T606-phosphorylated Kaiso interacting with 14-3-3 and p120ctn is sequestered in the cytoplasm of human cells

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Abstract

Kaiso is a transcription factor in the nucleus and p120ctn-binding protein in the cytoplasm. Although it is known that p120ctn is involved in Kaiso cytoplasmic-nuclear transportation, regulatory mechanisms of Kaiso transportation remain to be explored. We firstly found that Kaiso could directly interact with 14-3-3 family proteins, depending on the phosphorylation at the 606 threonine residue (**T**606) within the RSS**T**IP motif of Kaiso. AKT1 could phosphorylate Kaiso at T606. T606A mutation abolished most Kaiso–14-3-3 interaction. Notably, we found that the phosphorylated Kaiso (pT606-Kaiso) could also bind to p120ctn in the cytoplasm and block the cytoplasmic–nuclear transportation of Kaiso. The present study indicates, for the first time, that Kaiso can be phosphorylated by AKT1 at T606 and that pT606-Kaiso can bind both 14-3-3 and p120ctn proteins in the cytoplasm. The pT606-Kaiso–p120ctn (and 14-3-3) complexes cannot shift to the nucleus and accumulate in the cytoplasm. T606 phosphorylation regulates intracellular transportation of Kaiso.

Background

Kaiso protein is encoded by the ZBTB33 gene as a classic transcription repressor containing a zinc-finger domain and a BTB/POZ domain (1). The zinc-finger domain of nuclear Kaiso can bind to both methylated CGCG-containing sequences and non-methylated Kaiso binding sequences (KBSs) in the genome whereas the BTB/POZ domain can recruit the complex of NCoR1 corepressor and histone deacetylases to target genes and repress their transcription (2-4). Recent studies show that Kaiso acts as a transcription repressor or activator in the promoter context-dependent manner (5,6). Kaiso is also a cytoplasm protein, which regulates WNT-related pathway through interacting with p120ctn (7).

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 (8-11). The amount of nuclear Kaiso protein is correlated with the invasion or prognosis of cancers (12-15). Kaiso-deficient mice show resistance to intestinal cancer (16). Apparently, the expression and subcellular locations of Kaiso determine its normal functions and roles in cancer development.

However, current reports on the intracellular localization of Kaiso are controversial. Differences of subcellular localizations of Kaiso were observed between cultured cell lines and tissues (12, 17). It was reported that p120ctn might be a Kaiso binding protein and p120ctn–Kaiso complex could shift from the nucleus to the cytoplasm of cells (18). Cigarette smoke was also reported to affect Kaiso nuclear–cytoplasmic trafficking through MUC1 and p120ctn binding pathway (19). However, detailed regulation machinery for the compartmentalization of Kaiso is far from clear.

The 14-3-3 proteins are originally identified by Moore and Perez in 1967 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) in the RSS**T**IP motif by the protein serine-threonine kinase AKT1 and that T606-phosphorylated Kaiso (pT606-Kaiso) could efficiently interact with 14-3-3 and p120ctn in the cytoplasm, block the p120ctn-mediated cytoplasmic–nuclear transportation

of Kaiso.

Results

Kaiso is phosphorylated in the cytoplasm

Immunoaffinity profiling of phosphorylated proteins in cancer cells has found putative phosphorylation sites of Kaiso in proteomic mass spectrometry analysis (30). We wondered if the different compartmental localizations of Kaiso were related to the status of phosphorylation. In Phos-tag SDS-PAGE assay, Phos-tag binds 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 (31). Because there was no specific antibody for the putative phosphorylated Kaiso at the beginning, the Phos-tag SDS-PAGE assay was utilized to analyze the phosphorylation status of Kaiso and 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 (Figure 1A). And the delayed Kaiso migration was partially concealed by CIAP-dephosphorylation treatment, suggesting that the endogenous cytoplasmic Kaiso in these cells was mostly phosphorylated.

To validate the differential phosphorylation states of Kaiso in the cytoplasm and nuclear, the anti-phosphoserine/threonine/tyrosine universal antibody was used to precipitate global phosphorylated proteins and the Kaiso-specific antibody was then used to visualize them, or vice versa (Figure 1B). Again, phosphorylated Kaiso was observed only in the cytoplasmic precipitates, but not in the nuclear counterpart. Furthermore, in the mouse xenograft derived from MGC803 cells stably transfected with GFP-Kaiso, the phosphorylated GFP-Kaiso was still detected and no phosphorylated GFP-Kaiso was detected post the CIAP-dephosphorylation treatment in the Phos-tag SDS-PAGE analyses (Figure 1C). All the above results suggest that a certain portion of Kaiso is phosphorylated in the cytoplasmic.

The phosphorylation of Kaiso at T606 by AKT1

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 (32-35). Human Kaiso contains a conservative RSSTIP motif

with the threonine 606 (T606) residue that could be a potential phosphorylation candidate site for AKT1. (Fig.2A).

With various AKT signaling stimulations (36), including insulin, IL-6, and fetal bovine serum (FBS) after overnight starvation, the phosphorylation level of endogenous Kaiso was increased in MGC803 cells (Fig. 2B). In the Co-IP analyses, endogenous Kaiso could be identified in MGC803 cells using antibody against phosphorylated AKT substrate (pAKT-Sub) and vice versa (Fig. S1A). Consistent with that, the amount of pAKT-Sub was significantly increased by insulin stimulation (Fig. 2C) or *AKT1* overexpression (Fig. 2D), and decreased with AKT inhibitor MK2206 treatment (Fig. 2C). We found that active AKT1 recombinant kinase could phosphorylate GST-Kaiso recombinant protein (aa346-638 in Kaiso-wt) in an *in vitro* kinase assay while the AKT1 recombinant kinase was unable to phosphorylate most Kasio-T606A mutant control. (Fig. 2E). Together, these results suggest that AKT1 could directly phosphorylate Kaiso at T606.

To confirm that, we used the phosphor-peptide LSD**RSS<u>pT</u>IP**AM as antigen to prepare pT606-Kaiso-specific antibody. The amount of pT606-Kaiso was significantly increased in MGC803 and BGC823 cells with insulin stimulation (Fig. 2F) or *AKT1* overexpression (Fig. 2G). Using the pT606-Kaiso antibody, most pT606-Kaiso was detected in the cytoplasm of four human cancer cell lines (MGC803, BGC823, HCT116, and RKO) and CIAP dephosphorylation treatment markedly decreased the amount of pT606-Kaiso in the cytoplasm in Western blotting (Fig. 3A). Confocal microscopy confirmed the cytoplasmic accumulation of pT606-Kaiso in MGC803 cells (Fig. 3B). Together, these results suggest that AKT1 phosphorylate Kaiso at T606 and pT606-Kaiso accumulated in the cytoplasm of MGC803 cells.

Kaiso interacted with 14-3-3 family depending on pT606 of Kaiso

It is well-characterized that RSXpSXP is a 14-3-3 phosphoserine binding consensus motif (37). To examine whether Kaiso can bind to 14-3-3 using the RSSpTIP motif, in the first step, an 14-3-3 binding motif antibody (14-3-3 BM mAb; prepared from mice by immunizing with phospho-(Ser) 14-3-3 binding motif peptides) was employed in an immunoprecipitation (IP) assay. IP results demonstrated that the 14-3-3 binding-specific motif antibody could precipitate endogenous Kaiso, or vice versa (Fig. 4A). Further, we performed GST-pull down experiment using purified GST-Kaiso recombinant protein and incubated the GST-Kaiso with the lysate of MGC803 cells. As a result,

GST-Kaiso could pull down pan-14-3-3 proteins in the lysate of these cells (Fig. 4B top). Further detection showed that GST-Kaiso could pull down all of seven tested 14-3-3 isoforms (Fig. 4B bottom). The Co-IP results also confirmed that endogenous Kaiso and endogenous 14-3-3 proteins indeed interacted with each other in MGC803 cells (Fig. 4C). As expected, the T606A mutation abolished most of the Kaiso–14-3-3 interaction (Fig. 4D), indicating that T606 residue within RSSTIP motif is the main phosphorylation site of Kaiso protein, although T606A mutation couldn't conceal all the phosphorylated Kaiso form (Fig. S1B). In addition, AKT1 promoted the Kaiso–14-3-3 interaction (Fig. S1C). These data indicate that Kaiso interacted with 14-3-3 proteins in a T606 phosphorylation-dependent manner.

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

Kaiso usually distributes in both the cytoplasm and the nucleus. Intracellular Kaiso compartmentalization is affected by growth conditions (13, 17). Our indirect immunofluorescence analyses showed that endogenous Kaiso was mostly observed in the nucleus of MGC803 cells (81% in the nucleus vs. 19% in the cytoplasm); Compared to the mCherry empty vector, overexpression of mCherry-labeled 14-3-3γ or 14-3-3σ significantly induced the accumulation of endogenous Kaiso in the cytoplasm (from 19% to 37% or 36%; Fig.5A-B). Notably, Kaiso was mainly co-located with mCherry-14-3-3γ or mCherry-14-3-3σ in the cytoplasm of MGC803 cells. Western blotting confirmed that 14-3-3σ overexpression increased the cytoplasmic accumulation of endogenous Kaiso in these cells (Fig. 5C). In addition, 14-3-3σ only promoted the cytoplasmic accumulation of wildtype Kaiso (Kaiso-wt), but not the T606A mutant (Fig. 5D). 14-3-3γ or 14-3-3σ overexpression induced more cytoplasmic pT606-Kaiso in these two cell lines (Fig. S1D and S1E). 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.

pT606-Kaiso–14-3-3 complex binds to p120ctn in the cytoplasm

As a well-known Kaiso interacting protein, p120ctn is essential for the nuclear to cytoplasmic trafficking of Kaiso (18, 19). Thus, we further studied whether p120ctn is involved in the 14-3-3-mediated cytoplasmic pT606-Kaiso accumulation. Here, we found that 14-3-3σ overexpression markedly increased Kaiso–p120ctn interaction (Fig. 6A) while the levels of total Kaiso and p120ctn proteins were not changed in MGC803 cells (Fig. 6B). 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. 6C). Indirect immunofluorescence confocal microscopy showed that p120ctn, Kaiso, and 14-3-3σ were co-localized in the cytoplasm (Fig. 6D). Moreover, when p120ctn was knocked down by siRNA, the cytoplasmic Kaiso accumulation promoted by 14-3-3σ disappeared in the MGC 803 cells (Fig. 6E) while the level of total Kaiso was not changed (Fig. 6F). These results suggest that the p120ctn interaction is essential for 14-3-3σ to increase the pT606-Kaiso accumulation in the cytoplasm.

Discussions

The subcellular locations of Kaiso determine its normal functions and roles in 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 and the pT606-Kaiso could interact with 14-3-3 in the cytoplasm, and promoted the cytoplasmic accumulation of Kaiso in a p120ctn binding-dependent manner.

It's well known that 14-3-3 proteins bind to common phosphoserine/phosphothreonine-containing peptide motifs corresponding to Mode-1 (RSXpSXP) or Mode-2 (RXY/FXpSXP) sequences (37). We observed that Kaiso contains a very conservative motif RSSTIP that could be phosphorylated by AKT1 in both *in vivo* and cell-free system at RSSTIP-T606. The pT606-Kaiso could directly bind 14-3-3 family proteins and T606A mutation abolish most Kaiso–14-3-3 binding. However, T606A mutation couldn't conceal all the phosphorylated Kaiso form, suggesting a few phosphorylation existing at other sites. In proteomics studies by mass spectrometry, Kaiso phosphorylation was previously detected at other sites, for example Y442, within Kaiso without information on biological function (30). Our study results showed that Y442F mutation could not affect the subcellular distribution pattern and total phosphorylation level of Kaiso, suggesting Y442 not to be a phosphorylation site in MGC803 cells (data not shown).

It has been reported that a region consisting of 1-200 amino acid residues of Kaiso directly interacts with p120ctn (1). Kaiso–p120cnt interaction may affect nuclear to cytoplasmic trafficking of Kaiso (19). Usually, p120ctn binds to E-cadherin and modulates E-cadherin function and stability (38). WNT-stimulated p120ctn phosphorylation promotes p120ctn releasing from the E-cadherin–p120ctn complexes, enhances Kaiso–p120ctn interaction (39). Here, we found that the T606 phosphorylation

status of Kaiso not only affects its interaction with 14-3-3, but also affects its interaction with p120ctn in the cytoplasm. Further study is needed to see whether pT606-Kaiso–p120ctn binding and subsequent accumulation of pT606-Kaiso in the cytoplasm is related to the phosphorylation status of p120ctn.

Most 14-3-3σ protein localizes in the cytoplasm (40) while Kaiso mainly localizes in the nucleus. In our study, Kaiso–14-3-3σ binding is dependent on Kaiso–p120ctn binding. It is unknown how 14-3-3σ influences the p120ctn-Kaiso binding. In confocal analysis, we observed that Kaiso, 14-3-3σ, and p120ctn were co-localized in the cytoplasm, suggesting that Kaiso, 14-3-3σ, and p120ctn might form a triplex and sequester Kaiso in the cytoplasm (Fig. 7). Although we didn't find any mutual binding motif in p120ctn or 14-3-3σ protein, direct p120ctn–14-3-3σ interaction could not be excluded. How Kaiso, 14-3-3σ, and p120ctn interacting with each other is worth studying.

There is controversy of Kaiso's role in cancer development. In the absence of the tumor suppressor *APC*, Kaiso-deficient mice were susceptible to intestinal cancer, suggesting that Kaiso might be an oncogene (16). On the contrary, Kaiso has also been suggested to be a potential tumor suppressor, which repressed transcription of *MMP7*, *CCND1*, and *WNT11* genes involved in oncogenesis and metastasis (7, 8, 10, 41). Functions of Kaiso are tightly related and significantly influenced by microenviromental factors (17). Smoking also affects compartmentalization of Kaiso (18). Our data indicate that increase of the level of Kaiso phosphorylation was significantly coupled with decrease of transcription repression of Kaiso target genes including *CDKN2A* and *CDH1* in reporter assays (data not shown). It is interesting to study if the status of phosphorylation and subcellular localization of Kaiso determines the role of Kaiso in cancer cells.

In conclusion, Kaiso protein can be phosphorylated by AKT1 at the very conservative motif RSSTIP. pT606-Kaiso directly interacts with 14-3-3 family proteins and promotes pT606-Kaiso accumulation in the cytoplasm in the p120ctn binding-dependent way. The phosphorylation status of Kaiso may determine its functions in human cells.

Materials and Methods

Cell lines and culture

The gastric cancer cell line MGC803 and BGC823 were kindly provided by Dr. Yang Ke at Peking University Cancer Hospital & Institute. The human embryonic kidney 293T cell line was kindly

provided by Professor Yasuhito Yuasa at Tokyo Medical and Dental University. The human colon cancer cell line HCT116 and RKO were respectively kindly provided by Dr. Yuanjia Chen, Peking Union Hospital (ATCC CCL-247) and Dr. Guoren Deng, University of California (ATCC-CRL-2577). 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., Ltd. before they were used in this study. Short tandem repeat (STR) patterns were analyzed using GoldeneyeTM20A STR Identifiler PCR Amplification Kit.

Plasmids, antibodies and reagents

The full-length *Kaiso* coding sequence was amplified from human cDNA of MGC803 cells with primers 5'-attaaactcgaggcatggagagtagaaaactga-3' and 5´-cgcttcgaattcgtttagtaagactctggtattat-3', then inserted between *Xho*I and *Eco*RI sites of pEGFP-C1 vector to generate pEGFP-C1-Kaiso. pEGFP-C1-Kaiso-T606 mutations were obtained by mutation PCR using primers: forward primer 5'-gatagatcaagcgctattcctgcaatg -3' and reverse primer 5'-cattgcaggaatagcgcttgatctatc-3' for 606Thr → Ala (T606A) mutation. Plasmid pCMV-3Tag-2C-Kaiso was generated by inserting full-length *Kaiso* into *BamH*I and *Eco*RI sites of pCMV-3Tag-2C vector. To construct pGEX-4T-1-Kaiso (aa346-638), BTB/POZ domain-deleted Kaiso was amplified with primers 5'-tatcggaattccctgactcggccgtcagt-3' and 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-M98-14-3-3γ-GFP, pEZ-M56-14-3-3σ-mCherry vectors were purchased from FulenGen Co., Ltd. (EX-T4084-M56, EX-T4084-M98-5, EX-C0507-M98, Guangzhou, China).

Antibodies used in Western blot or IP for Kaiso (sc-365428, Santa Cruz, USA), p120 (66208-1, Proteintech, IL, USA), Pan-14-3-3 (sc-629, Santa Cruz, USA), 14-3-3 family (α/β, ε, η, γ, τ, ζ, σ)kit (#9769, CST, USA), 14-3-3σ (sc-100638, Santa Cruz, USA)，14-3-3 binding motif antibody (14-3-3 BM mAb; #9606, CST, USA), Ser/Thr/Tyr phosphor-protein (ab15556, Abcam, UK), AKT substrate antibody (#9611, CST USA), Lamin B1 (66095-1, Proteintech, IL, USA), β-Tublin (66240-1, proteintech, IL, USA), HA (M20003, Abmart, Shanghai, China), FLAG (66008-2, Proteintech, IL,

USA), GFP (NB100-1614, Novus, CO, USA), GST (66001-1, Proteintech, IL, USA), GAPDH (660004-1, Proteintech, IL, USA) were purchased from respective companies.

siRNAs for 14-3-3σ was purchased from Santa Cruz (sc-29590, USA). For p120, the siRNA sequences were #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' (Genepharma, Shanghai, China)

Insulin (P3376, Beyotime, Shanghai, China), IL-6 (Cat. 200-06, Proteintech, NJ, USA), EGF (PHG6045, Thermo Fisher Scientific, MA, USA), and MK2206 (HY-10358, MedChemExpress, NJ, USA) were purchased from respective companies and used to treated MGC803 cells at the concentration described for 15 min before detecting. Active, recombinant full-length human Akt1 was purchased from merck (14-276, merck, Darmstadt, Germany).

Cell transfection, **stable cell line generation and *in vivo* xenografts**

X-tremeGENE siRNA Transfection Reagent or X-tremeGENE HP DNA Transfection Reagent (Cat. 04476093001, Cat. 06366236001, Roche, Mannheim, Germany) were used in cell transfection of siRNAs for p120 and 14-3-3 σ (final concentration, 100 nM) or plasmids for Kaiso and mutations (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 cell line generation, 750μg/ml G418 was used for MGC803 to select GFP-Kaiso positively expressed cells. For Kaiso dectecting in xenografts, MGC803 cells stably expressed GFP-Kaiso were resuspended in PBS (1×10^7 /ml) and 0.2 ml were inoculated subcutaneously into the right/left pads of 6 week-old male BALB/c-nu mice. Mice were sacrificed on days 21 after inoculation, and xenografts were separated and cut into about 1-mm3 pieces to grind for protein fractionation.

Subcellular fractionation and dephosphorylation treatment (42, 43)

To prepare cytoplasmic and nuclear extracts, cells at 80% confluence were homogenized in ice-cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1 mM DTT, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$ and 1 × protease inhibitor cocktail (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 buffer B (10 mM Tris-HCl, pH 7.6, 420 mM NaCI, 0.5% NP-40, and 1 mM DTT, 2 mM $MgCI_2$, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$ and 1 × protease inhibitor cocktail) incubated 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 dephosphorylation, 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 (100 mM NaCl, 50mM Tris-HCl, 10 mM $MgCl_2$, 1Mm DTT, pH 7.9; final concentration) and incubated for 1 hr at 37°C.

Immunoprecipitation (IP) and Western blotting

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 lysates were 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 control. The precipitates were washed six times with lysis buffer, and boiled in 1 × loading buffer. Protein samples were resolved by SDS–PAGE or Phos-tag SDS-PAGE (Phos-tag Acrylamide AAL-107, WAKO, Japan), and electroblotted onto nitrocellulose membranes, which were blocked in 5% skim milk in PBST and probed with the indicated antibodies according to the protocol in the instruction manual. For Western blot detection, we utilized primary antibodies as described above and horseradish-peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (SE134, Solarbio, Beijing, China) or goat anti-mouse secondary antibody (SE131, Solarbio, Beijing, China). Protein bands were further detected with HRP Substrate Luminol Reagent (Cat. WBKLS0500, Millipore, MA, USA).

Phos-tag SDS-PAGE assay

This is a modified SDS-PAGE method based on the novel Phos-tag (31), which can bind to phosphorylated proteins and decrease their migration speed. It can be used to distinguish dephosphorylated proteins from phosphorylated proteins based on their migration difference.

GST-Pull down and Co-IP

GST or GST-Kaiso protein was expressed using pGEX-4T1 plasmid in BL21 *E. coli* induced with

IPTG 0.5 mM at 37℃. Glutathione sepharose beads (17-0756-01, GE healthcare, Sweden) were incubated with *E. coli* bacteria lysates expressing GST-Kaiso at 4 ℃ 8 hrs. After washed with PBS, 1 μg GST or GST-Kaiso protein with Glutathione sepharose beads was added with cell lysate from 10 × 10^7 MGC803 cells and incubated at 4 ℃ overnight. Beads were washed six time with 500 μL cell lysis buffer. After the last centrifuging, remove all the supernatant as clean as possible and subjected to 20 μL 1 × SDS sample buffer. Antibodies for Pan-14-3-3 and 14-3-3 family were used to detected the pulled down precipitant.

For Co-IP, most procedures was the same as the IP assay. After pre-cleared with protein A/G-coupled Sepharose beads for 2 hrs, the soluble protein from whole cell lysate were immunoprecipitated with anti-Kaiso (Santa Cruz, sc-365428) or anti-GFP (Abcam, ab290) or anti-14-3-3 binding motif (Cell Signal Technology, 9606) or other antibodies plus protein A/G Sepharose overnight at 4°C. Mouse IgG or rabbit IgG was used as control. To immunoprecipitate overexpressed FLAG-tagged 14-3-3 isoforms, FLAG Affinity Gels (FLAGIPT1-1KT, Sigma-Aldrich, MO, USA) was used. 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** (44,45)

1 μg of the substrate GST or GST-Kaiso-wt/T606A protein per reaction was expressed through pGEX-4T1 plasmid system in *BL21 E. coli*, induced with IPTG 0.5 mM at 37 ℃ and purified with Glutathione sepharose beads. Kinase AKT1 (PKBα) Protein, active, 15 μg, was purchased from Merck (14-276, Darmstadt, Germany). 10× Kinase Buffer (1×concentration: 25 mM Tris (pH 7.5), 5 mM b-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 suspend pellet in 30 μL of 1 × Kinase Buffer supplemented with 1 μl of 10 mM ATP and 0.5 μg AKT1 kinase wereincubated for 30 min at 30°C. Reaction was terminated with 30 μL 2 × SDS Sample Buffer. Samples were vortex, then microcentrifuge for 30 sec at 12,000 *g*. The reaction products were then resolved through SDS-PAGE gel and Western blot to detect the phosphorylated Kaiso with AKT1 substrate antibody.

Preparation of pT606-phosphorylated Kaiso-specific antibody

Anti-pT606-Kaiso polyclonal antibodies were raised in rabbits challenged with the synthesized phosphor-peptide antigen LSD**RSSpTIP**AM, a sequence corresponding to amino acids 600-610 of wildtype Kaiso (Kaiso-wt), and purified with non-phosphor-peptide LSD**RSSTIP**AM, followed by phosphor-peptide LSD**RSSpTIP**AM. Peptide synthesis and immunization of the animals were done at YouKe (Shanghai, China). The nonphosphor-peptide was synthesized and immunized at same time as negative control. Specificity of pT606-Kaiso and control antibodies against the peptides of LSDRSSpTIPAM or LSDRSSTIPAM was detected through ELISA assay (Fig. S2A and S2B) and Western blot (Fig. S2C).

ELISA analysis for polyAb against pT606-Kaiso

Polystyrene plates were coated with 1μg/ml synthetic phosphorylated peptide C- L S D R S S（pT）I P A M - N H 2 or nonphosphorylated peptide C- L S D R S S T I P A M - N H 2 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 h. The purified antibodies, diluted as described in figure were added (100μl/well) and incubated at 37°C for 1 h. After being washed with 0.05% Tween/PBS, plates were added HRP-labeled goat anti-Rabbit IgG (100μl /well) and incubated at room temperature for 30 min. Peroxidase activity was measured with 0.15 mg/ml TMB substrate solution (100μl /well). After 15 min at room temperature, the reaction was stopped by 2M H_2SO_4 (50μl /well). Optical density absorbance (OD) at 450 nm was determined using a model 550 microplate reader.

Indirect immunofluorescence

For mCherry-14-3-3 and GFP-Kaiso, direct fluorescence was detected with laser confocal microscope assay. MGC803 cells with overexpression of mCherry-14-3-3 and GFP-Kaiso were rinsed for three times with PBS, fixed with 1% paraformaldehyde in PBS 30 min at 37°C, 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 T606-phosphorylated Kaiso (1 μg/μL, 1:100, YouKe, Shanghai, China), for total endogenous Kaiso (1 μg/μL, 1:100 YouKe, Shanghai, China; and commercial antibody (0.2μg/μl, 1:20, sc-365428, Santa Cruz, USA), for p120ctn (1:100, 66208-1, Proteintech, IL, USA) were used as the primary antibodies; the FITC-labeled antibody against Rabbit

IgG (1:100, ab6717, Abcam, UK) and CY5-labeled antibody against Mouse IgG (1:100, Cat. No. 072-02-18-06, KPL Gaithersburg, MD, USA) were used as the secondary antibodies for observation under laser confocal microscope and were analyzed with ImageXpress Micro high content screening System.

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Competing Interest

The authors declare that they have no conflict of interest.

Author contribution

Cell culturing and molecular cell biology experiment, WT, SQ, BZ; Western blot and indirect immunofluorescence, LG, WT; Data analysis, JZ; Writing—original draft, WT, DD; Writing—review & editing, SQ; Supervision, DD;

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Figure Legends

Fig.1. Differential phosphorylation status of Kaiso in cytoplasm and nucleus in cultured cells and xenografts. (**A**) Differential phosphorylation status of cytoplasmic and nuclear fractions of endogenous Kaiso in MGC803 and HEK293T cells examined using the Phos-tag and regular SDS-PAGE. The purities of cytoplasmic and nuclear extracts were respectively verified by anti-β-tubulin and anti-Lamin B antibodies. The bands of increased migration speed after the CIAP treatment indicated dephosphorylated state of Kaiso. (**B**) The phosphorylation states of cytoplasmic and nuclear Kaiso in MGC 803 validated using anti-phosphoserine/ threonine/ tyrosine (Ser/ Thr/ Tyr) antibody for Kaiso's immuprecipiatates, or vice verse. (**C**) The phosphorylation states of GFP-Kaiso both in cultured MGC803 cells and in xenografts cells detected using the Phos-tag (50 μM) SDS-PAGE.

Fig.2. AKT1 increases the phosphorylation of Kaiso at T606. **(A**) A conservative RSXTXP motif in Kaiso. (**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 (10 μmol/mL) for 30 min blocked the promotion of Insulin on Kaiso phosphorylation as AKT 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.

Fig.3. Subcellular localization of endogenous pT606-Kaiso. (**A**) Amount of pT606-Kaiso in cytoplasmic and nuclear proteins, with and without dephosphorylation treatment of CIAP for 30 min,

from 4 cancer cell lines MGC803, BGC823, HCT116, and RKO. (**B**) Location of pT606-Kaiso and total Kaiso in the cytoplasm and nucleus with indirect immunofluorescence confocal assay.

Fig.4. Kaiso interacted with 14-3-3 family, depending on Kaiso-pT606. (**A**) The 14-3-3 binding motif (14-3-3 BM) antibody could immnoprecipitate endogenous Kaiso (identified by Kaiso-specific antibody; top chart) whereas the Kaiso antibody could immnoprecipitate 14-3-3 binding motif containing proteins (identified by 14-3-3 binding motif-specific antibody; bottom chart). (**B**) Pan-14-3-3 was pulled down by purified recombinant GST-Kaiso fragment from MGC803 cell lysate, but not by GST control. GST-Kaiso pulled down various isoforms of 14-3-3 family. (**C**) Endogenous Kaiso immunoprecipitated 14-3-3 protein in MGC803 cell lysate in Co-IP analyses. Endogenous 14-3-3 σ immunoprecipitated Kaiso in MGC803 cell lysate in Co-IP analyses. (**D**) T606A mutation of Kaiso concealed its interaction with 14-3-3 in MGC803 cell lysate.

Fig.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.

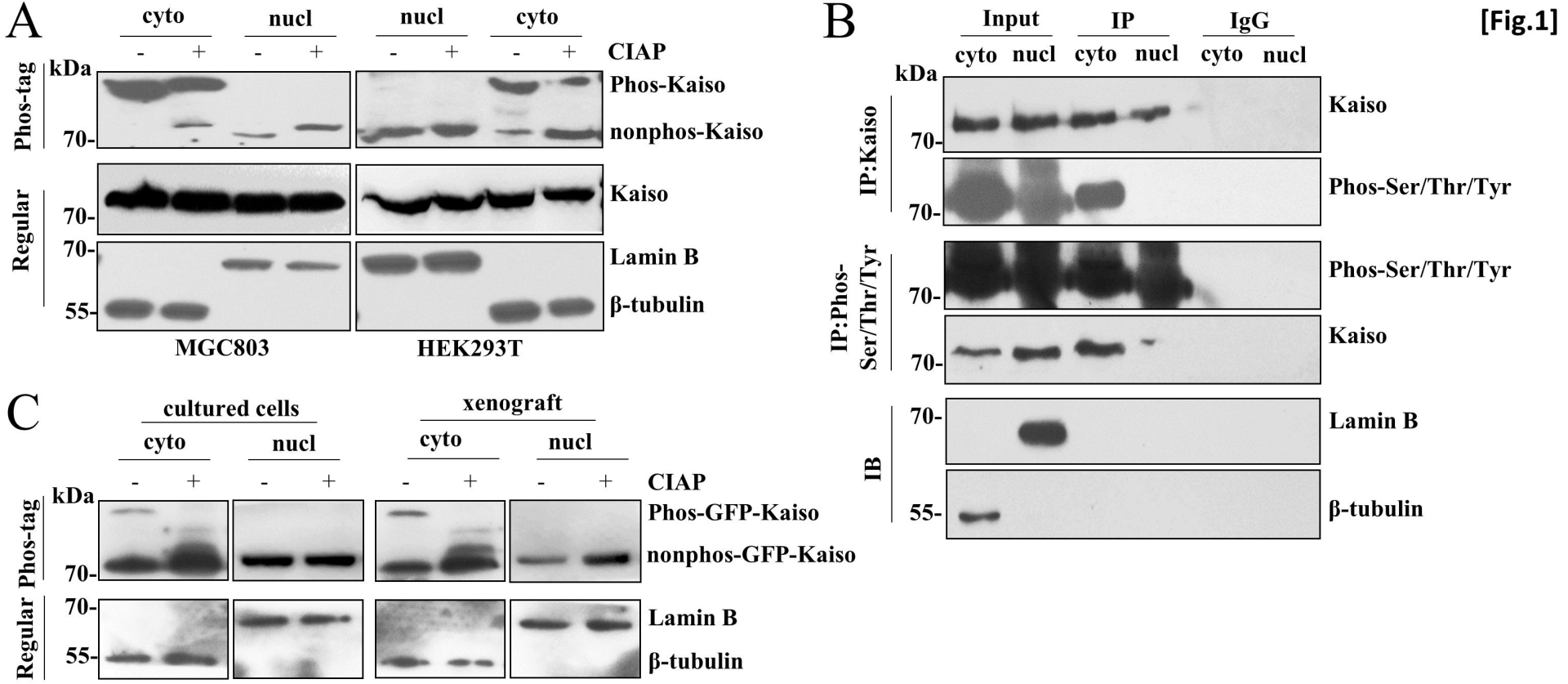
Fig.6. 14-3-3σ promotes the interaction of Kaiso and p120ctn in the cytoplasm. (**A**) More Kaiso-p120 complex was immunoprecipitated by Kaiso antibody in MGC803 cells with 14-3-3σ overexpression in Co-IP assay. (**B**) Total protein levels of p120ctn and 14-3-3σ in the lysate of MGC803 cells with overexpression of *14-3-3σ* or siRNA silencing of *14-3-3σ*. (**C**) Alterations of interactions between Kaiso and p120ctn proteins in the cytoplasm and nucleus in the Co-IP assay using Kaiso antibody. Heavy chain of IgG (HC) was used as control. (**D**) The subcellular locations of Kaiso, p120ctn, and mCherry-14-3-3σ in MGC803 cells. (**E**) Western blotting images for detecting effect of *p120ctn* knockdown on distribution of Kaiso in the cytoplasm and nucleus of MGC803 cells. (**F**) The levels of

total p120ctn and Kaiso proteins in the lysate of MGC803 cells with *14-3-3σ* overexpression and siRNA-knockdown of *p120ctn* expression.

Fig.7. model of AKT- 14-3-3-Kaiso interaction

Fig. S1. 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**) T606A mutation decreased the phosphorylation level of GFP-Kaiso in Phos-tag SDS-PAGE gel and Western blotting analyses. **(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.

Fig. S2 Characterizing the phosphorylation of Kaiso at T606 and the specificity of its polyclonal antibody. (**A**) ELISA assay for the specificity of pT606-Kaiso and control antibodies against pT606-Kaiso peptide (LSDRSSpTIPAM). (**B**) ELISA assay for the specificity of pT606-Kaiso and control antibodies for nonphosphorylated control peptide (LSDRSSTIPAM). (**C**) The binding activity of pT606-Kaiso antibody to Kaiso was significantly altered by T606A mutation comparing with wildtype.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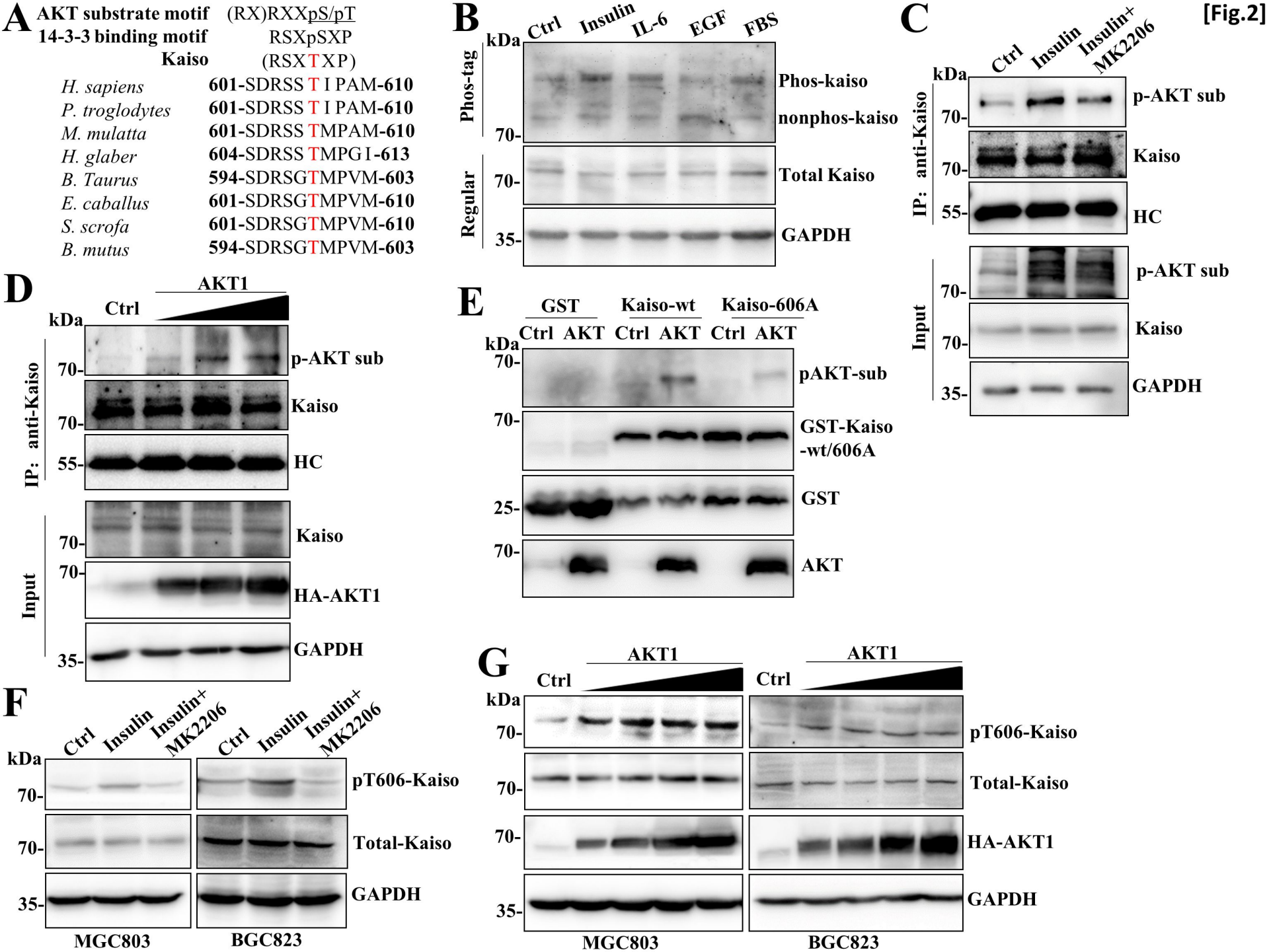
[Fig.1]

[Fig.2]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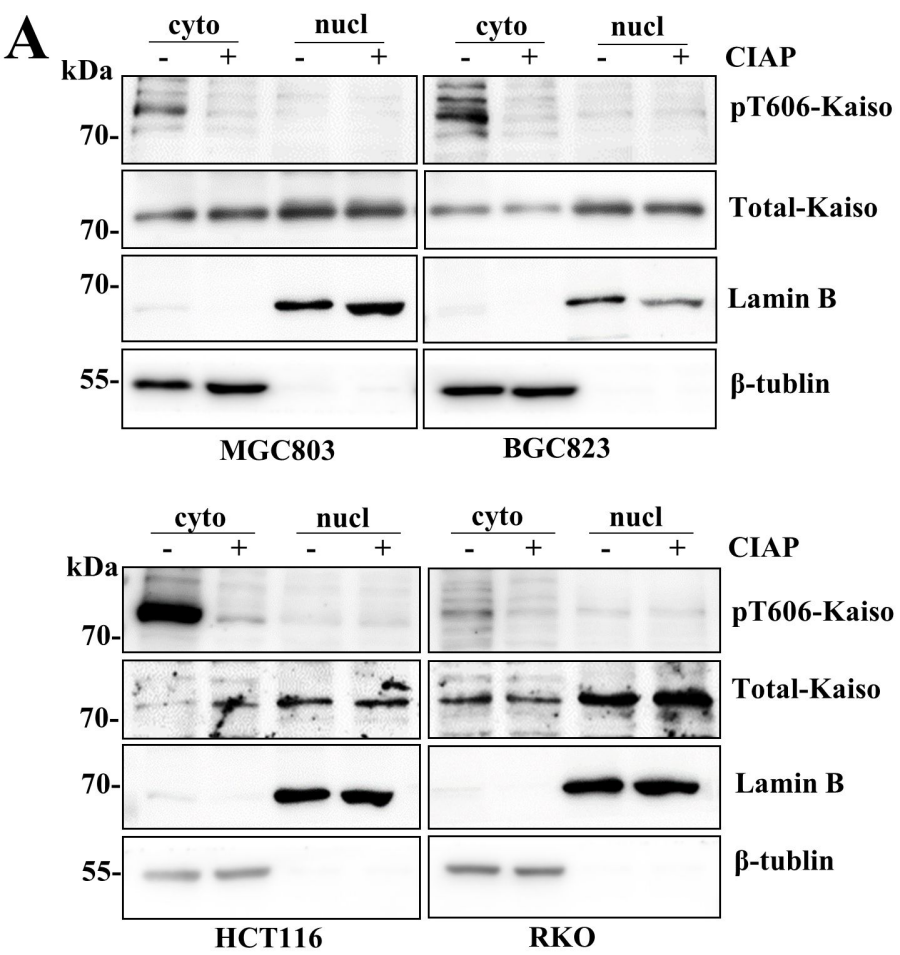
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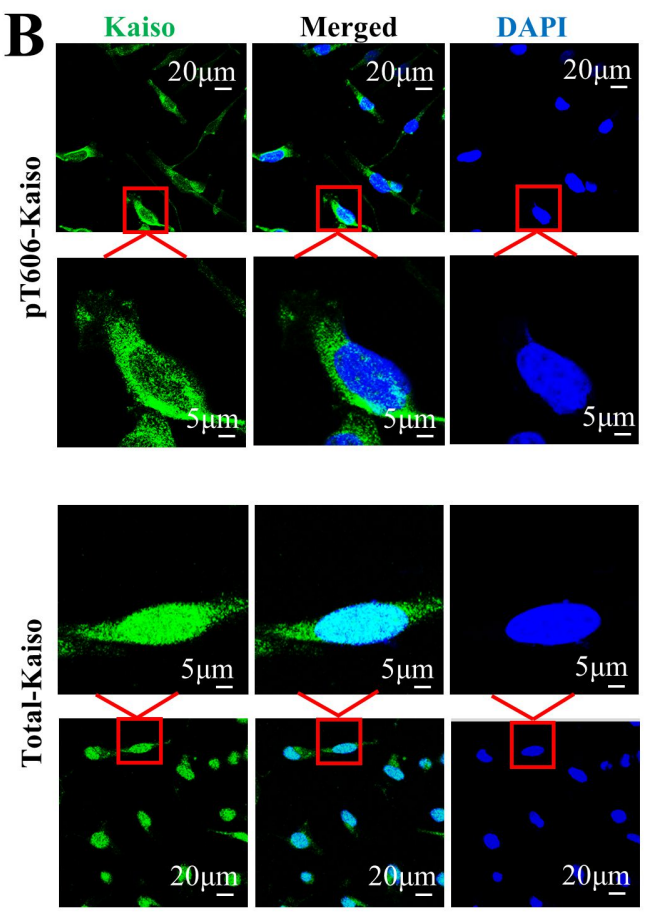
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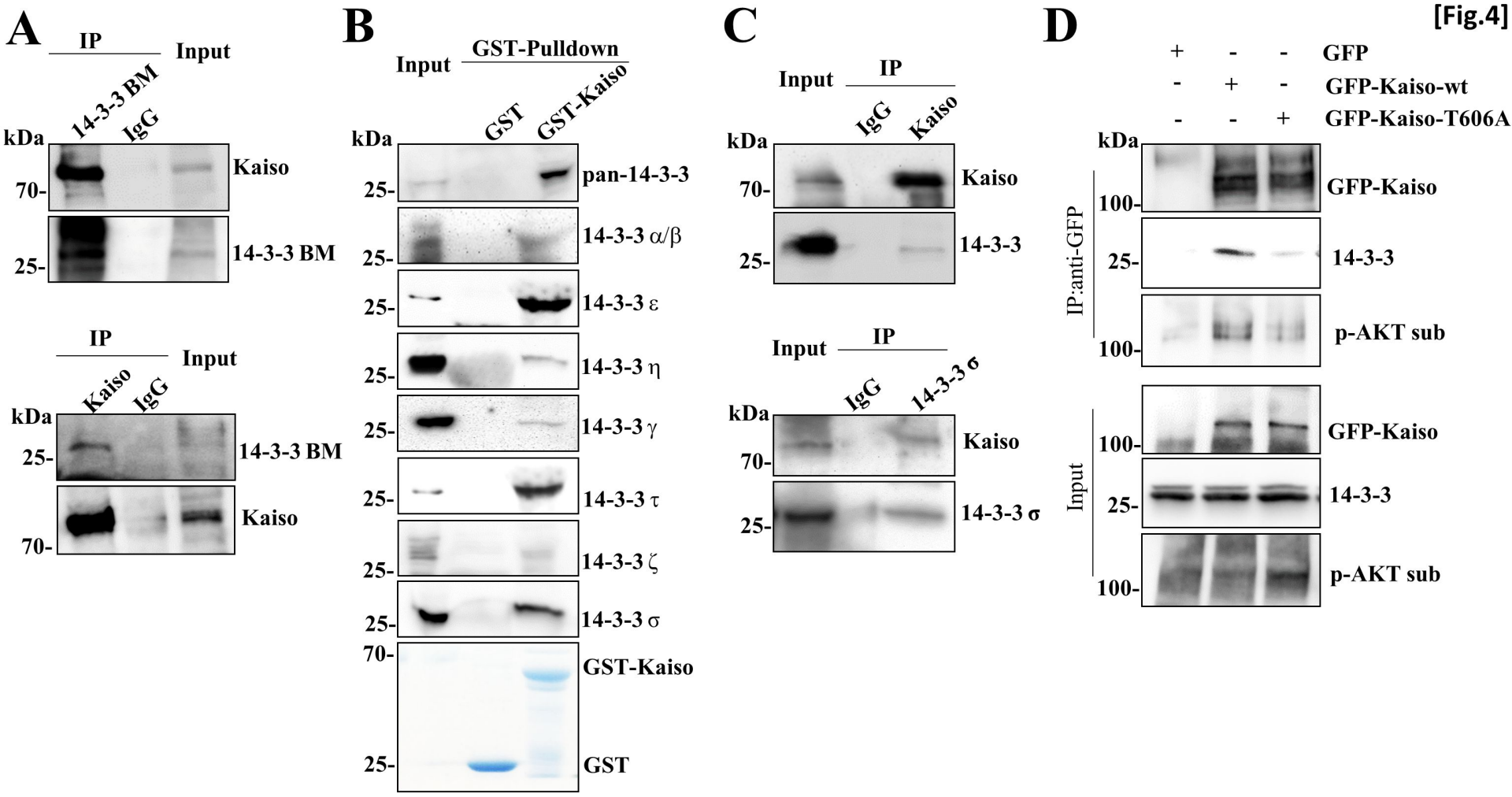
[Fig.3]

[Fig.4]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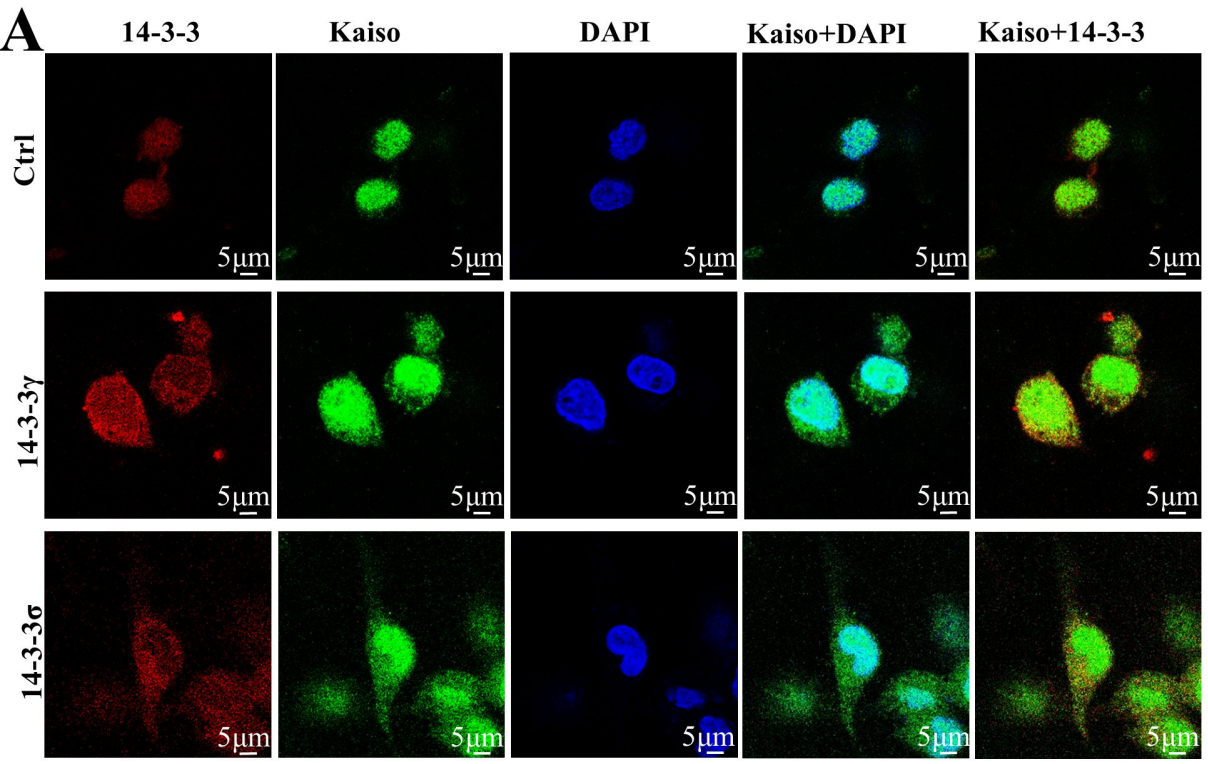
[Fig.5]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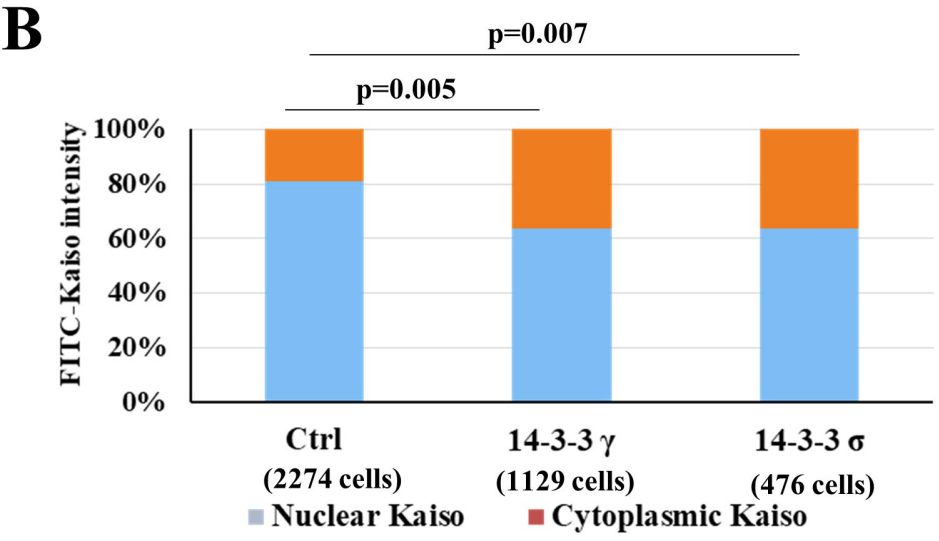
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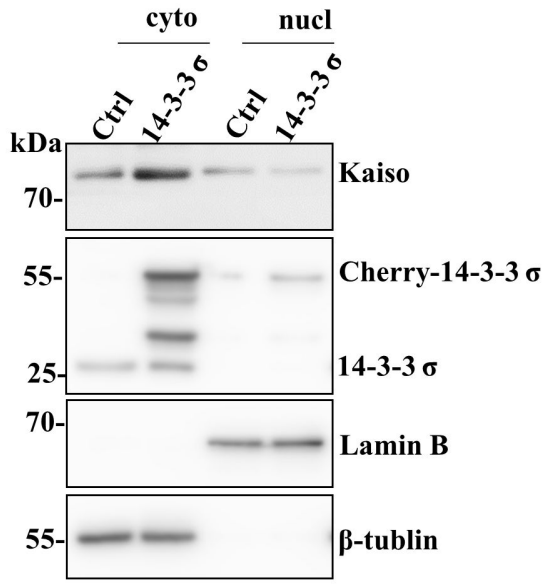
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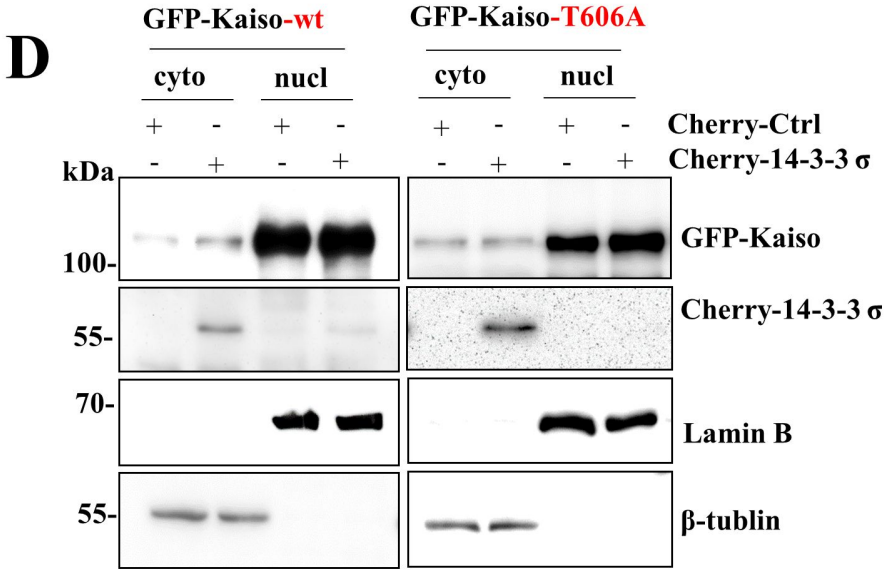
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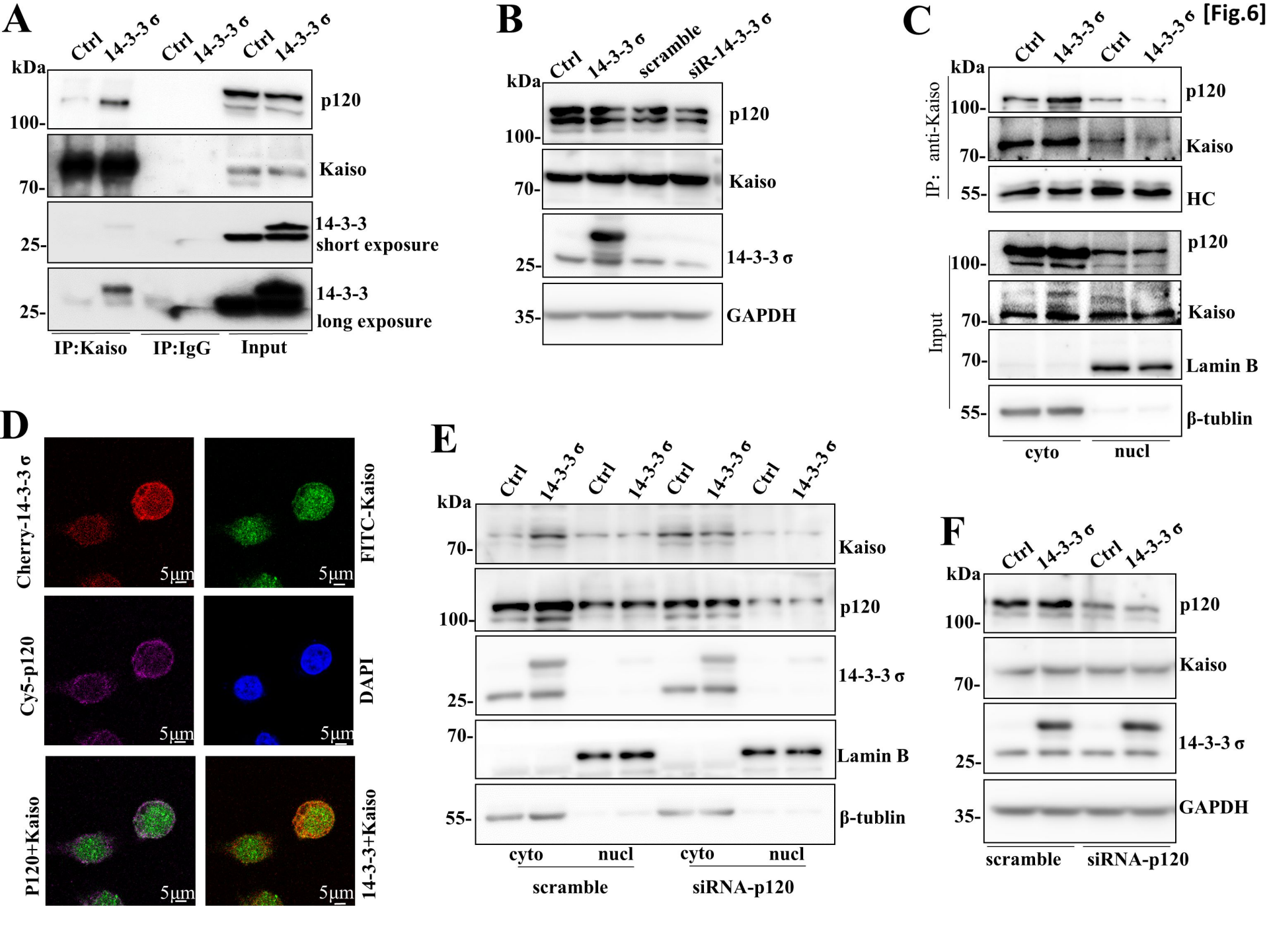
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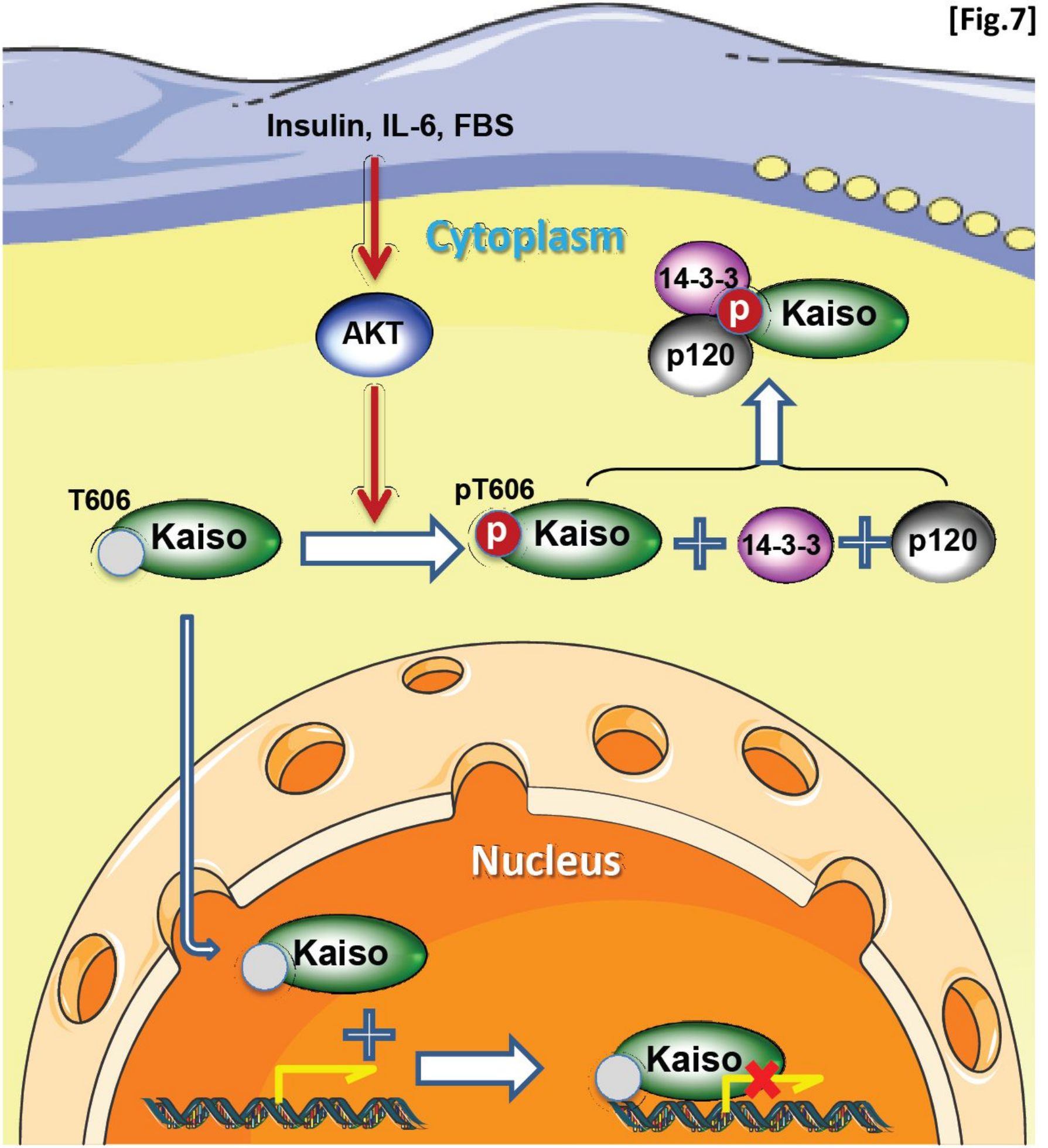
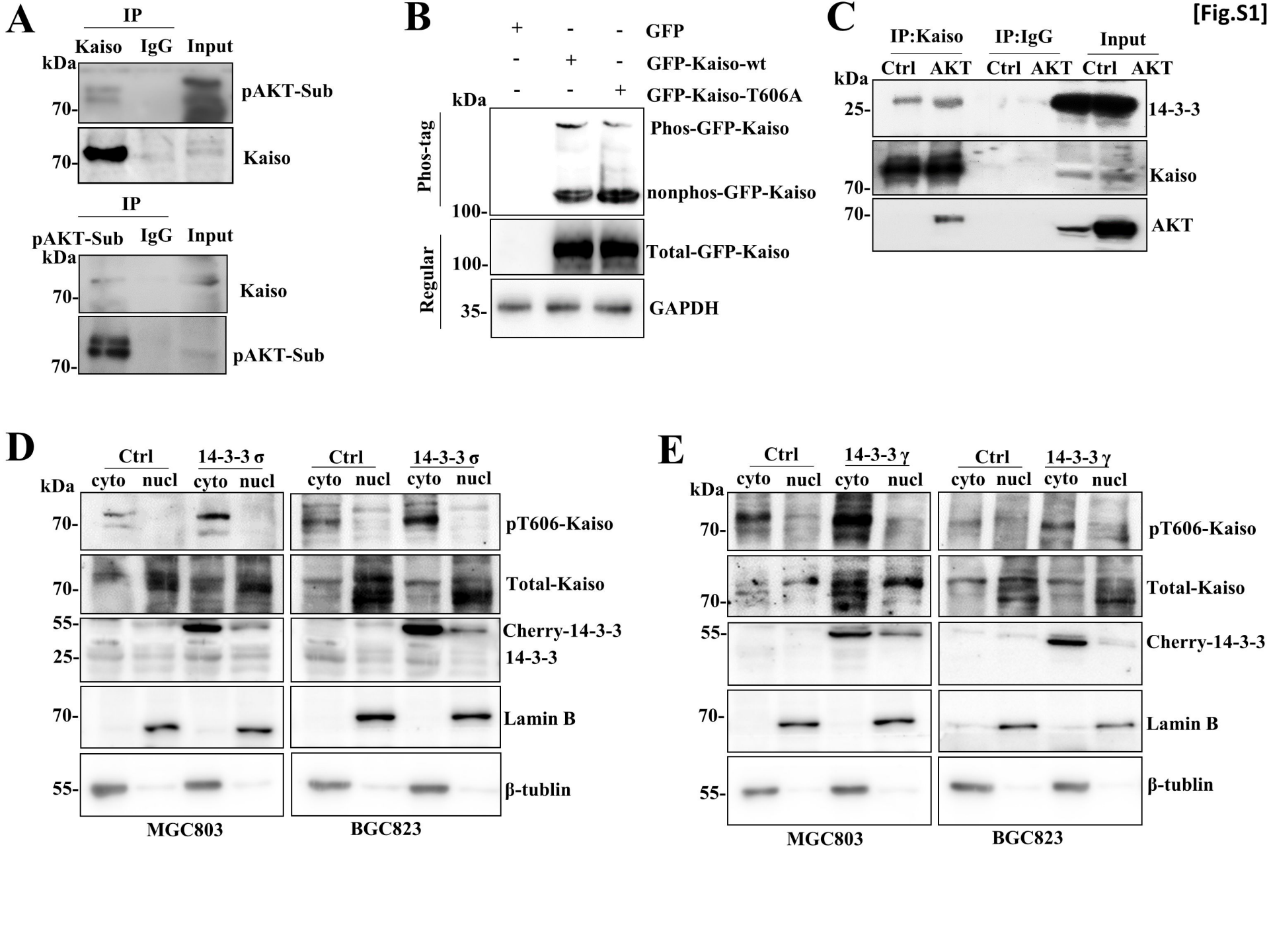
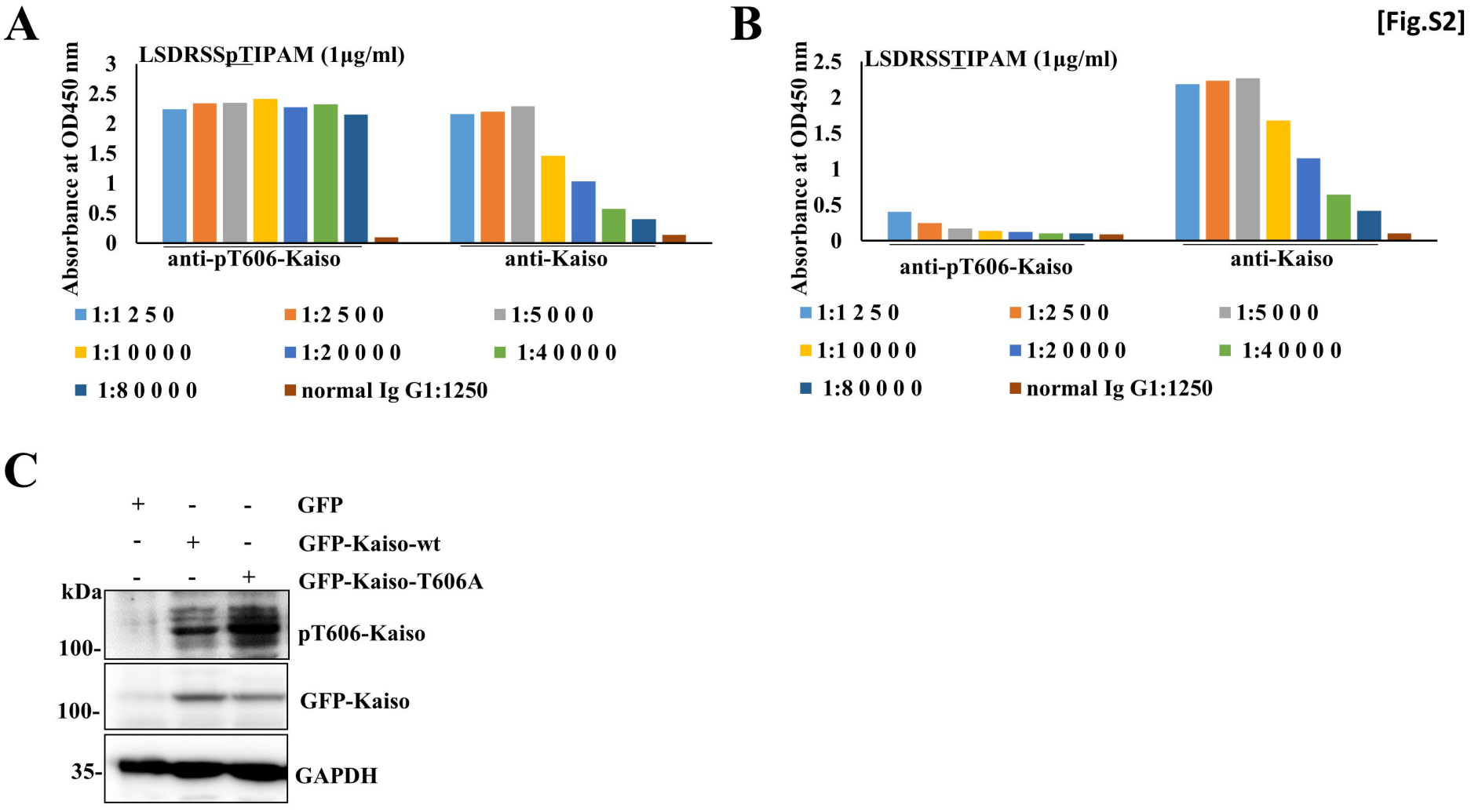
14-3-3 σ
GAPDH
C
[Fig.6]
Ctrl
14-3-3σ
Ctrl
14-3-3σ
kDa
IP: anti-Kaiso
100-
70-
55-
p120
Kaiso
HC
Input
100-
70-
70-
55-
p120
Kaiso
Lamin B
β-tublin
cyto
nucl
D
Cherry-14-3-3 σ
FITC-Kaiso
Cy5-p120
DAPI
P120+Kaiso
14-3-3+Kaiso
5μm
E
Ctrl
14-3-3σ
Ctrl
14-3-3σ
Ctrl
14-3-3σ
Ctrl
14-3-3σ
kDa
70-
100-
25-
70-
55-
Kaiso
p120
14-3-3 σ
Lamin B
β-tublin
cyto
nucl
cyto
nucl
scramble
siRNA-p120
F
Ctrl
14-3-3σ
Ctrl
14-3-3σ
kDa
100-
70-
25-
35-
p120
Kaiso
14-3-3 σ
GAPDH
scramble
siRNA-p120

[Fig.7]


Insulin, IL-6, FBS
Cytoplasm
AKT
14-3-3
p
Kaiso
p120
T606
Kaiso
pT606
p
Kaiso
14-3-3
p120
Nucleus
Kaiso
Kaiso


[Fig.S1]
A
IP
Kaiso IgG Input
kDa
70-
pAKT-Sub
70-
Kaiso
IP
pAKT-Sub IgG Input
kDa
70-
Kaiso
70-
pAKT-Sub
B
+ - - GFP
- + - GFP-Kaiso-wt
- - + GFP-Kaiso-T606A
kDa
Phos-tag
Phos-GFP-Kaiso
100-
nonphos-GFP-Kaiso
Regular
100-
Total-GFP-Kaiso
35-
GAPDH
C
IP:Kaiso IP:IgG Input
Ctrl AKT Ctrl AKT Ctrl AKT
kDa
25-
14-3-3
70-
Kaiso
70-
AKT
D
Ctrl 14-3-3 σ Ctrl 14-3-3 σ
cyto nucl cyto nucl cyto nucl cyto nucl
kDa
70-
pT606-Kaiso
70-
Total-Kaiso
55-
Cherry-14-3-3
25-
14-3-3
70-
Lamin B
55-
β-tublin
MGC803
BGC823
E
Ctrl 14-3-3 γ Ctrl 14-3-3 γ
cyto nucl cyto nucl cyto nucl cyto nucl
kDa
70-
pT606-Kaiso
70-
Total-Kaiso
55-
Cherry-14-3-3
70-
Lamin B
55-
β-tublin
MGC803
BGC823

[Fig.S2]


A
LSDRSSpTIPAM (1μg/ml)
Absorbance at OD450 nm
3
2.5
2
1.5
1
0.5
0
anti-pT606-Kaiso
anti-Kaiso
1:1 2 5 0
1:2 5 0 0
1:5 0 0 0
1:1 0 0 0 0
1:2 0 0 0 0
1:4 0 0 0 0
1:8 0 0 0 0
normal Ig G1:1250
B
LSDRSSTIPAM (1μg/ml)
Absorbance at OD450 nm
2.5
2
1.5
1
0.5
0
anti-pT606-Kaiso
anti-Kaiso
1:1 2 5 0
1:2 5 0 0
1:5 0 0 0
1:1 0 0 0 0
1:2 0 0 0 0
1:4 0 0 0 0
1:8 0 0 0 0
normal Ig G1:1250
C
+ - - GFP
- + - GFP-Kaiso-wt
- - + GFP-Kaiso-T606A
kDa
100-
pT606-Kaiso
100-
GFP-Kaiso
35-
GAPDH