadherin endocytosis.

4. Discussion

δ-Catenin and p120-catenin bind to the juxtamembrane domain regions of E-cadherin, whereas β-catenin binds to the distal region of cytoplasmic domain of E-cadherin [12]. E3-ligase function of Hakai mediates the ubiquitination of E-cadherin and β-catenin [21]. It has been also reported that competitive binding between p120-catenin and ubiquitination of E-cadherin by Hakai at the juxtamembrane domain regulate E-cadherin degradation [35]. Though interactions of Hakai with p120 catenin and β-catenin have been reported, we found no report on interaction between Hakai and δ-catenin.

This study reports that Hakai stabilizes δ-catenin regardless of its E3 ligase activity. Furthermore, we showed that Src kinase activity is essential for Hakai mediated stabilizing effect of δ-catenin. This increased stability of δ-catenin by Hakai suggests possible downregulation of the negative regulators of δ-catenin due to E3-ligase activity of Hakai. However, we found that the Hakai RING and PEST domain deletion mutants could still stabilize δ-catenin. The RING finger domain is important for ubiquitin-ligase activity, whereas the PEST sequence motif is important for degradation of target proteins [27]. These findings suggest that there is no involvement of target protein degradation by Hakai on its stabilizing effect on δ-catenin. However, it was found that Hakai decreased the expression of δ-catenin constructs lacking Thr1078 (Fig. 5F). This

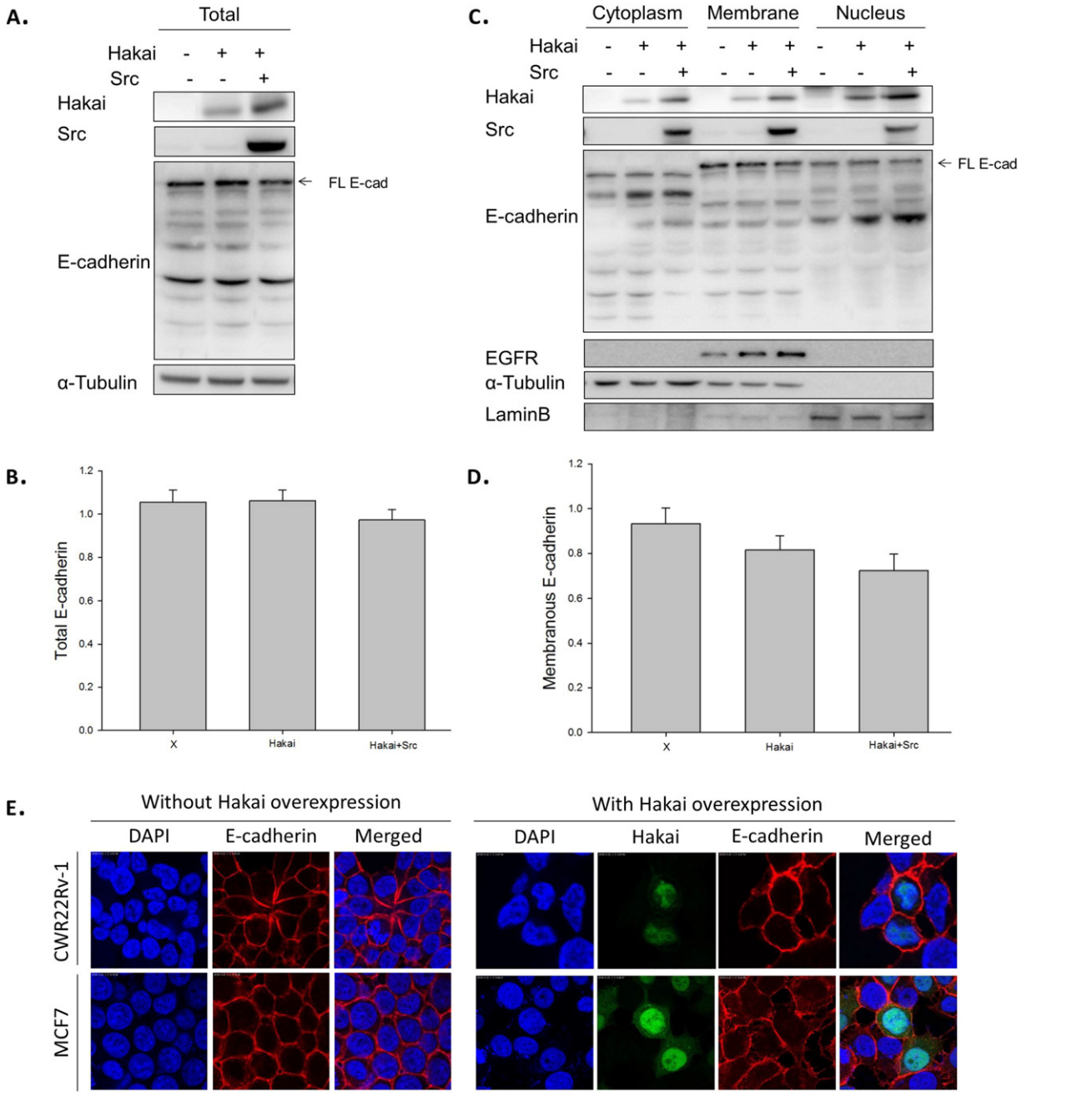

Fig. 7. Effect of Hakai overexpression on E-cadherin. CWR22Rv-1 cells were transiently transfected with Hakai-Fl and Src. After 24 h of transfection, protein lysates from different subcellular fractions were collected. (A, B) Western blot analysis of total cell lysate shows no significant decrease in E-cadherin level with Hakai overexpression. (C, D) Membranous E-cadherin was decreased with overexpression of Hakai. This effect was higher in the presence of exogenous Src. (E) Immunostaining shows that Hakai overexpression does not significantly affect the endocytosis of E-cadherin in CWR22Rv-1 and MCF7 cells.

suggests the possibility of Hakai's negative regulatory effect on δ-catenin through some other mechanisms but its stabilizing effect through inhibiting GSK-3β mediated ubiquitination is dominant over its negative regulatory effect. Furthermore, we showed that Hakai and δ-catenin do not bind physically, suggesting the stabilizing effect of Hakai on δ-catenin is not due to physical interaction between these two protein. Src kinase phosphorylates δ-catenin on its tyrosine residues, and δ-catenin Y1073, Y1112, and Y1176 are the predominant sites responsible for tyrosine phosphorylation mediated by c-Src [31]. As Hakai is a known E3 ubiquitin ligase that interacts with E-cadherin in a tyrosine phosphorylation-dependent manner by Src, our previously published data on tyrosine phosphorylation of δ-catenin by Src suggests that Hakai may act as a δ-catenin E3 ligase. In this study, we showed that Hakai decreases ubiquitinated δ-catenin level and increases total δ-catenin level, and this effect was greater in presence of Src. Thus, we concluded that Hakai decreases ubiquitination, which stabilizes δ-catenin, and this stabilizing effect is enhanced by Src kinase. This finding raises a question about the relationship between Src and Hakai. We found out that Hakai stabilizes Src. Our previously published data show that c-Src-mediated Tyr-phosphorylation of δ-catenin increases its stability by decreasing its affinity to GSK-3β [31], suggesting a role for Src kinase in the Hakai mediated δ-catenin stabilization. We propose a model that Hakai stabilizes δ-catenin via Src-mediated Tyr-phosphorylation (Fig. 9). Further investigation will be required to define the possible mechanisms for Hakai mediated Src stabilization and its possible functional effects.

Hakai regulates cell-cell contacts and cell proliferation through E-cadherin dependent or independent manner. Studies show that Hakai

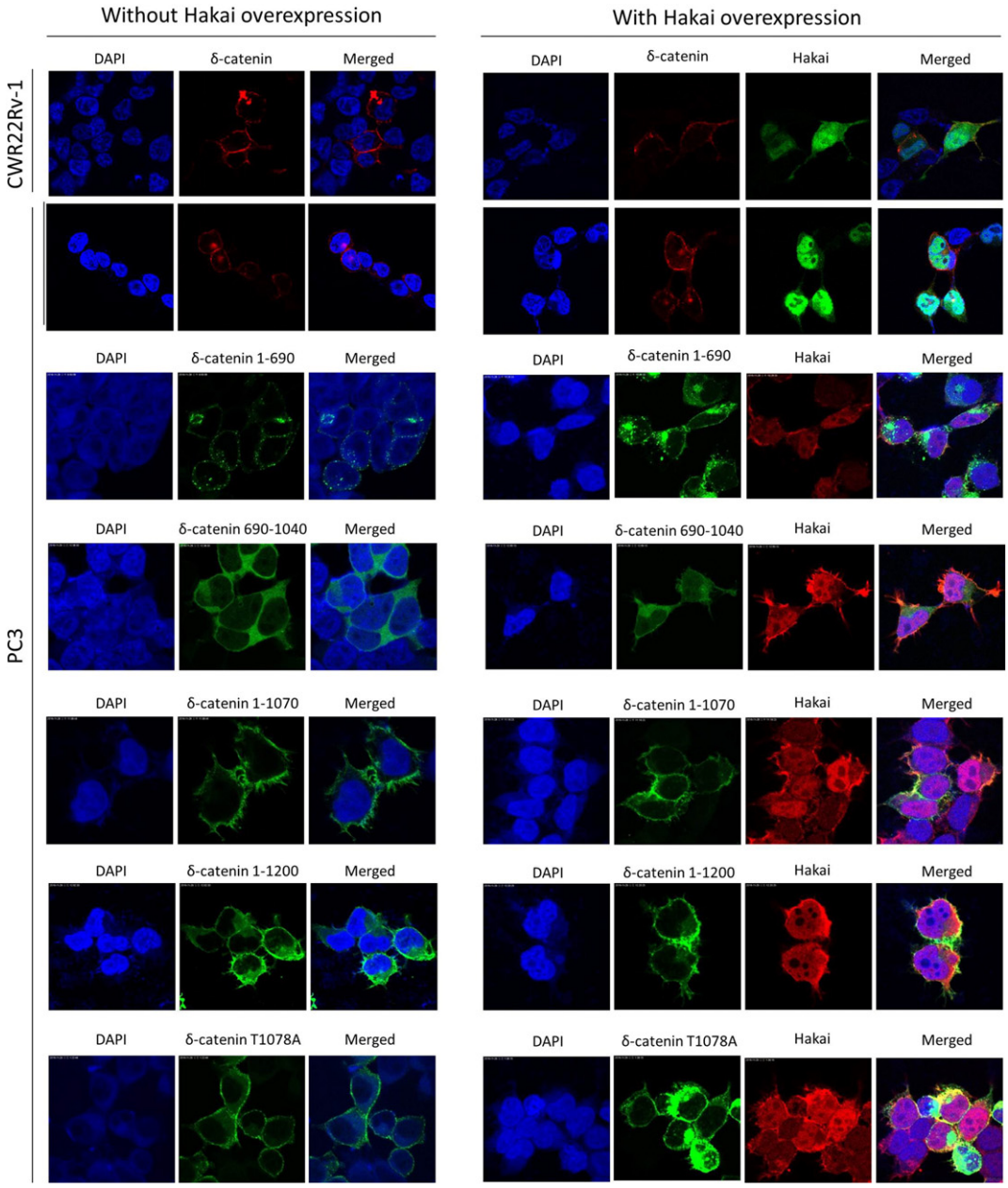

Fig. 8. δ-Catenin-RFP and Hakai-GFP were transfected in CWR22Rv-1 and PC3 cells and subjected to confocal microscopy. Plasma membrane-localized δ-catenin remained in the plasma membrane regardless of E-cadherin endocytosis after Hakai overexpression. Different δ-catenin constructs were also transfected with and without Hakai in PC3 cells. δ-Catenin 1–690, 1–1070, 1–1200 and T1078A were found to be localized in plasma membrane whereas δ-catenin 690–1040 was in cytoplasm. δ-Catenin constructs which localize in plasma membrane also remained in plasma membrane regardless of Hakai overexpression.

may exert either positive or negative control of cell proliferation in different conditions. For example, overexpression of Hakai increased cell proliferation in MDCK cells whereas Hakai inhibited estrogen dependent growth of MCF-7 cells [27]. The study of correlation between Hakai and E-cadherin in several adenocarcinoma showed a low expression of Hakai and E-cadherin or an inverse correlation between these two proteins. On the other side, δ-catenin which is known to be overexpressed in some tumors has been found to be colocalized with E-cadherin at the plasma membrane. δ-Catenin binds to the juxtamembrane domain of E-cadherin [34] and progresses cancer through E-cadherin processing [12]. Further investigation will be required to define the possible competition between δ-catenin and Hakai for binding with E-cadherin. As Hakai is a well-known E-cadherin E3 ligase that binds E-cadherin and promotes endocytosis and destruction of the E-cadherin complex, further investigation is required to understand the fate of E-cadherin-bound catenins after endocytosis of E-cadherin to clarify the influence of Hakai on cell phenotype, including cell-substrate and invasion capability into tumor cells. More investigations are needed to address the apparent relevance of the ubiquitin-independent functions of Hakai that may influence cell phenotype, to highlight the influence of different signaling pathways on Hakai, and to investigate its clinical potential usefulness as a therapeutic target for cancer.

5. Conclusion

In this study we show Hakai stabilizes δ-catenin. We found out that Hakai-induced stabilization of δ-catenin is independent of Hakai's E3 ligase activity. Furthermore, we show that Hakai stabilizes δ-catenin through Src kinase. Hakai stabilizes Src which in turn phosphorylates

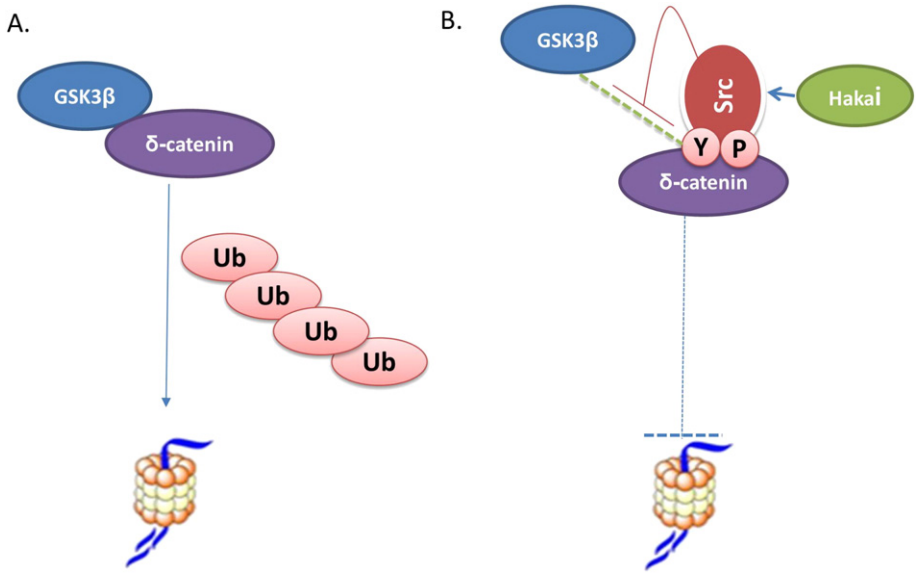

Fig. 9. A schematic diagram for the role of Hakai-induced stabilization of δ-catenin. (A) GSK-3β binding to δ-catenin leads to ubiquitination and degradation of δ-catenin through proteosomal degradation pathway. (B) Hakai stabilizes Src kinase which in turn phosphorylates tyrosine residues of δ-catenin and causes inhibition of GSK-3β mediated ubiquitination and degradation of δ-catenin.

δ-catenin which leads to less GSK-3β mediated ubiquitination and eventually stabilization of δ-catenin.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2017.01.009.

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