T606-phosphorylation deprives the function of Kaiso as a transcription repressor

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Abbreviations

KBS: Kaiso binding sequence

pT606: T606-phosphorylated

Phos-tag: Phos-tag molecular binds specifically to phosphate group in proteins via metal ions

CIAP: calf intestinal alkaline phosphatase

pAKT-Sub: phosphorylated AKT substrate

wt: wildtype

T606A: replacement of the 606th amino acid residue threonine with alanine

Y442F: replacement of the 442th tyrosine with phenylalanine

14-3-3 BM mAb: 14-3-3 binding motif antibody

IP: immunoprecipitation

ELISA: enzyme-linked immunosorbent assay

BSA: bovine serum albumin

PBS: Phosphate-buffered saline

DAPI: 4', 6-diamidino-2-phenylindole

FITC: fluorescein isothiocyanate

CY5: Cyanine5

HC: Heavy chain of IgG

Abstract

It is well known that Kaiso protein encoded by *ZBTB33* gene is a transcription repressor and that Kaiso-P120ctn interaction increases the shift of Kaiso from the nucleus into the cytoplasm. However, the regulatory mechanisms of Kaiso compartmentalization are far from clear. Here, we reported that AKT1 could phosphorylate 606-threonine residue (T606) within the RSSTIP motif of Kaiso in the cytoplasm. The pT606-Kaiso could directly bind to 14-3-3 family proteins and the loss of T606 phosphorylation byT606A mutation abolished most of the Kaiso-14-3-3 binding. In addition, the Kaiso-P120ctn interaction was essential for the pT606-Kaiso accumulation in the cytoplasm. Notably, enforced *14-3-3σ* overexpression could increase the pT606-Kaiso accumulation in the cytoplasm and de-repress the transcription of Kaiso target genes, including *CDH1*. Decreased amounts of both T606-phosphorylated Kaiso (pT606-Kaiso) and CDH1 were frequently observed in human gastric and colon cancer tissues relative to paired normal controls. The mRNA levels of *14-3-3σ/SFN* and Kaiso target gene *CDH1* were positively and significantly correlated with each other in bioinformatics analyses using publicly available RNA-seq datasets for human normal tissues (n=11688, r=0.60, p<0.001) in the GTEx project and for cancer cell lines (n=1156, r=0.41, p<0.001) in the CCLE project. In conclusion, Kaiso could be phosphorylated by AKT1 at the T606 and the pT606-Kaiso accumulates in the cytoplasm through binding to 14-3-3/P120ctn that de-represses the expression of Kaiso target genes and may contribute to the development of gastrointestinal cancer.

Keywords: Kaiso, phosphorylation, AKT1, 14-3-3, P120ctn, transcription factor, cancer

Introduction

Kaiso protein encoded by the *ZBTB33* gene is a classic transcription repressor containing a zinc-finger domain and a BTB/POZ domain (1). The zinc-finger domain of Kaiso can bind to both methylated CGCG-containing and non-methylated Kaiso binding-specific sequences of Kaiso target genes, and the BTB/POZ domain can further recruit the complex of NCoR1 corepressor and histone deacetylases to target genes and repress their transcription in the nucleus (2-4). Recent studies show that Kaiso may also act as a transcription activator in the promoter context-dependent manner (5, 6).

As a transcription repressor, Kaiso controls the cell cycle through repressing *CCND1* and *CCNE1* expression, affects Notch signaling pathway in intestinal cells through targeting *DLL1* and *JAG1* promoter, and inhibits the proliferation and invasion of tumor cells through downregulating *MMP7*, *MTA2* and other genes (7-10). Kaiso also represses *CDH1* and *CDKN2A* expression (11,12). Interestingly, it has been reported that the amount of nuclear Kaiso, but not total Kaiso, is correlated with the invasion or prognosis of cancers (13, 14). Kaiso-deficient mice show resistance to intestinal cancer (15). Apparently, the expression and subcellular location states of Kaiso determine its normal functions and roles in cancer development.

Kaiso is also a cytoplasm protein, which regulates WNT-related pathway through interacting with P120ctn protein (16). Differences of subcellular locations of Kaiso are observed between cultured cells and tissues (12, 17). The P120ctn-Kaiso complexes could shift from the nucleus to the cytoplasm (18). Kaiso nuclear-cytoplasmic trafficking could be affected by environmental factors, such as cigarette smoke, through MUC1 and P120ctn binding (19). However, detailed regulation machinery for the compartmentalization of Kaiso remains far from clear.

The 14-3-3 proteins are originally identified in the brain (20). There are seven human 14-3-3 isoforms (α/β, ε, η, δ/γ, τ, ζ, σ). These 14-3-3 isoforms are homologous proteins with approximately 50% amino acid identity, capable of forming either homo- or hetero-dimers (21-24). Recent findings have implicated 14-3-3 as a key regulator of signal transduction events (25). Among the family, 14-3-3γ and 14-3-3σ have been confirmed to play important roles in cancer development (26-29).

In the present study, we found, for the first time, that Kaiso could be phosphorylated at Thr-606 (T606) within the RSSTIP motif by the protein serine-threonine kinase AKT1 and

T606-phosphorylated Kaiso (pT606-Kaiso) could efficiently interact with 14-3-3 family member and P120ctn proteins, and accumulate in the cytoplasm that in turn blocks transcription repressor function of Kaiso and de-repressed the expression of its target gene *CDH1*. Relative to the paired normal control tissues, the level of pT606-Kaiso was markedly decreased in human gastric and colon cancer tissues that could lead to transcriptional repression of the *CDH1* gene.

Materials and Methods

Cell lines and culture

The gastric cancer cell lines MGC803, BGC823 and SGC7901 were kindly provided by Dr. Yang Ke at Peking University Cancer Hospital; MKN45 cell line was purchased from the National Infrastructure of Cell Line Resource (Beijing, China). The human embryonic kidney HEK293T cell line was kindly provided by Professor Yasuhito Yuasa at Tokyo Medical and Dental University. The human colon cancer cell line HCT116 was kindly provided by Dr. Yuanjia Chen, Peking Union Hospital; RKO cell line, by Dr. Guoren Deng, University of California. HEK293T cells was cultured in DMEM medium containing 10% FBS, and all the others were cultured in RPMI 1640 medium containing 10% FBS and 100 U/mL penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified incubator with 5% CO_2. These cell lines were tested and authenticated by Beijing JianLian Gene Technology Co., before use. Short tandem repeat (STR) patterns were analyzed using GoldeneyeTM20A STR Identifiler PCR Amplification Kit.

Gastric and colon carcinoma tissues and ethical issue

Gastric and colon cancer tissues and the paired normal surgical margin tissues were respectively collected from 4 patients with gastric carcinoma and 4 patients with colon carcinoma at Peking University Cancer Hospital from 2000 to 2001 and stored at −70 °C. The Institutional Review Board of the Peking University Cancer Hospital approved the study. All of the patients provided written informed consent.

Plasmids and reagents

The full-length Kaiso-coding sequence of the *ZBTB33* gene was amplified from human cDNA of

MGC803 cells with a primer set (forward 5'-attaaactcgaggcatggagagtagaaaactga-3' and reverse 5′-cgcttcgaattcgtttagtaagactctggtattat-3'), then inserted into *Xho*I and *Eco*RI sites of pEGFP-C1 vector to generate pEGFP-C1-Kaiso expression vector. pEGFP-C1-Kaiso-T606 mutants were obtained by mutation PCRs using a primer set (forward 5'-gatagatcaagcgctattcctgcaatg -3' and reverse 5'-cattgcaggaatagcgcttgatctatc-3') for 606Thr → Ala (T606A) mutation. Plasmid pCMV-3Tag-2C-Kaiso was generated by inserting the full-length Kaiso-coding sequence into *BamH*I and *Eco*RI sites of pCMV-3Tag-2C vector. Plasmid pEBG-GST-Kaiso was generated by inserting the full-length Kaiso coding sequence into *BamH*I and *Not*I sites of pEBG vector. Kaiso fragment (aa346-638) coding sequence without BTB/POZ domain was amplified with a primer set (forward 5'-tatcggaattccctgactcggccgtcagt-3' and reverse 5'-attgcctcgagcattggcttgttctgagt-3'), then inserted into *Eco*RI and *Xho*I sites of vector pGEX-4T-1.

pcDNA3.1-HA-AKT1 vector was purchased from Addgene (#9008, MA, USA); pEZ-M56-14-3-3γ-mCherry, pEZ-M56-14-3-3σ-mCherry vectors, from FulenGen (EX-T4084-M56, EX-T4084-M98-5, EX-C0507-M98, Guangzhou, China); pENTER-Flag-14-3-3 isoforms (α /β, ε, η, δ/γ, τ, ζ, σ) were purchased from Vigene Bioscience (CH867785, CH897212, CH845486, CH898602, CH878525, CH824520, CH890307, Shandong, China); Insulin (P3376, Beyotime, Shanghai, China), IL-6 (Cat. 200-06, Proteintech, NJ, USA), EGF (PHG6045, Thermo Fisher Scientific, MA, USA), MK2206 (HY-10358, MedChemExpress, NJ, USA) were also used in the study. *P120ctn*-specific siRNAs (#1: sense 5'-gaaugugaugguuuaguuuu-3' and antisense 5'-aacuaaaccaucacauucuu-3'; #2: sense 5'-uagcugaccuccugacuaauu-3' and antisense 5'-uuagucaggaggucagcuauu-3'; #3: sense 5'-ggaccuuacugaaguuauuuu-3' and antisense 5'-aauaacuucaguaagguccuu-3') were synthesized by Genepharma (Shanghai, China). The quantitative RT-PCR primer sequences for detection the level of *CDH1* mRNA were: forward 5'-gaacgcattgccacatacac-3' and reverse 5'-gaattcgggcttgttgtcat-3' (Tm=58 °C). The *Alu* RNA was used as the reference (forward 5'-gaggctgaggcaggagaatcg-3' and reverse 5'-gtcgcccaggctggagtg-3', Tm=60°C), as previously described (30).

Cell transfection and xenografts in nude mice

X-tremeGENE siRNA Transfection Reagent or X-tremeGENE HP DNA Transfection Reagent (Cat.

04476093001, Cat. 06366236001, Roche, Mannheim, Germany) were used in cell transfection with siRNAs for *p120ctn* (final concentration, 100 nM) or Kaiso or its mutant expression vectors (2 μg/well in 6 wells plate) following manufacturer's instructions. The efficiency of gene overexpression or knockdown was determined 48 or 72 hrs post transfection by Western blotting. For stable transfection, G418 was added into the medium to select consistent GFP-Kaiso expressing MGC803 cells (final concentration, 750 μg/mL). These cells resuspended in 0.2 mL PBS (1×10^7 cells/mL) were inoculated subcutaneously into the right/left pads of 6 week-old male BALB/c nude mice. Mice were sacrificed on days 21 after inoculation, and xenografts were separated and cut into about 1-mm^3 pieces for protein fractionation.

Subcellular fractionation and de-phosphorylation treatment (31, 32)

To prepare cytoplasmic and nuclear extracts, small pieces of tissue or cultured cells at 80% confluence were homogenized in ice-cold buffer A (REF04693159001, Roche, Mannheim, Germany) on ice for 20 min. Samples were then supplemented with NP-40 to 0.1% (v/v), vortexed, and centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatants were recovered to obtain the cytosolic extracts. The pellets were washed sequentially with ice-cold buffer A and then incubated in buffer B on ice for 20 min. After centrifuging at 14,000 g for 15 min, the extract was collected as nuclear proteins. The purities of cytoplasmic and nuclear extracts were respectively verified by probing with anti-β-Tubulin and anti-Lamin B antibodies.

For the calf intestinal alkaline phosphatase (CIAP)-catalyzed de-phosphorylation, the cytoplasmic and nuclear extracts were aliquoted into two centrifuge microtubes. CIAP (p4978, Merck, Darmstadt, Germany) (1 U/μg) of protein was added into one aliquot in the CIAP buffer and incubated for 30 min at 37 °C.

Immunoprecipitation (IP) and Western blotting

Antibodies for Kaiso (sc-365428, Santa Cruz, USA), P120ctn (66208-1, Proteintech, IL, USA), Pan-14-3-3 (sc-629, Santa Cruz), 14-3-3 family (α/β, ε, η, δ/γ, τ, ζ, σ) kits (#9769, CST, USA), 14-3-3σ (sc-100638, Santa Cruz), Ser/Thr/Tyr phosphor-protein (ab15556, Abcam, UK), phosphorylated AKT substrate [(R/K)X(R/K)XX(pT/pS)] (pAKT-sub; #9611, Cell Signaling

Technology), CDH1 (#3195, CST), Lamin B1 (66095-1, Proteintech), β-Tublin (66240-1, Proteintech), HA (M20003, Abmart, Shanghai, China), FLAG (66008-2, Proteintech), GFP (NB100-1614, Novus, CO, USA), GST (66001-1, Proteintech), GAPDH (660004-1, Proteintech) were used in the IP and Western blotting analyses.

After being pre-cleared with protein A/G-coupled Sepharose beads (Cat. 11134515001 and 11243233001, Roche, Mannheim, Germany) for 2 hrs, the nuclear or cytoplasmic lysate was immunoprecipitated with mouse anti-Kaiso antibody or anti-Phosphoserine/threonine/tyrosine antibody plus protein A/G Sepharose for 8 hrs at 4 °C. Mouse IgG was used as a negative control. The precipitates were washed six times with lysis buffer, and boiled in 1 × loading buffer. Protein samples were resolved by SDS–PAGE, and electroblotted onto nitrocellulose membranes, which were blocked in 5% skim milk in PBST and probed with antibodies according to the Instruction Manual.

Phos-tag SDS-PAGE assay

This is a modified SDS-PAGE method based on the novel Phos-tag (33), which can bind to phosphorylated proteins and decrease their migration speed. Thus, this assay is often used to distinguish dephosphorylated proteins from phosphorylated proteins on the case of that a phosphorylation-specific antibody is not available. 50 μM Phos-tag (final concentration, Phos-tag Acrylamide AAL-107, WAKO, Japan) and 100 μM $MnCl_2$ were mixed to prepare SDS-PAGE gel.

GST-Pull down (34)

The day after pEBG-GST-Kaiso transfection, MGC803 cells (in 10-cm dishes, eight dishes/group) were further transfected with pENTER-Flag-14-3-3 members (α/β, ε, η, δ/γ, τ, ζ, σ) or negative control vector, respectively. 48 hrs post transfection, MGC803 cells were harvested and used to prepare lysate with cell lysis buffer with 1× Protease Inhibitor Cocktail (REF04693159001, Roche, Mannheim, Germany). The lysate was incubated with Glutathione Sepharose beads (20 μL for one group, 17-0756-01, GE healthcare, Sweden) at 4 °C overnight. Beads were washed six times with 500 μL cell lysis buffer. After the last centrifuging, the supernatant was removed as clean as possible, and the pellet was suspended in 20 μL 1×SDS sample buffer. Antibodies for GST-Kaiso and Flag-14-3-3 family proteins were used to detect the pulled down precipitant.

Co-immunoprecipitation (Co-IP)

After pre-cleared with protein A/G-coupled Sepharose beads for 2 hrs, the soluble proteins from whole cell lysate were immunoprecipitated with anti-Kaiso (sc-365428, Santa Cruz) or anti-GFP (ab290, Abcam) or other antibodies plus protein A/G Sepharose overnight at 4 °C. Mouse IgG or rabbit IgG was used as a negative control. The precipitates were washed six times with lysis buffer, and boiled after 1 × SDS loading buffer was added. Protein samples were resolved by SDS–PAGE, and electroblotted onto nitrocellulose membranes, which were blocked with 5% skim milk in PBST and probed with the interacted protein antibodies.

***In vitro* kinase assay** (35)

1 μg of the substrate (GST or GST-Kaiso-wt/T606A) protein per reaction was extracted from pGEX-4T1 plasmid transfected *BL21 E. coli* induced with IPTG 0.5 mM at 37 °C and purified with Glutathione Sepharose beads. Active kinase AKT1 protein (PKBα, 15 μg) was purchased from Merck (#14-276, Darmstadt, Germany). 10× Kinase buffer (1 × concentration: 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) was prepared when used. Glutathione Sepharose beads were washed with substrate twice with 500 μL of 1 × Kinase Buffer on ice. The suspended pellet in 30 μL of 1 × Kinase buffer supplemented with 1 μL of 10 mM ATP and 0.5 μg AKT1 kinase were incubated at 30°C for 30 min. Reaction was terminated with 30 μL 2 × SDS Sample buffer. Samples were vortexed, then microcentrifuged for 30 sec at 12,000 *g*. The reaction products were then resolved in SDS-PAGE gel and Western blotted to detect the phosphorylated Kaiso using AKT1 substrate antibody.

Preparation of pT606-Kaiso-specific antibody

Anti-pT606-Kaiso polyclonal antibodies were raised in rabbits challenged with the synthesized phosphorylated peptide LSD**RSS<u>pT</u>IP**AM, a sequence corresponding to amino acids 600-610 of wildtype Kaiso (Kaiso-wt), absorbed with non-phosphorylated peptide LSD**RSS<u>T</u>IP**AM, and enriched by the phosphorylated peptide LSD**RSS<u>pT</u>IP**AM. Peptide synthesis and immunization of the animals were done by YouKe (Shanghai, China). The non-phos-peptide was also used to prepare the control polyclonal antibodies against total Kaiso at the same time. The specificity of pT606-Kaiso and control

antibodies against the corresponding peptide (LSDRSSpTIPAM or LSDRSSTIPAM) was detected with ELISA assay (Fig. S1).

ELISA analyses for polyclonal antibodies against pT606-Kaiso and total Kaiso

Polystyrene plates were coated with 1 μg/mL synthetic phosphorylated peptide LSDRSSpTIPAM or non-phosphorylated peptide LSDRSSTIPAM link-coupled by bovine serum albumin (BSA) in 1 × CBS buffer overnight at 4 °C, respectively, and were washed three times with PBS containing 0.05% Tween 20. Unbinding sites were blocked with 5% milk at room temperature for 2 hrs. The purified antibodies, diluted as described in Figure S1, were added (100 μL/well) and incubated at 37 °C for 1 hr. After being washed with 0.05% Tween/PBS, plates were added HRP-labeled goat anti-Rabbit IgG (100 μL/well) and incubated at room temperature for 30 min. Peroxidase activity was measured with 0.15 mg/mL TMB substrate solution (100 μL/well). After 15 min at room temperature, the reaction was stopped by 2M H_2SO_4 (50 μL/well). Optical density absorbance (OD) at 450 nm was determined using a model 550 microplate reader.

Immunohistochemical staining (IHC)

Rabbit pT606-Kaiso polyclonal antibody (1:100) and mouse monoclonal antibody for total Kaiso (1:50, sc-23871, Santa Cruz) were used in the IHC analysis. The REAL™ EnVision™ Detection system including anti-rabbit/mouse IgG secondary antibodies and peroxidase/diaminobenzidine (Dako, Agilent Technologies) was used to visualize the primary antibody-binding cells according to the manufacturer's protocol. Briefly, paraffin sections (4 μm) were dewaxed and rehydrated in xylene and ethanol. For antigen retrieval these sections were autoclaved for 3 min in 10 mM sodium citrate buffer containing 0.05% Tween-20 (pH 6.0) for pT606-Kaiso and in 1 mM EDTA buffer (pH 8.0) for total Kaiso. Then, these sections were immersed in 3% H_2O_2 for 10 min to block endogenous peroxidase. Following submerging in 5% bovine serum albumin (BSA) (cat. no. A1933; Sigma Life Science; Merck KGaA) for 60 min, the sections were incubated with the primary antibody overnight at 4 ˚C. The PBS washed sections were then treated with the REAL™ EnVision™ Detection system and counterstained with hematoxylin (0.125%; ZhongShan Jinqiao Biotechnology) at room temperature for 1 min. Normal rabbit IgG and normal mouse IgG (cat. no. ZDR 5003 and cat. no. ZDR 5006; ZhongShan Jinqiao

Biotechnology) were used as negative controls. The former was diluted and incubated as for the pT606-Kaiso antibody, and the latter was diluted and incubated as for total Kaiso antibody).

Confocal analysis

For mCherry-14-3-3, direct fluorescence was detected with laser confocal microscope assay. MGC803 cells with mCherry-14-3-3 overexpression were rinsed for three times with PBS, fixed with 1% paraformaldehyde in PBS 30 min at 37 °C, punched with 0.5% triton X-100 for 10 min at 37 °C, incubated with respective primary and secondary antibodies, washed for three times in PBS, counterstained with DAPI (1 μg/mL) for 5 min, and then examined with Leica SP5 Laser Scanning Confocal Microscopy. The antibodies for pT606-Kaiso (1 μg/μL, 1:100), for total Kaiso (commercial antibody (0.2 μg/μL, 1:20, sc-365428, Santa Cruz), and for p120ctn (1:100, 66208-1, Proteintech) were used as the primary antibodies; the FITC-labeled antibody against rabbit IgG (1:100, ab6717, Abcam) and CY5-labeled antibody against mouse IgG (1:100, Cat. No. 072-02-18-06, KPL Gaithersburg, MD, USA) were used as secondary antibodies for observation under Leica SP5 Laser Scanning Confocal Microscope and were analyzed with ImageXpress Micro high content screening System.

For the detection of Kaiso in tissues, fresh cryostat sections (4 μm) from gastric carcinoma and the paired normal tissues were fixed with 4% paraformaldehyde for 10 min at 37°C and treated with 0.5% triton X-100 for 10 min at 37 °C, and then incubated with respective primary, secondary antibodies, DAPI, and then examined as described above.

Publicly available RNA-Seq datasets

The RNA sequencing datasets in CCLE and GTEx projects were downloaded from official websites (portals.broadinstitute.org/ccle and www.gtexportal.org) (36, 37). All raw data were transferred to Transcripts per kilobase of exon model per Million mapped reads (TPM) using uniform names for each protein-coding gene in the human genome. The Pearson correlation coefficient (r) of genes expression was calculated by R statistical software (version 3.6.1) with a set of procedures (38). Briefly, raw data was read through "read.table()" function, and an array was established to store data by "array()" function. Then the Pearson correlation coefficient was calculated by "cor()" function, and finally saving the data to files in csv format by the function of “write.table()”.

Results

Discovery of phosphorylated Kaiso in the cytoplasm

Immunoaffinity profiling of phosphorylated proteins in cancer cells suggests there may be putative phosphorylation sites within Kaiso in proteomic mass spectrometry analysis (Fig. S2) (39, 40). We wondered if Kaiso compartmentalization was related to the statuses of the putative phosphorylation. In Phos-tag SDS-PAGE analyses, a Phos-tag could bind specifically to a phosphate group in proteins via metal ions, such as Zn^{2+} or Mn^{2+}, which could be used to separate phosphorylated proteins from non-phosphorylated proteins (33). Thus, the Phos-tag assay was preliminarily utilized to analyze the phosphorylation status of Kaiso in human cells. We found that while the cytoplasmic and nuclear Kaiso from MGC803 and HEK293T cells migrated at the same speed in regular SDS-PAGE gel, approximately all the cytoplasmic Kaiso migrated much slower than the nuclear Kaiso in the Phos-tag gel (Fig. 1A). While the migration of nuclear Kaiso was not affected by the CIAP de-phosphorylation treatment, the delayed cytoplasmic Kaiso migration was partially concealed by the CIAP treatment, suggesting that the endogenous cytoplasmic Kaiso in these cells was mostly phosphorylated.

To validate the different phosphorylation statuses of Kaiso in the cytoplasm and nucleus, an anti-phosphoserine/threonine/tyrosine universal antibody was then used to precipitate global phosphorylated proteins and a Kaiso-specific antibody was used to visualize the possible phosphorylated Kaiso, and vice versa (Fig. 1B). Again, phosphorylated Kaiso was observed only in the cytoplasmic precipitates, but not in the nuclear counterpart. Furthermore, the phosphorylated GFP-Kaiso was consistently detected in the cytoplasm of MGC803 cells stably transfected with GFP-Kaiso, whether these cells were cultured *in vitro* or transplanted into nude mice as a xenograft in the Phos-tag SDS-PAGE analyses, and no phosphorylated Kaiso was detected with the CIAP de-phosphorylation (Fig. 1C). All the above results suggest there may be phosphorylation of Kaiso in the cytoplasm. Thus, Kaiso phosphorylation kinase and target site were characterized in details as described below.

Kaiso is phosphorylated at T606 by AKT1 kinase

Protein kinase B AKT1 is a typical kinase for the (RX)RXXpS/pT motif in multiple proteins such as mTOR, GSK-3β, AMPKA, Catenin-β1 (41-44). Human Kaiso contains a conservative RSSTIP motif with the threonine-606 (T606) residue (Fig.2A). According to the results of liquid chromatography-mass spectrum (LC/MS) analyses for protein modifications, the T606 was one of phosphorylation sites in Kaiso with a low frequency (Fig. S2) (40). To verify if Kaiso T606 could be a true phosphorylation site for the Kinase AKT1, we stimulated the activity of AKT signaling of MGC803 cells with insulin, IL-6, and FBS after overnight starvation, as Hong et al. reported (45). As expected, the phosphorylation level of endogenous Kaiso was markedly increased in MGC803 cells stimulated with insulin, IL-6, and FBS, but not with EGF (Fig. 2B).

Using the immunoprecipitation assay employing a phospho-(Ser/Thr) AKT substrate (pAKT-sub)-specific antibody, endogenous Kaiso signal could also be detected in the immunoprecipitated pAKT-sub protein complexes, and vice versa (Fig. S3A). The amounts of both global pAKT-sub and Kaiso-antibody-immunoprecipitated pAKT-sub were also significantly increased by insulin stimulation and reversed by AKT inhibitor MK2206 treatment (Fig. 2C). A similar effect was also observed in MGC803 cells with *AKT1* overexpression (Fig. 2D).

We further found that active AKT1 recombinant kinase could directly phosphorylate GST-Kaiso recombinant protein (aa346-638 in Kaiso-wt) in an *in vitro* kinase assay. When the phosphorylation site T606 was mutated as A606, which is not a phosphorylation site for any kinase, most Kasio-T606A protein could not be phosphorylated by the AKT1 recombinant kinase (Fig. 2E). Together, these results suggest that AKT1 could directly phosphorylate Kaiso at T606.

Then, a pT606-Kaiso-specific polyclonal antibody was prepared from rabbit using the phos-peptide LSD**RSSpTIP**AM as antigen (Fig. S1) and used to directly detect phosphorylated Kaiso. As expected, the amount of pT606-Kaiso was significantly increased in MGC803 and BGC823 cells with the insulin stimulation (or *AKT1* overexpression) and reversed by the AKT inhibitor MK2206 treatment (Fig. 2F-G).

In consistent with the results described above, most pT606-Kaiso was detected in the cytoplasm of human cancer cell lines (MGC803, BGC823, HCT116, and RKO) and the CIAP de-phosphorylation

treatment markedly decreased the amount of pT606-Kaiso in the cytoplasm in Western blotting (Fig. 3A). The confocal microscopy results further confirmed the cytoplasmic compartmentalization of pT606-Kaiso in MGC803 cells (Fig. 3B). While total Kaiso was mainly detected in the nucleus of human gastric mucosal tissues, pT606-Kaiso was only detected in the cytoplasm in the IHC and confocal microscopy analyses (Fig. 3C-D). Collectively, the above results demonstrate that AKT1 can phosphorylate Kaiso at T606 and pT606-Kaiso mainly located in the cytoplasm of human cells.

pT606-Kaiso interacted with 14-3-3 family members

It is well known that RSXpSXP is a 14-3-3 phosphoserine binding consensus motif (49). To study whether the Kaiso RSSpTIP is a 14-3-3 binding motif, we performed GST-pull down and Co-IP experiments. The results of GST-pull down assay showed that GST-Kaiso could strongly pull down 14-3-3σ, moderately pull down 14-3-3ε, 14-3-3γ, 14-3-3ζ, and weakly pull down 14-3-3η in MGC803 cells lysate (Fig. 4A). The Co-IP results confirmed that endogenous Kaiso could bind to endogenous pan-14-3-3 or 14-3-3σ (SFN) protein in MGC803 cells (Fig. 4B). As expected, the T606A mutation abolished most Kaiso-14-3-3 interaction (Fig. 4C), suggesting that the phosphorylation site T606 within the RSSTIP motif may play a main role in determining the Kaiso-14-3-3 interaction. In addition, AKT1 overexpression could slightly increase the Kaiso-14-3-3 interaction (Fig. S3C). These data indicate that Kaiso could interact with 14-3-3 family proteins in a T606 phosphorylation-dependent manner.

Increased cytoplasmic accumulation of pT606-Kaiso by the 14-3-3σ interaction

Kaiso usually distributes in both the cytoplasm and the nucleus. Intracellular Kaiso compartmentalization is affected by growth conditions (13, 17). Our indirect immunofluorescence confocal microscope analyses showed that 81% endogenous Kaiso was observed in the nucleus of MGC803 cells (Fig.5A-B). Notably, enforced mCherry-14-3-3γ or -14-3-3σ overexpression significantly increased the proportion of endogenous Kaiso in the cytoplasm, compared to the mCherry control vector (from 19% to 37% or 36%) and Kaiso was mainly co-localized with mCherry-14-3-3γ or -14-3-3σ in the cytoplasm of these cells in the confocal analysis. Western blotting confirmed that mCherry-14-3-3σ overexpression increased the cytoplasmic accumulation of endogenous Kaiso (Fig.

5C). In addition, 14-3-3σ only promoted the cytoplasmic accumulation of wildtype Kaiso (Kaiso-wt), but not the T606A mutant (Fig. 5D, red arrowed). 14-3-3σ or 14-3-3γ overexpression also induced more cytoplasmic pT606-Kaiso in MGC803 and BGC823 cell lines in Western blotting (Fig. S3D-E). These results solidly confirm that the pT606-Kaiso can interact with 14-3-3 and in turn accumulate in the cytoplasm of human cancer cells.

The P120ctn interaction is essential for pT606-Kaiso-14-3-3 accumulation in the cytoplasm

As a given Kaiso interacting protein, the P120ctn binding is essential for the trafficking of Kaiso from the nucleus to the cytoplasm (18, 19). Thus, we further studied whether 14-3-3 bound to the pT606-Kaiso-P120ctn complex or individual pT606-Kaiso molecule in the cytoplasm. Interestingly, we found that 14-3-3σ overexpression markedly increased the amount of P120ctn in the anti-Kaiso antibody precipitated complexes, while the levels of total endogenous Kaiso and P120ctn proteins were not changed in MGC803 cells (Fig. 6A). Further analysis showed that more Kaiso-P120ctn binding was detected in the cytoplasm, but not in the nucleus of MGC803 cells with 14-3-3σ overexpression (Fig. 6B). Immunofluorescence confocal microscopy showed that P120ctn, Kaiso, and 14-3-3σ were co-localized in the cytoplasm (Fig. 6C). Moreover, when P120ctn was knocked down by siRNA, the cytoplasmic Kaiso accumulation promoted by 14-3-3σ disappeared in the MGC 803 cells (Fig. 6D) while the level of total Kaiso was not changed (Fig. 6E). These results suggest that the P120ctn interaction is essential for the pT606-Kaiso-14-3-3σ accumulation in the cytoplasm.

De-repression of Kaiso target genes by T606 phosphorylation

It was reported that Kaiso could bind to both methylated and non-methylated Kaiso binding sequences in target gene promoters and suppress their transcription (11). We wondered whether T606 phosphorylation of Kaiso and its bindings with 14-3-3/P120ctn proteins could affect expression of Kaiso target genes through the cytoplasmic accumulation. Through re-analyzing the RNA-seq datasets for 14-3-3 family members and Kaiso target genes (including *CDH1*, *CCND1*, *CCNE1, MTA2, DLL1,* and *DAG1*) from Genotype-Tissue Expression (GTEx) and Cancer Cell Line Encyclopedia (CCLE) (38-40), we found that the level of 14-3-3σ mRNA was positively and most significantly correlated with that of *CDH1* in both normal human tissues (n=11688, r=0.60, P<0.001 in Pearson correlation

analysis) in GTEx datasets and cancer cell lines in CCLE datasets (n=1156, r=0.41, P<0.001) (Fig. 7A and Fig. S4A), suggesting an exact effect of 14-3-3σ expression on regulation of *CDH1* transcription, probably through the pT606-Kaiso-14-3-3σ interaction. Interestingly, association between the levels of *CDH1* and *Kaiso* mRNA was only observed in normal tissues in the GTEx datasets, but not in cancer cell lines in the CCLE datasets (Fig. S4B), suggesting a dysregulation of *CDH1* transcription by Kaiso in cancer cells.

Then, two gastric cancer cell lines SGC7901 and MKN45 with active *CDH1* expression were used to study effects of Kaiso and 14-3-3σ expression changes on the function of Kaiso as a transcription repressor. As expectedly, Kaiso overexpression alone indeed decreased the amount of CDH1 whereas 14-3-3σ co-overexpression could abolish Kaiso-induced *CDH1* repression in SGC7901 and MKN45 cells in Western blotting (Fig. 7B). These results were confirmed in qRT-PCR analyses (Fig. 7C). A similar relationship was observed in gastric and colon carcinoma tissues. While the amount of pT606-Kaiso was much lower in these cancer tissues than in the paired normal tissues, the amount of CDH1 was also much lower in these cancer tissues than in the paired normal tissues (Fig. 7D and Fig. S5). Taken together, the above results indicate that pT606-Kaiso-14-3-3σ binding in the cytoplasm can deprive the function of Kaiso as a transcription repressor and lead to de-repression of the transcription of Kaiso target gene *CDH1*.

Discussions

The subcellular locations of Kaiso determine its roles in normal cell differentiation and cancer development. However, detailed regulation machinery for the compartmentalization of Kaiso is far from clear. In this study we demonstrated, for the first time, that Kaiso could be phosphorylated at T606 by AKT1, the pT606-Kaiso could interact with 14-3-3 and P120ctn in the cytoplasm and promote the shift of Kaiso from the nucleus to the cytoplasm. The phosphorylation of Kaiso finally leads to deprivation of Kaiso as a transcription repressor and de-repression of Kaiso target gene *CDH1*.

It's well known that 14-3-3 family proteins bind to common phosphoserine/phosphothreonine -containing peptide motifs corresponding to Mode-1 (RSXpSXP) or Mode-2 (RXY/FXpSXP) sequences (46). We found that Kaiso contains a very conservative RSS<u>T</u>IP motif that could be phosphorylated by AKT1 in both *in vivo* and cell-free system at RSS<u>T</u>IP-T606 site. This is consistent

with the report that T606 is one of phosphorylation sites of Kaiso in mass spectrometry analysis (40). That pT606-Kaiso could directly bind 14-3-3 family proteins and the T606A mutation could abolish most Kaiso-14-3-3 binding indicate the T606 phosphorylation is essential for Kaiso-14-3-3 binding.

It has been reported that a region consisting of 1-200 amino acid residues of Kaiso directly interacts with P120ctn (1) and Kaiso-P120cnt interaction promotes cytoplasmic-nuclear trafficking of Kaiso (18, 19). In addition, P120ctn could bind to CDH1 and modulate its function and stability (47) and WNT-stimulated P120ctn phosphorylation could promote P120ctn releasing from the E-Cadherin-P120ctn complexes, enhances Kaiso-P120ctn interaction (48). It is well known that most 14-3-3σ proteins localize in the cytoplasm (49) while Kaiso mainly localizes in the nucleus (50). Here, we observed that pT606-Kaiso, 14-3-3, and P120ctn proteins could collocate in the cytoplasm and siRNA-knockdown of *P120ctn* abolished the 14-3-3-induced cytoplasmic Kaiso-14-3-3 binding. These results demonstrate that Kaiso-14-3-3σ interaction may be dependent on the Kaiso-P120ctn binding and that Kaiso, 14-3-3σ, and P120ctn may form a triplex in the cytoplasm. How Kaiso, 14-3-3σ, and P120ctn interacting with each other is worth studying.

It is controversy on Kaiso's roles in cancer development. In the absence of the tumor suppressor *APC*, Kaiso-deficient mice were resistant to intestinal cancer, suggesting that Kaiso might be an oncogene (15). On the contrary, Kaiso has also been suggested to be a potential tumor suppressor, which repressed the transcription of *MMP7*, *CCND1*, and *WNT11* genes involved in oncogenesis and metastasis (7, 9, 51). Functions of Kaiso are tightly related and significantly influenced by microenviromental factors (17). We found here that increase of the level of pT606-Kaiso or pan-14-3-3 (especially 14-3-3σ) expression could lead to Kaiso accumulation in the cytoplasm, deprive transcriptional function of Kaiso, and de-repression of Kaiso target gene *CDH1*. This indicates that Kaiso phosphorylation is involved in cancer development through de-repression of tumor related genes.

In conclusion, Kaiso can be phosphorylated by AKT1 at T606 within the very conservative motif RSS<u>T</u>IP in the cytoplasm. pT606-Kaiso can directly interact with 14-3-3 and P120ctn proteins and accumulated in the cytoplasm that consequently lead loss of Kaiso's function as a transcription repressor. The decreased phosphorylation of Kaiso may contribute to cancer development through downregulation of *CDH1* expression in the stomach and colon.

Acknowledgements

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Competing Interest

The authors declare that they have no conflict of interest.

Author contribution

D. Deng, W. Tian and S. Qin. designed research; W. Tian, S. Qin and B. Zhang performed research; D. Deng , W. Tian, L. Gu and J. Zhou analyzed data; D. Deng, W. Tian and S. Qin wrote the paper.

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Figures and Legends

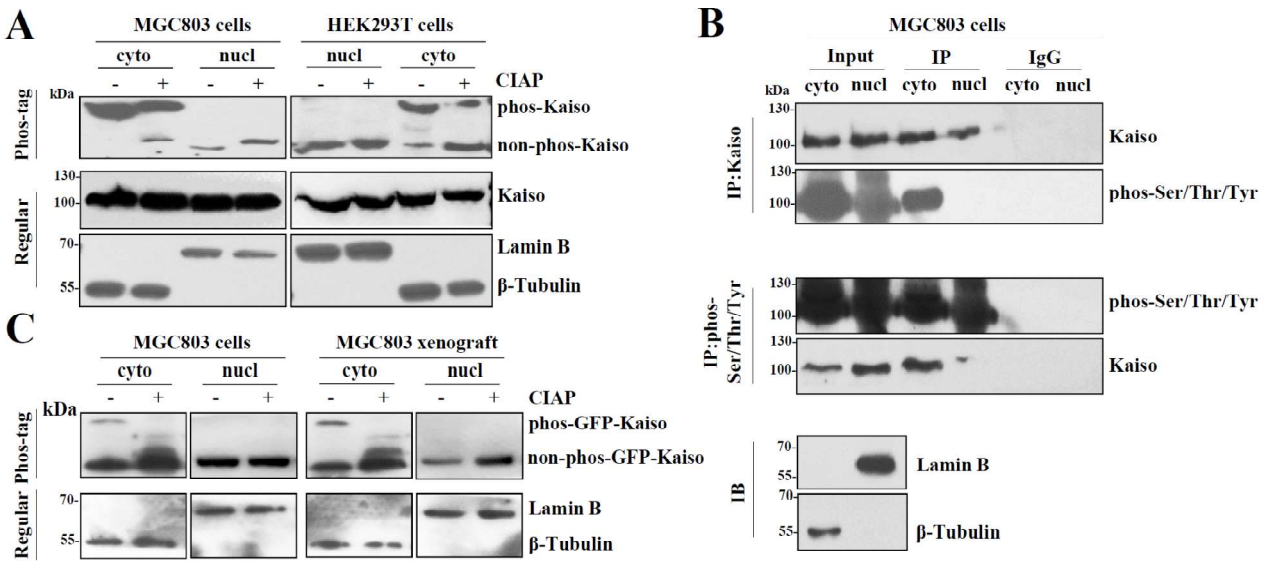

Figure 1. Different phosphorylation states of Kaiso protein in the cytoplasm and nucleus of cells *in vitro* and *in vivo*. (**A**) Different phosphorylation statuses of endogenous Kaiso in the cytoplasm and nucleus of MGC803 and HEK293T cells in the Phos-tag and regular SDS-PAGE analyses. The purities of cytoplasmic and nuclear extracts were respectively verified using anti-β-Tubulin and anti-Lamin B antibodies. Kaiso in both protein extracts with and without the de-phosphorylation treatment by CIAP were showed. (**B**) The phosphorylation statuses of endogenous cytoplasmic and nuclear Kaiso in MGC803 cells were validated using anti-phosphoSer/Thr/Tyr universal antibody. (**C**) The phosphorylation statuses of GFP-Kaiso stably transfected MGC803 cells and the corresponding xenograft tissues in the Phos-tag SDS-PAGE analysis.

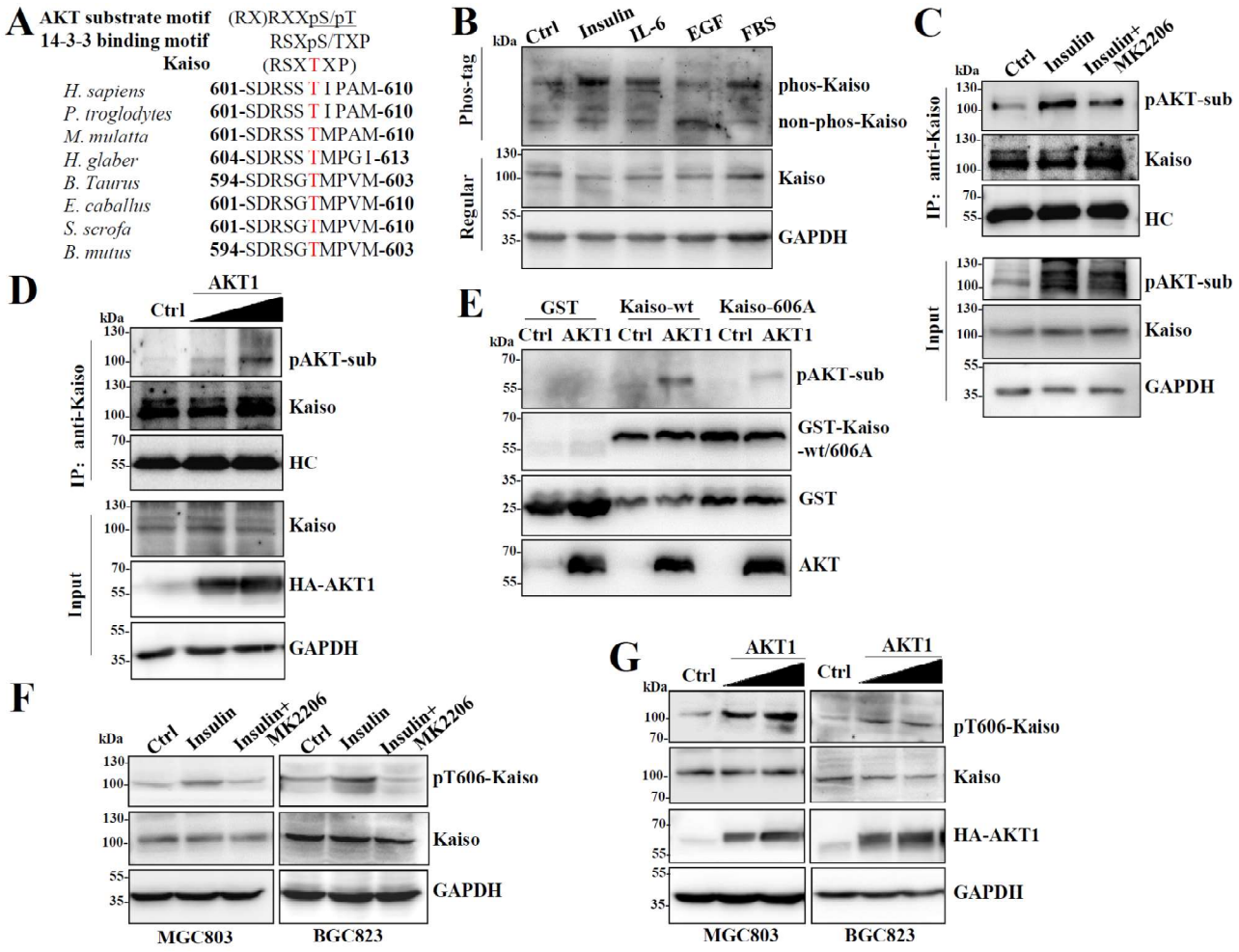

Figure 2. AKT1 increases the phosphorylation of Kaiso at T606. (**A**) A conservative RSXTXP motif within Kaiso of human and other species (**B**) After starvation overnight, treatments of Insulin (100 ng/mL), IL-6 (10 ng/mL) and fetal bovine serum (FBS, 1:10000 v/v) for 15 min increased the phosphorylation level of endogenous Kaiso in MGC803 cells. (**C**) Effects of AKT inhibitor MK2206 treatment (10 μmol/mL) for 30 min blocked the promotion of Insulin induced Kaiso phosphorylation as pAKT substrate in MGC803 cells. (**D**) *AKT1* overexpression at different doses increased the amount of phosphorylated AKT substrate (pAKT-sub) in Kaiso complexes immunoprecipitated by Kaiso antibody in MGC803 cells. (**E**) GST-Kaiso-wt recombinant treated with *AKT1* was identified by AKT substrate-specific antibody in an *in vitro* kinase assay and T606A mutation abolished AKT1's impact on Kaiso. (**F**) The T606-phosphorylation status of endogenous Kaiso in MGC803 and BGC823 with treatment of Insulin and its combination with AKT inhibitor MK2206 after starvation overnight. (**G**) The T606-phosphorylation status of endogenous Kaiso in MGC803 and BGC823 with *AKT1* overexpression after starvation overnight.

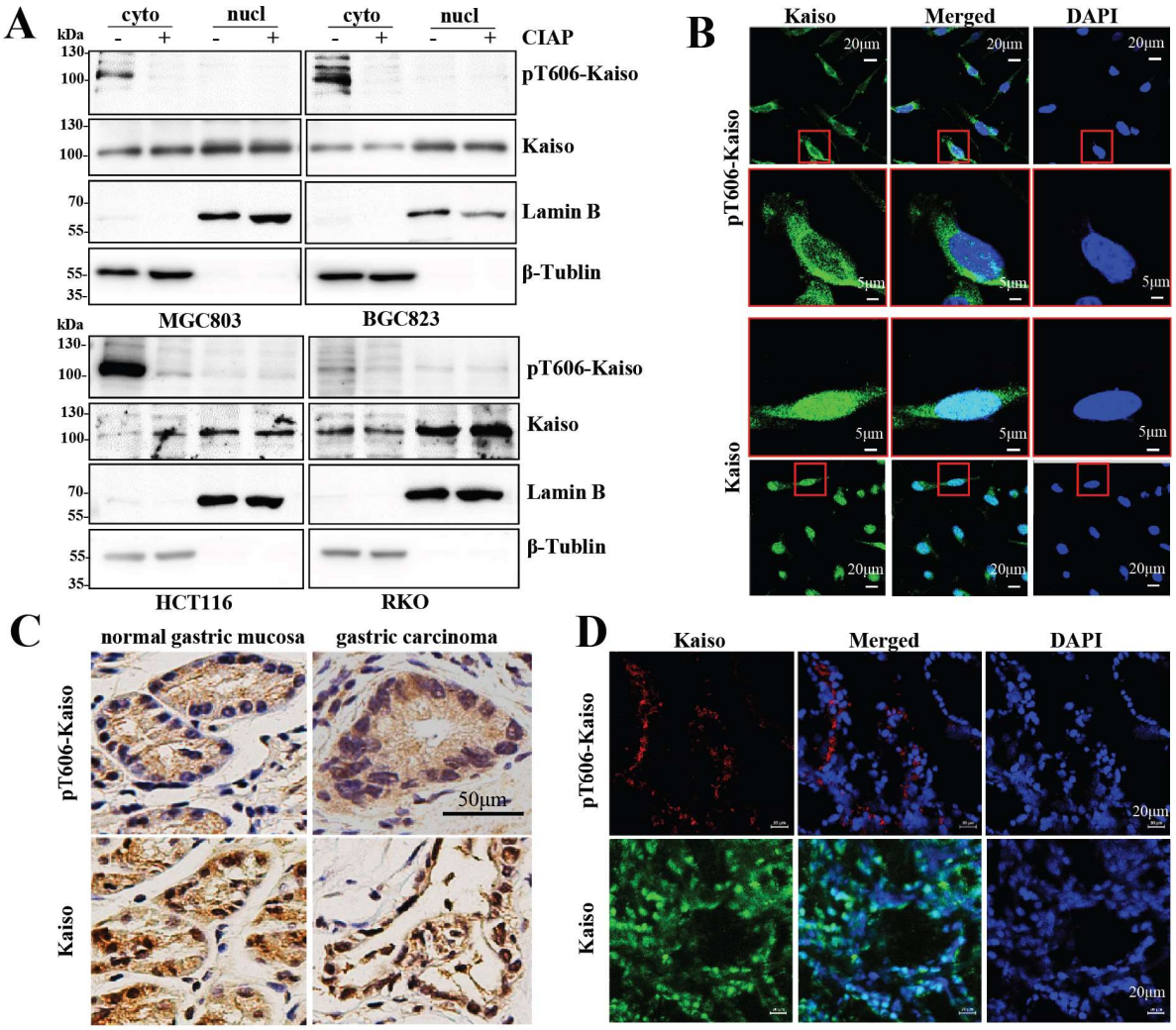

Figure 3. Subcellular location of endogenous pT606-Kaiso and total Kaiso. (**A**) Amounts of pT606-Kaiso and total Kaiso in cytoplasmic and nuclear proteins (with and without de-phosphorylation treatment by CIAP for 30 min) extracted from 4 cancer cell lines in Western blotting. (**B**) Locations of pT606-Kaiso and total Kaiso in the cytoplasm and nucleus in an indirect immunofluorescence confocal analysis. (**C**) Locations of pT606-Kaiso and total Kaiso in a representative gastric carcinoma and the paired normal tissues in IHC analysis. (**D**) Locations of pT606-Kaiso and total Kaiso in human gastric tissues in the indirect immunofluorescence confocal analysis.

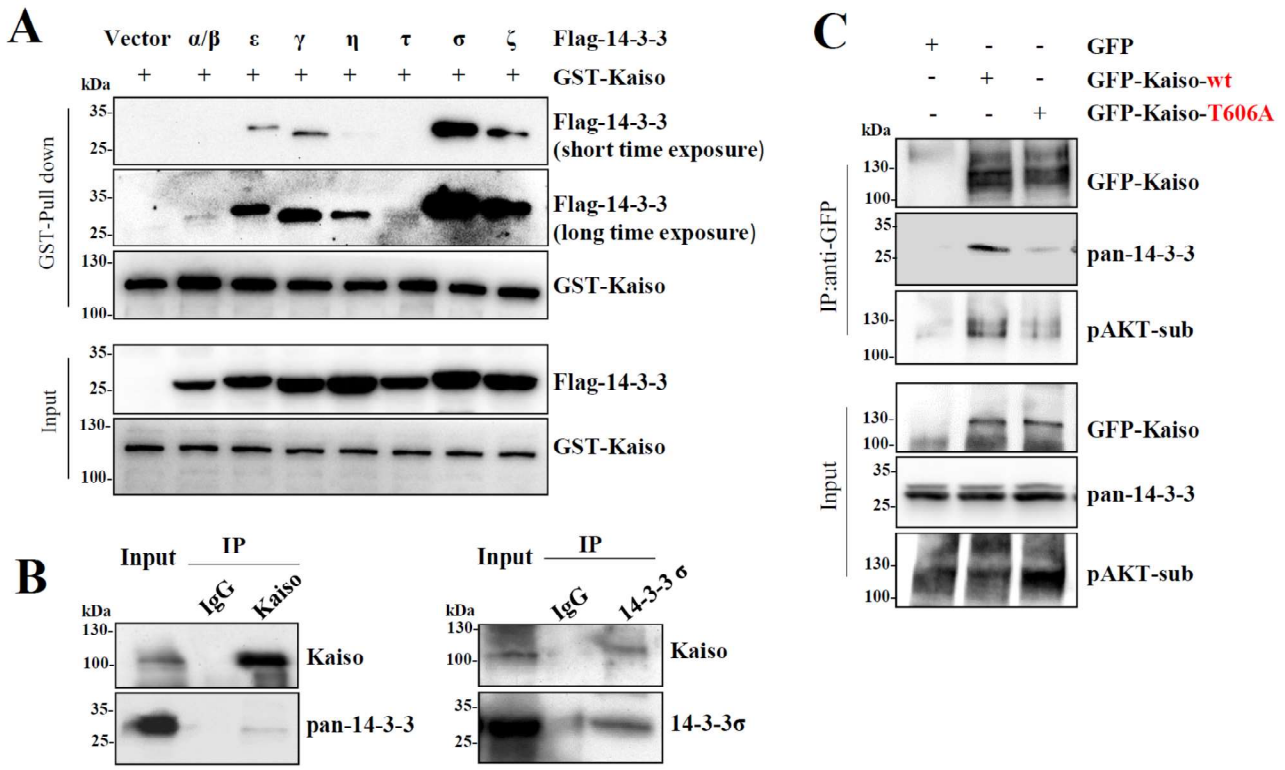

Figure 4. Kaiso interacted with 14-3-3 family members depending on Kaiso pT606. (**A**) After the plasmids of 14-3-3 family members and GST-Kaiso were co-transfected into MGC803 cells, GST-Kaiso pulled down various isoforms of 14-3-3 family, especially 14-3-3 σ. (**B**) Endogenous Kaiso immunoprecipitated pan-14-3-3 or 14-3-3σ protein immunoprecipitated endogenous Kaiso in MGC803 cell in Co-IP analysis. (**C**) T606A mutation of Kaiso concealed its interaction with pan-14-3-3 in MGC803 cells in Co-IP analysis.

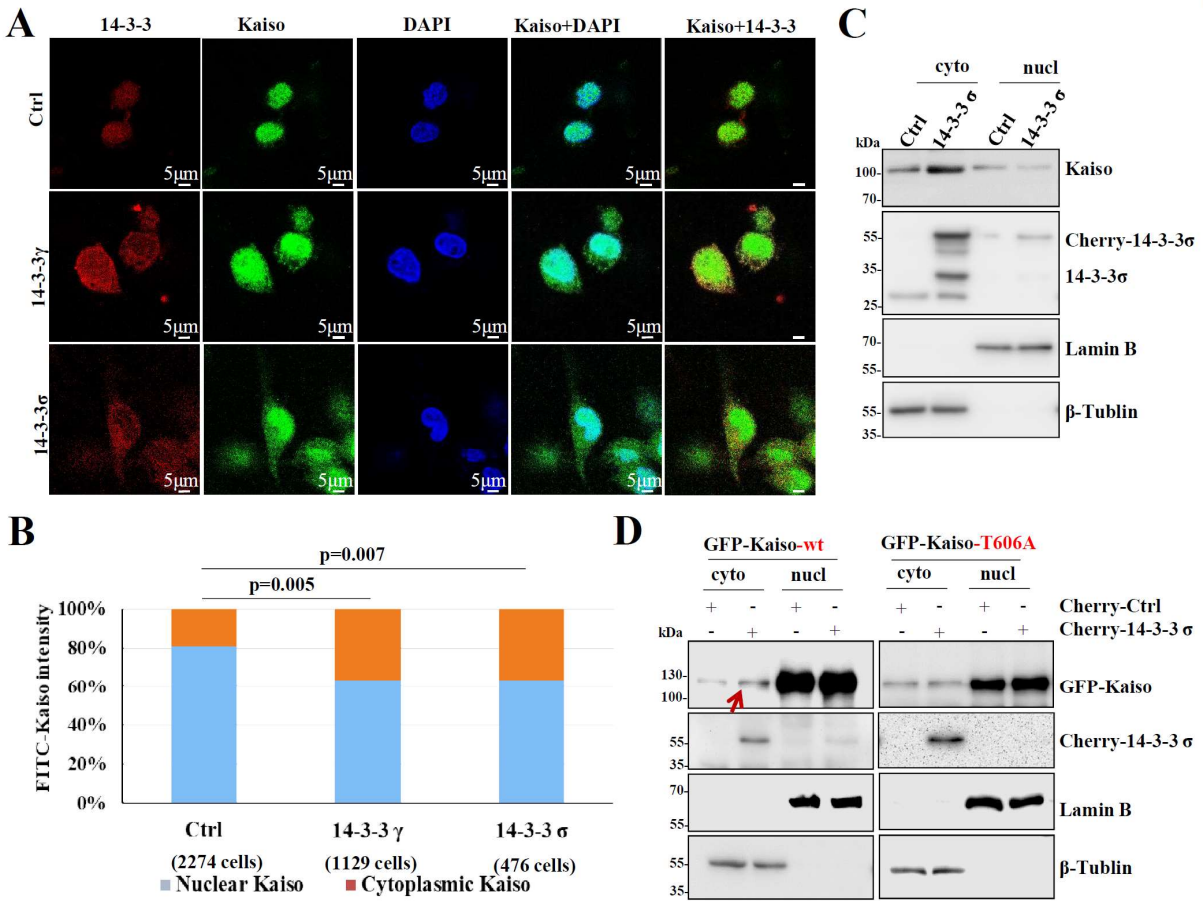

Figure 5. 14-3-3 proteins promote the cytoplasmic accumulation of Kaiso in Kaiso T606 phosphorylation-dependent manner. (**A**) The subcellular location of endogenous Kaiso in MGC803 cells with or without 14-3-3γ or 14-3-3σ overexpression by indirect immunofluorescence staining assay. (**B**) Proportion of Kaiso in the nucleus and in cytoplasm of MGC803 cells with and without 14-3-3γ or 14-3-3σ overexpression. (**C**) Western blot for detecting the amounts of endogenous Kaiso in cytoplasm and nucleus protein in MGC803 cells with 14-3-3σ overexpression. (**D**) Comparison of the levels of GFP-Kaiso (wild type) or T606A mutant in the cytoplasm and nucleus in MGC803 cell with and without 14-3-3σ overexpression. Lamin B and β-Tublin were used as nuclear and cytoplasmic protein controls.

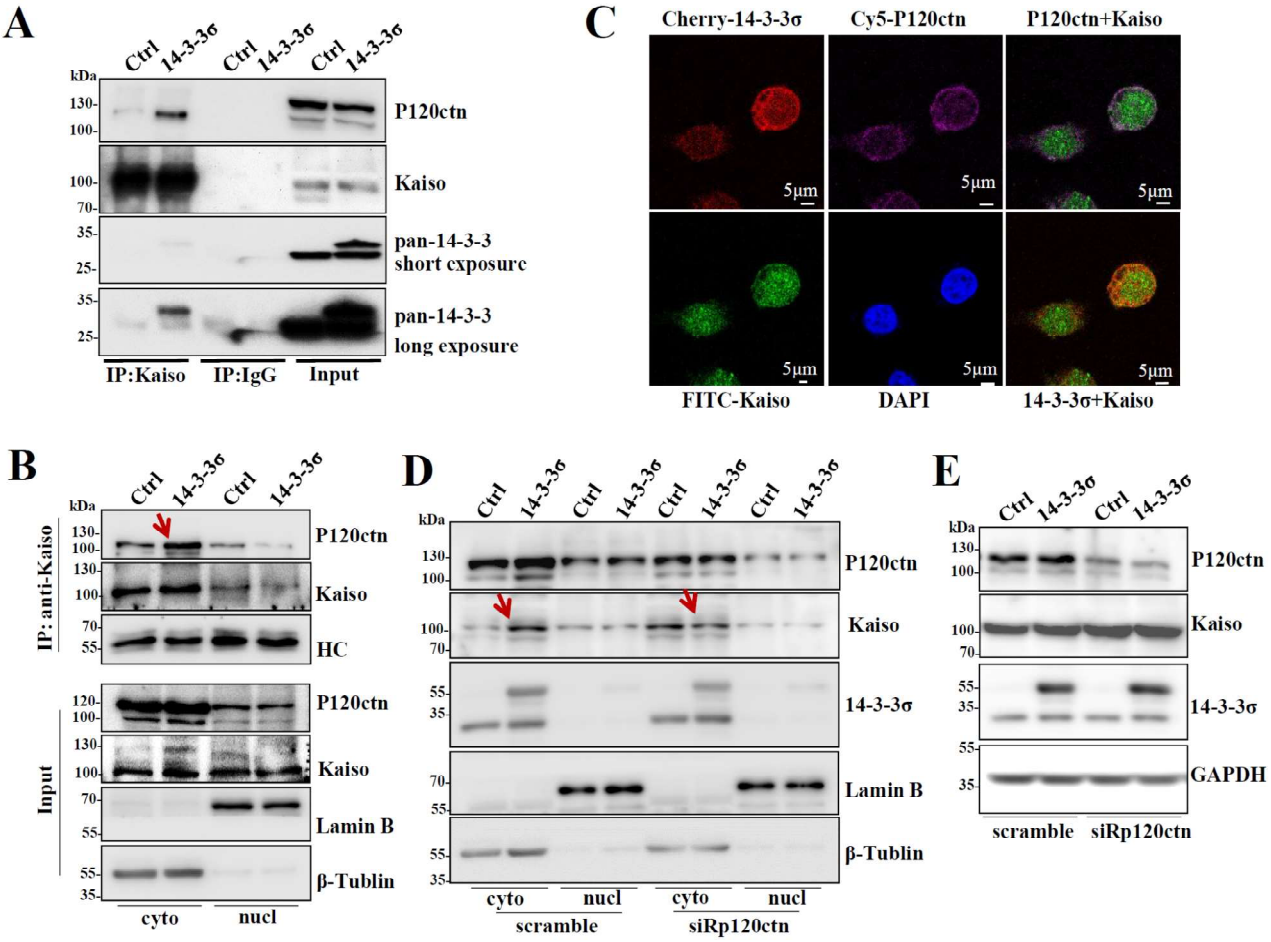

Figure 6. 14-3-3σ promotes the interaction of Kaiso and p120ctn in the cytoplasm. (**A**) More Kaiso-p120 complex was immunoprecipitated by Kaiso antibody in MGC803 cells with 14-3-3σ overexpression in Co-IP assay. Total protein levels of p120ctn and 14-3-3σ in the lysate of MGC803 cells with *14-3-3σ* overexpression were displayed in the right side. (**B**) Alterations of interactions between Kaiso and p120ctn proteins in the cytoplasm and nucleus of these cells with *14-3-3σ* overexpression in the Co-IP assay using Kaiso antibody. (HC: Heavy chain of IgG). (**C**) The subcellular locations of Kaiso, p120ctn, and mCherry-14-3-3σ in MGC803 cells. (**D**) Western blotting images for detecting effect of *p120ctn* knockdown and *14-3-3σ* overexpression on distribution of Kaiso in the cytoplasm and nucleus of MGC803 cells. (**E**) The levels of total p120ctn and Kaiso proteins in the lysate of MGC803 cells with *14-3-3σ* overexpression and siRNAs knockdown of *p120ctn* expression.

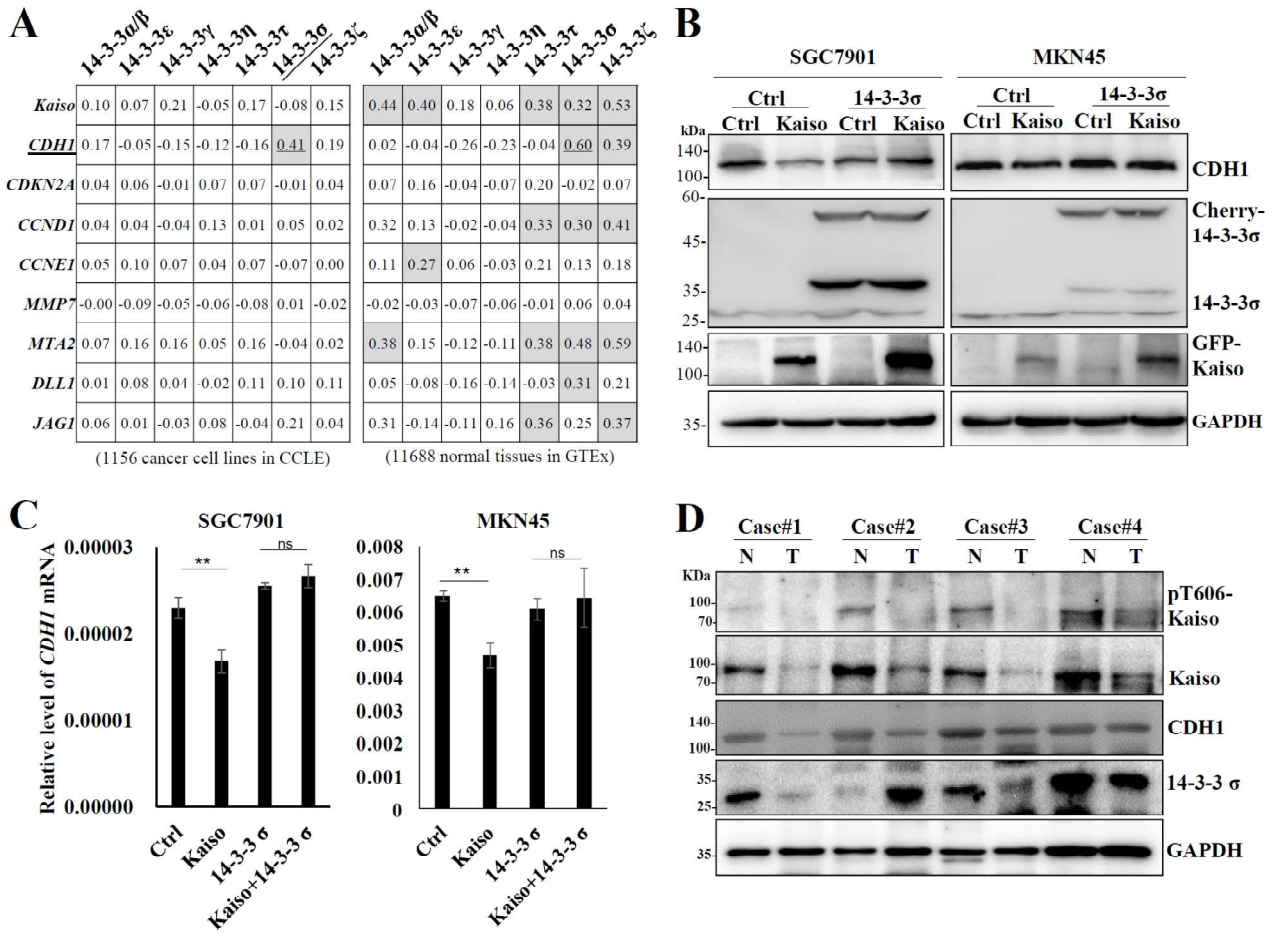

Figure 7. Effects of 14-3-3σ on inhibition of Kaiso's target gene *CDH1* expression. (**A**) The Pearson correlation coefficient between mRNA levels of 14-3-3 family members and Kaisos' target genes in public RNA-seq datasets from Cancer Cell Line Encyclopedia (CCLE, 1156 cancer cell lines) and Genotype-Tissue Expression (GTEx, 11688 human normal tissues). (**B**) The level of *CDH1* in SGC7901 and MKN45 cells with or without wild type Kaiso and 14-3-3σ overexpression. (**C**) The level of the *CDH1* mRNA in SGC7901 and MKN45 cells with or without wild type Kaiso and 14-3-3σ overexpression (**D**) The amounts of pT606-Kaiso, total Kaiso, 14-3-3 σ, and CDH1 proteins in gastric carcinoma (T) and the paired normal tissues (N) from 4 patients by Western blotting

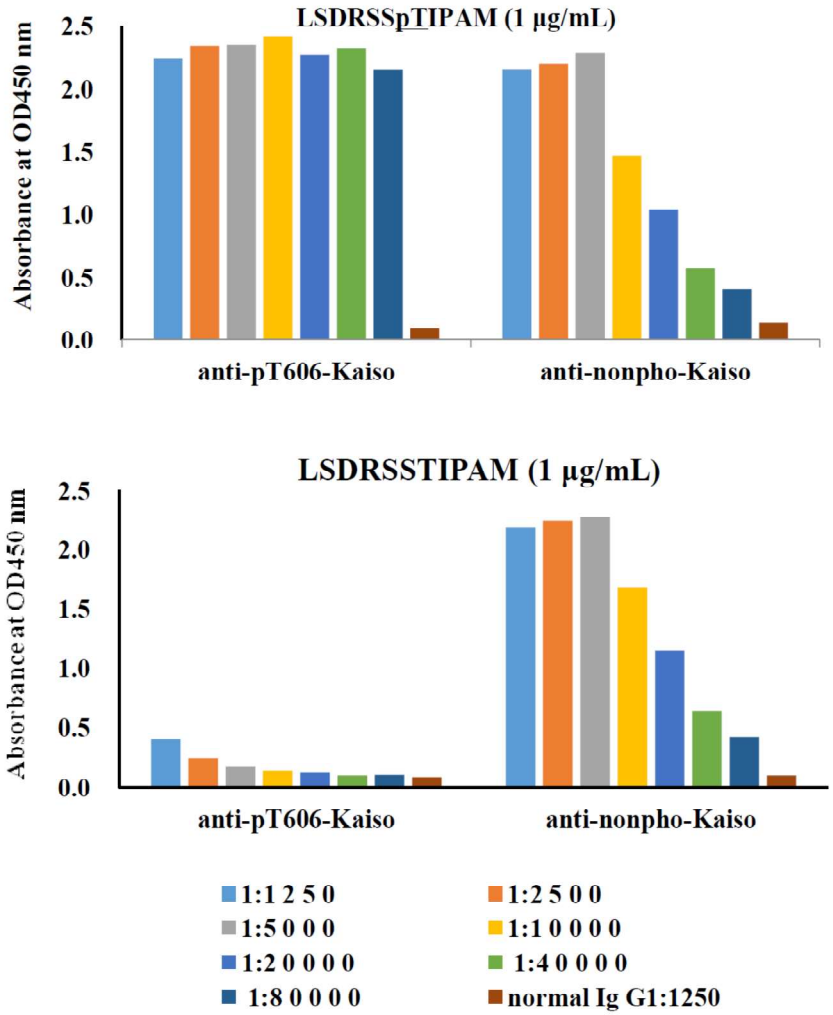

Figure S1. Characterizing the specificity of pT606-Kaiso polyclonal antibody. ELISA results for the specificity of pT606-Kaiso and control antibodies against the pT606-Kaiso peptide (LSDRSSpTIPAM, the top chart) and for the specificity of pT606-Kaiso and control antibodies for the nonphosphorylated control peptide (LSDRSSTIPAM, the bottom chart).

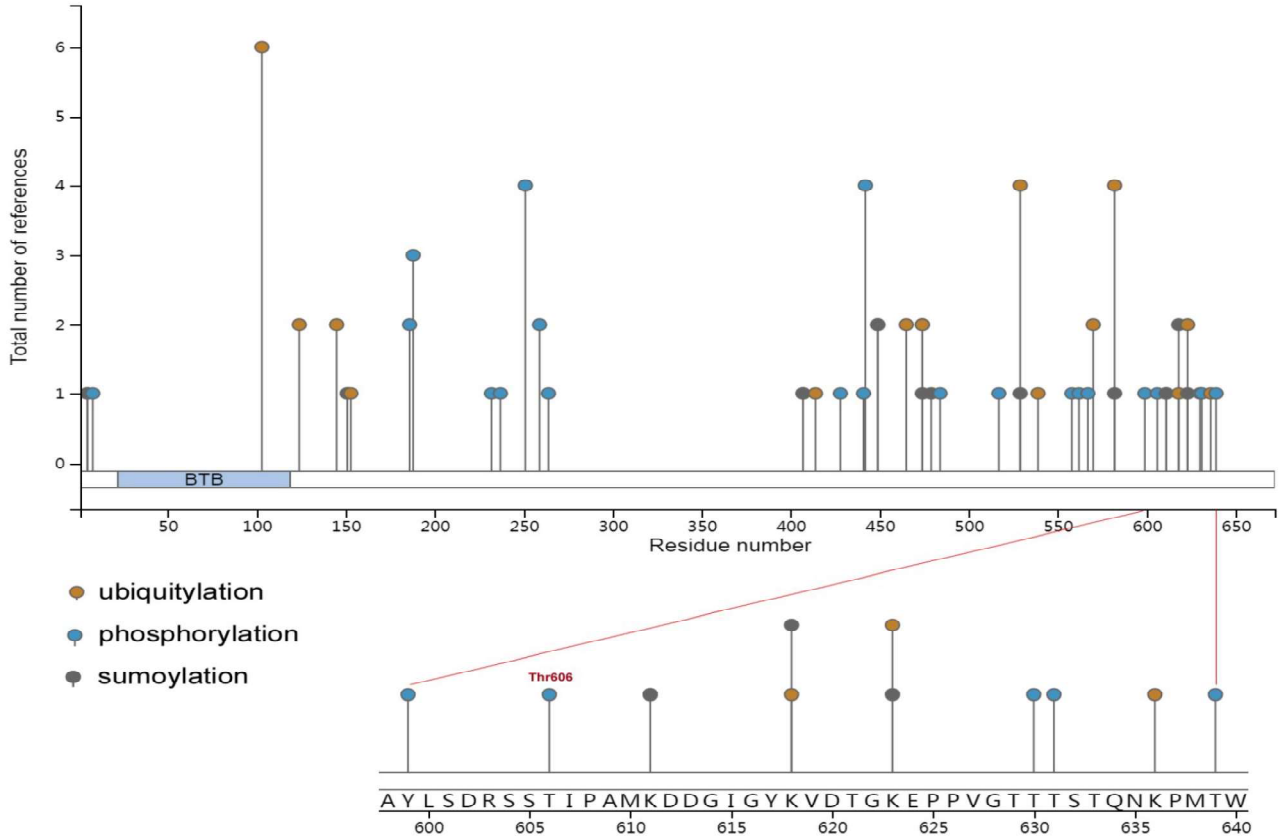

Figure S2. The status of Kaiso protein modifications detected by LC/MS. Phosphorylation at the Thr606 residue in the LSDRSSTIPAM motif was illustrated in the bottom chart in details. The image was adapted with graphs for Kaiso modifications from the web site (www.phosphosite.org; Ref.:40).

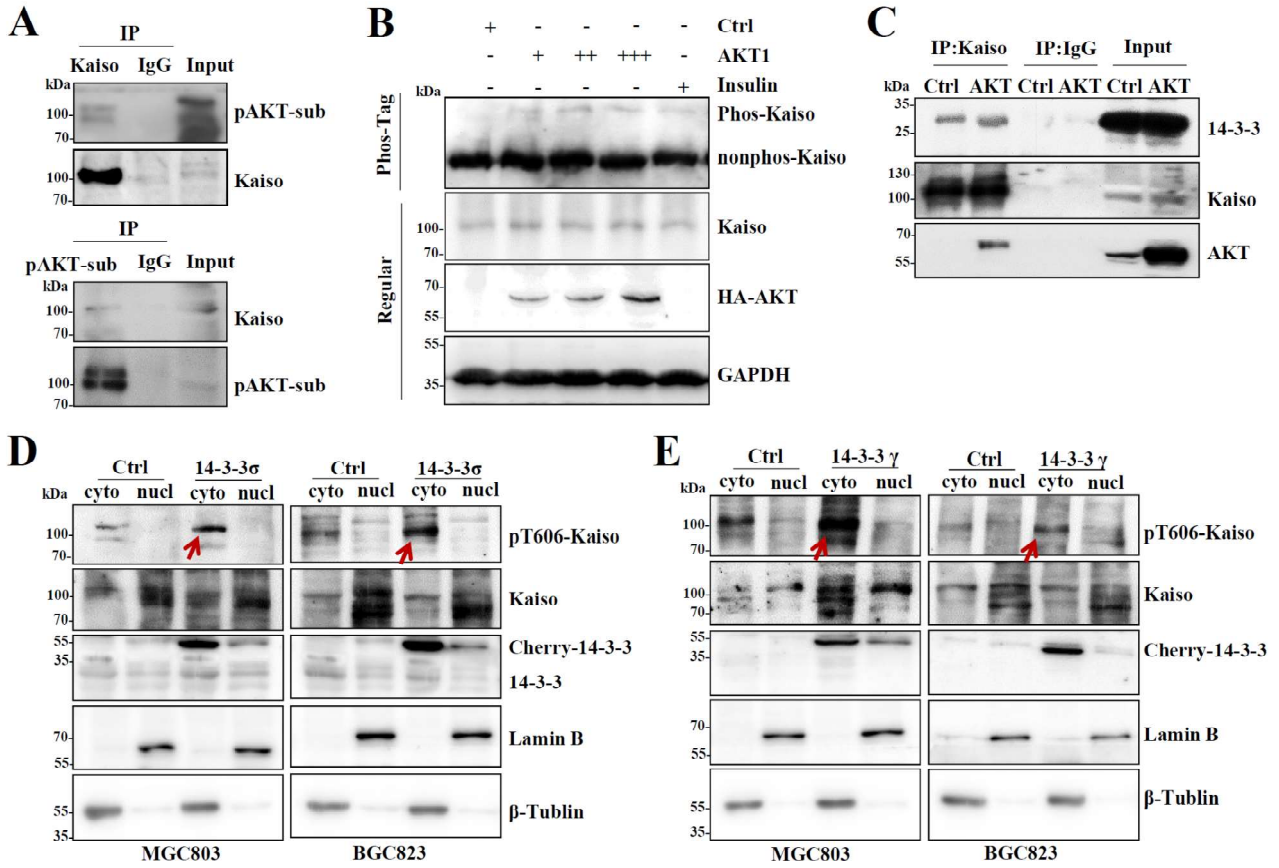

Figure S3. AKT1 and 14-3-3 regulate the T606-phosphorylation and subcellular localization of endogenous Kaiso. (**A**) Endogenous Kaiso in MGC803 cells immunoprecipitated by Kaiso antibody was identified by the antibody specific for AKT substrate motif, and the immunoprecipitation by AKT substrate antibody was identified by antibody against Kaiso. (**B**) After starvation overnight, overexpressed *AKT1* at different dose in HCT116 cells promoted the phosphorylation of Kaiso in phos-tag SDS PAGE gel. (**C**) *AKT1* overexpression increased endogenous Kaiso-14-3-3 interaction in MGC803 cells in Co-IP assay. (**D** and **E**) The T606-phosphorylation states of endogenous Kaiso in the cytoplasm and nucleus of MGC803 and BGC823 cells with 14-3-3σ and 14-3-3γ overexpression, respectively.

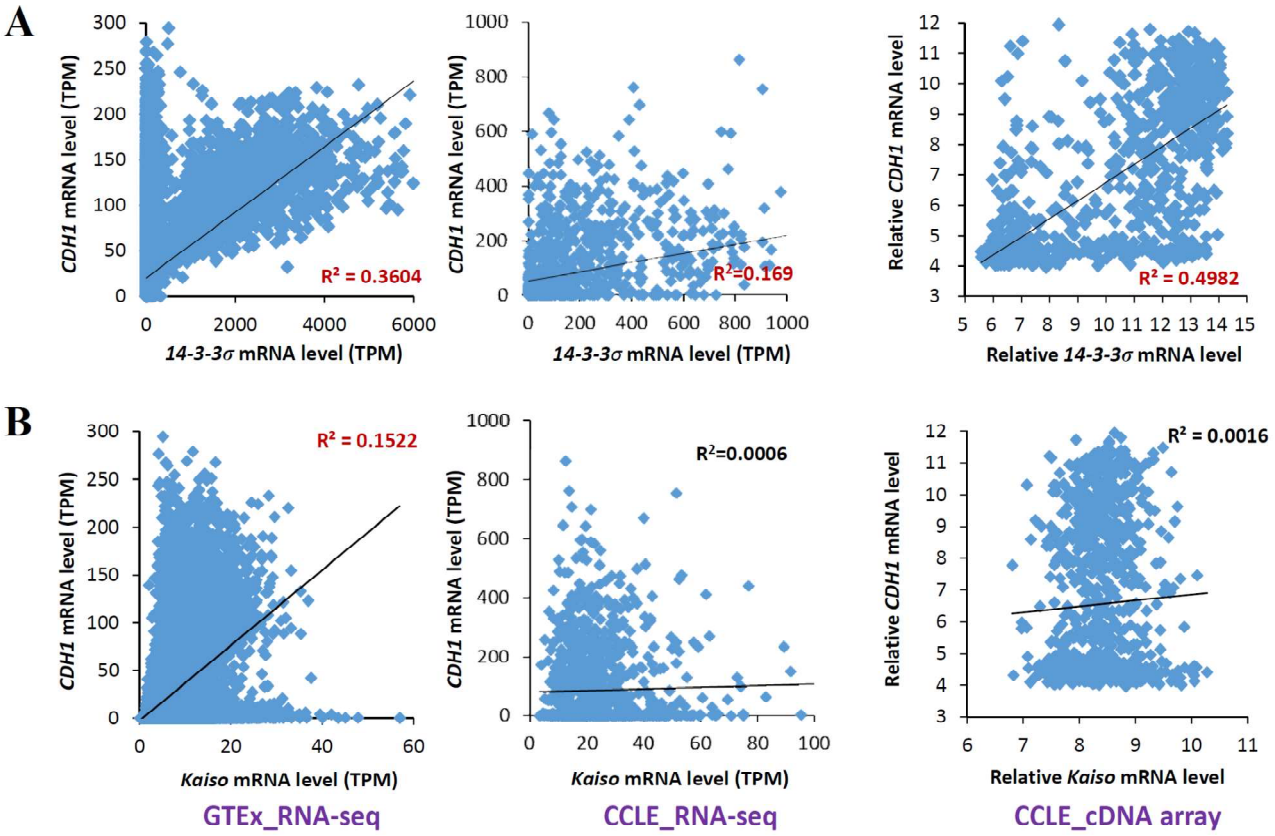

Figure S4. Correlation between the levels of *CDH1* and *14-3-3σ* (A) or *Kaiso* (B) mRNA in RNA-seq and cDNA array datasets for human normal tissues in the Genotype-Tissue Expression (GTEx) projects and cancer cell lines in Cancer Cell Line Encyclopedia (CCLE) project.

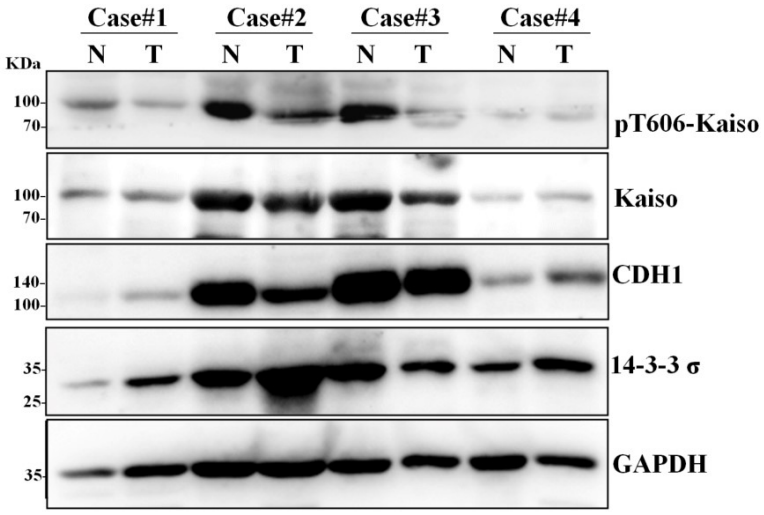

Figure S5. The amounts of pT606-Kaiso, total Kaiso, 14-3-3 σ, and CDH1 proteins in colon carcinoma (T) and the paired normal tissues (N) from 4 patients by Western blotting