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The CATS (FAM64A) protein is a substrate of the Kinase Interacting Stathmin (KIS)

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ABSTRACT

The CATS protein (also known as FAM64A and RCS1) was first identified as a novel CALM (PICALM) interactor that influences the subcellular localization of the leukemogenic fusion protein CALM/AF10. CATS is highly expressed in cancer cell lines in a cell cycle dependent manner and is induced by mitogens. CATS is considered a marker for proliferation, known to control the metaphase-to-anaphase transition during the cell division. Using CATS as a bait in a yeast two-hybrid screen we identified the Kinase Interacting Stathmin (KIS or UHMK1) protein as a CATS interacting partner. The interaction between CATS and KIS was confirmed by GST pull-down, co-immunopreciptation and co-localization experiments. Using kinase assay we showed that CATS is a substrate of KIS and mapped the phosphorylation site to CATS serine 131 (S131). Protein expression analysis revealed that KIS levels changed in a cell cycle-dependent manner and in the opposite direction to CATS levels. In a reporter gene assay KIS was able to enhance the transcriptional repressor activity of CATS, independent of CATS phophorylation at S131. Moreover, we showed that CATS and KIS antagonize the transactivation capacity of CALM/AF10.In summary, our results show that CATS interacts with and is a substrate for KIS, suggesting that KIS regulates CATS function.

1. Introduction

The rare but recurring t(10;11)(p13;q14) translocation leads to the fusion of *CALM* (*PICALM*) to *AF10* [1]. The *CALM/AF10* fusion is observed in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and in lymphoma [2–4]. *CALM/AF10* is the most frequent fusion found in T-ALL with T-cell receptor (TCR) gamma/delta rearrangement [5–7].

The *CALM/AF10* fusion gene leads to the development of leukemia in a murine bone marrow transplantation model [8,9], and in transgenic mice [10]. There is increasing evidence that CALM/AF10 exerts its leukemogenic potential mainly through two pathways: (i) transcriptional deregulation of target genes, including the HOXA gene cluster, therefore interfering with normal hematopoietic differentiation [8,10–13] and (ii) through the recently described novel pathway in which CALM/AF10 increases genomic instability through the global reduction of histone H3K79 methylation [14,15]. To date, several CALM/AF10 interacting proteins have been identified (e.g. CATS, DOTL1, IKAROS, FHL2), shedding light on the molecular mechanism relevant for CALM/AF10-mediated leukemogenesis and the possible involvement of these proteins in the transformation process [13,16–19].

We initially identified CATS (FAM64A) in a yeast-two-hybrid (Y2H) screen as a CALM interacting protein [16]. The CATS interaction region of CALM was mapped to amino acids 221 to 294 of CALM which is contained in the CALM moiety of both CALM/AF10 and the MLL/CALM fusion proteins. The findings that expression of CATS markedly increased the nuclear localization of CALM/AF10 [16] and that the murine *Cats* transcripts were up-regulated in hematopoietic cells ($B220^+$ lymphoid cells) transformed by CALM/AF10 in comparison to the same subpopulation from non-leukemic mice [8,20], suggested that CATS may play a role in CALM/AF10-mediated transformation.

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CATS codes for a nuclear protein enriched at the nucleoli. In a GAL4-based transactivation assay, CATS functions as a transcriptional repressor when fused to a heterologous DNA-binding domain (GAL4-DBD) [16]. In normal adult tissue, *CATS* expression is restricted to the lymphoid compartment; nevertheless detailed analysis of protein expression showed high expression of CATS in leukemia, lymphoma and tumor cell lines. The protein levels of CATS are cell cycle-dependent, induced by mitogens and correlate with the proliferative state of the cell. Thus CATS can be viewed as a marker for proliferation [20].

Satoh and colleagues identified CATS (referred as FAM64A) as one of the 47 prion protein (PrPC) interactors [21].

Moreover, CATS has been described as the Regulator of Chromosome Segregation, RCS1, a substrate of the anaphase-promoting complex/cyclosome (APC/C), whose loss results in faster progression from metaphase to anaphase and accelerated degradation of securin and cyclin B [22].

To further elucidate CATS function we searched for CATS interacting proteins using a Y2H approach. The Kinase Interacting Stathmin (KIS or UHMK1) was identified as one of the several CATS interacting partners. KIS is a nuclear serine/threonine kinase that possesses a U2AF homology motif (UHM) and phosphorylates and regulates the activity of RNA associated factors [23–26]. KIS positively regulates cell cycle progression through phosphorylation and inhibition of $p27^{KIP1}$ [27]. Here we show that CATS and KIS interact in *in vivo* and that CATS is a phospho-protein, which is phosphorylated at serine 131 (S131) by KIS *in vitro*. Our data suggest that KIS controls CATS function. The implications of these findings for our understanding of CALM-AF10-mediated leukemogenesis are discussed.

2. Material and methods

2.1. Plasmid construction

The bait plasmid for Y2H screen was constructed by inserting *CATS* cDNA into the pGBKT7 vector (Clontech, Heidelberg, Germany).

CATS expressing plasmids (pSfiExp-CATS-HA) were constructed by cloning the PCR-amplified *CATS* (both isoforms 1 and 2) in-frame with the C-terminal HA tag of the pSfiExpress expression vector [28]. The *CATS* ORF, and deletion mutants (N-termini and central domain of CATS, amino acids 1–143 and 136–187; respectively) were cloned into the modified bacterial expression vector pET28a-GST-Tev [29] to be expressed as the GST-CATS FL(1–238), GST-CATS NT(1–143) and GST-CATS (136–187) fusion proteins.

The phospho-defective mutation S131A was introduced into the constructs pET28a-GST-Tev-CATS NT(1-143) and pM1-CATS (the GAL4-DBD-CATS expressing construct) [16], and phospho-mimic mutation S131D was introduced into pM1-CATS (GAL4-DBD-CATSD131) using the QuickChange™ Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

The pEYFP-C1-CATS and -KIS expressing plasmids were constructed by cloning the respective PCR amplified human ORF into the pEYFP-C1 vector (Clontech). For a detailed description of the constructs contact the authors.

2.2. Yeast two hybrid screening

The AH109 yeast strain (Clontech) was serially transformed with the bait plasmid pGBKT7-CATS and with an HeLa S3 cDNA library cloned in the pGADGH vector (Clontech). The resulting colonies were plated on selective plates lacking histidine, adenine, leucine and tryptophan and colonies exhibiting a positive beta-galactosidase reaction were selected for further analysis.

2.3. Transfection of mammalian cells and protein extraction

1×10^6 HEK293 cells were transiently transfected with 8 μg of plasmid DNA and 12 μg polyethyleneimine (PEI) (Sigma-Aldrich, St Louis, MO, USA). After 24 h the cells were harvested, washed and lysed with 1 ml lysis buffer [50 mM Tris–HCl pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate and protease inhibitor cocktail (Roche, Basel, Switzerland)] for 30 min on ice. Whole-cell extracts were cleared by centrifugation.

2.4. Immunobloting and immunofluorescence

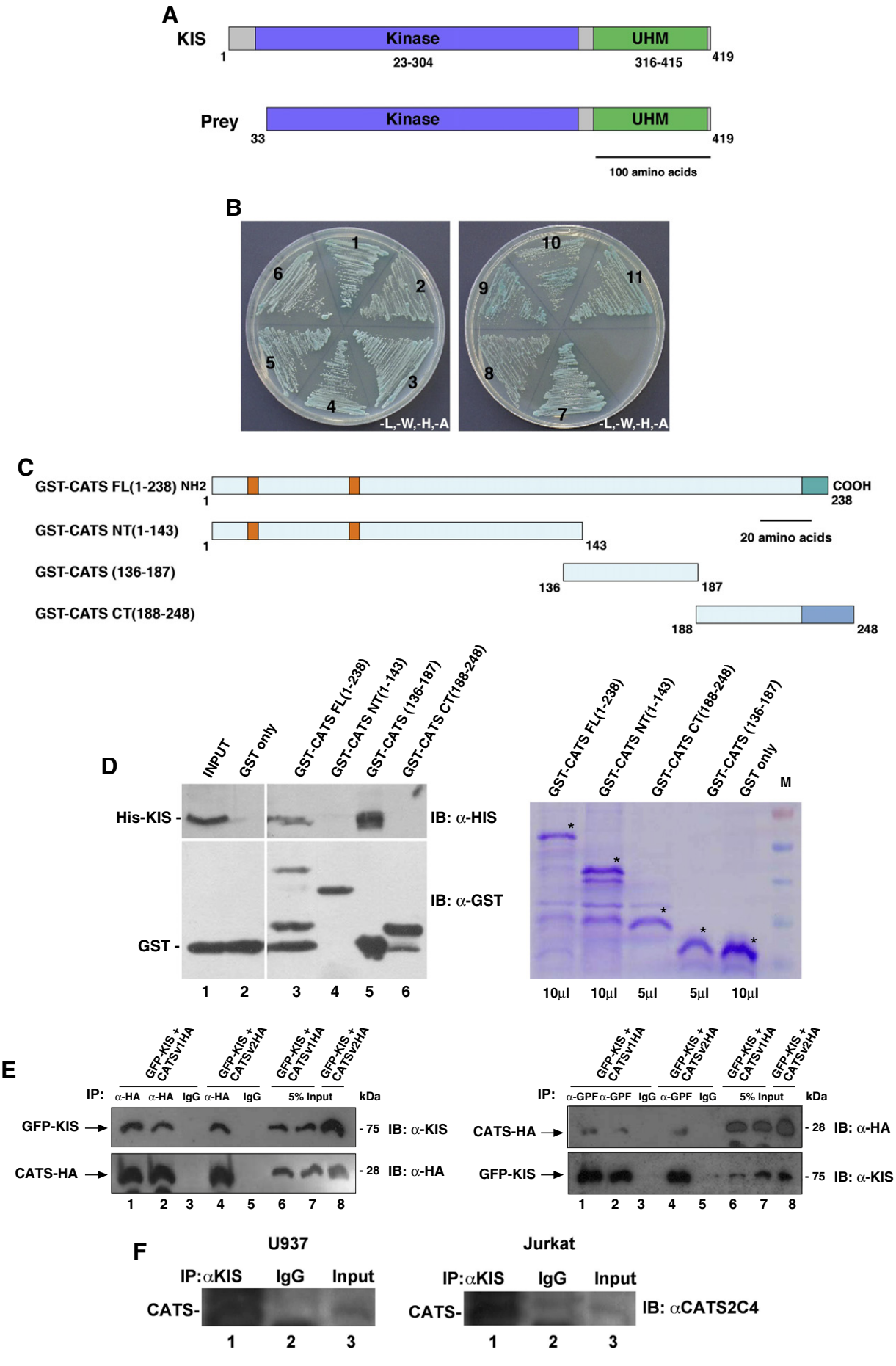
Immunocomplexes and cellular lysates were electrophoresed on 10–12% SDS-PAGE and transferred to nitrocellulose membrane (Hybond™ ECL™, GE Healthcare, Buckinghamshire, UK). The membranes were blocked and probed with specific antibody, followed by secondary antibodies conjugated to horseradish peroxidase. Proteins were detected with ECL (Pierce, Rockford, IL, USA).

For immunofluorescence, U2OS cells co-transfected with pECE-HA-KIS [23] and pEYFP-C1-CATS were grown on coverslips, fixed in 2% paraformaldehyde for 10 min, permeabilized with PBS/0.1% Triton X for 10 min at RT and blocked with PBS/10% FCS for 1 h. Coverslips were incubated with rabbit polyclonal anti-HA (diluted 1:200) (ab 9110; Abcam, Cambridge, MA, USA). Secondary Cy™3-conjugated antibodies diluted 1:500 were used for detection of the primary antibody. Nuclei were counterstained with DAPI (Sigma-Aldrich), cells were mounted on slides using Cytomat medium (DAKO, Glostrup, Denmark) and analyzed in a confocal fluorescence laser scanning system (TCS-SP2 and DM IRB inverted microscope, Leica, Germany).

2.5. Immunoprecipitation

Cellular extracts of HEK293 cells, co-transfected with GFP-KIS and pSfiExp-CATS-HA (500 μg) were incubated for 5 h with 5 μg rabbit anti-HA (sc-805, Santa Cruz Biotechnology) and mouse anti-GFP (sc-9996) (or respective isotype controls), which had been previously incubated with 30 μl Protein A-Sepharose (GE Healthcare) for 1 h. Beads were extensively washed with the lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40 and 0.5% sodium deoxycholate), resuspended in SDS sample buffer, boiled and separated on a 10% SDS-PAGE gel. Subsequent Western blot analysis was carried out with anti-KIS 3B12 [24] and anti-HA antibodies. For immunoprecipitation of the endogenous protein, 900 μg of U937 and Jurkat cellular extracts were incubated with 100 μl monoclonal rat anti-KIS 3B12 or normal rat IgG overnight. Protein G-Sepharose

Fig. 1. CATS interacts with the Kinase Interacting Stathmin, KIS. (A) Diagram representing the protein structure of KIS and the prey clone protein discovered in our Y2H screen. KIS contains an N-terminal serine/threonine kinase domain and a C-terminal noncanonical RNA recognition motif of the UHM (U2AF homology motif) type. The prey clone codes for residues 33–419 of KIS. (B) CATS and KIS interaction was tested in the Y2H system. The yeast strain AH109 was cotransformed with the CATS bait (GAL4-DBD-CATS) and prey plasmids (1–11) isolated in the screen. Co-transfection of GAL4-DBD-CATS and (1) GAL4-AD-KIS (aa 33–419); (2–11) GAL4-AD-clones containing other preys. Interaction was accessed for growth on an synthetic drop-out plate lacking leucine, tryptophan, histidine and adenine (-L, -W, -H, -A), and supplemented with X-α-GAL. (C) Diagram representing the CATS protein and deletion mutants expressed as glutathione S-transferase (GST)-fusions. (D) Confirmation of CATS-KIS interaction *in vitro*. Bacterially expressed (GST)-fusions of CATS (lower panel) incubated with His-KIS cellular extracts, washed and analyzed by SDS-PAGE (left panel). The right panel shows the Comassie blue staining of the input. Recombinant CATS, deletion mutants and GST-only control are indicated with an asterisk. (E) Co-immunoprecipitation of CATS and KIS. Lysates of HEK293 overexpressing HA-tagged CATS isoforms and GFP-KIS were precipitated with anti-HA and anti-GFP antibodies and respective isotype controls. WB analysis of the precipitation reactions with anti-HA and anti-KIS 3B12 antibodies is shown in the lower panels (left and right panels, respectively). WB analysis of the co-precipitated proteins with anti-KIS 3B12 and anti-HA is shown in the upper left and right panels, respectively. As input control, lanes 6, 7 and 8 were loaded with 5% of the protein extract used for each precipitation reaction. (F) Co-immunoprecipitation of the endogenous proteins. Lysates of U937 and Jurkat cells were precipitated with anti-KIS 3B12 (lane 1) or isotype control antibody (lanes 2) and analyzed by Western blot with anti-CATS 2C4. Lanes 3 were loaded with 10% input of the reaction.


A
KIS
Kinase
UHM
1
23-304
316-415
419
Prey
Kinase
UHM
33
419
100 amino acids
B
1
2
3
4
5
6
-L,-W,-H,-A
7
8
9
10
11
-L,-W,-H,-A
C
GST-CATS FL(1-238) NH2
1
COOH
238
GST-CATS NT(1-143)
1
143
20 amino acids
GST-CATS (136-187)
136
187
GST-CATS CT(188-248)
188
248
D
INPUT
GST only
GST-CATS FL(1-238)
GST-CATS NT(1-143)
GST-CATS (136-187)
GST-CATS CT(188-248)
His-KIS -
GST -
IB: α-HIS
IB: α-GST
1 2 3 4 5 6
GST-CATS FL(1-238)
GST-CATS NT(1-143)
GST-CATS CT(188-248)
GST-CATS (136-187)
GST only
M
10µl 10µl 5µl 5µl 10µl
E
GFP-KIS + CATSv1HA
GFP-KIS + CATSv2HA
GFP-KIS + CATSv1HA
GFP-KIS + CATSv2HA
IP: α-HA α-HA IgG α-HA IgG 5% Input kDa
GFP-KIS
CATS-HA
- 75 IB: α-KIS
- 28 IB: α-HA
1 2 3 4 5 6 7 8
GFP-KIS + CATSv1HA
GFP-KIS + CATSv2HA
GFP-KIS + CATSv1HA
GFP-KIS + CATSv2HA
IP: α-GPF α-GPF IgG α-GPF IgG 5% Input kDa
CATS-HA
GFP-KIS
- 28 IB: α-HA
- 75 IB: α-KIS
1 2 3 4 5 6 7 8
F
U937
IP:αKIS IgG Input
CATS-
1 2 3
Jurkat
IP:αKIS IgG Input
CATS-
IB: αCATS2C4
1 2 3

was added and incubated further for 90 min. Beads were washed and preceded as described above. Western blot analysis was carried out with anti-CATS 2C4 [16].

2.6. Expression of recombinant proteins in bacterial cells

Induction of GST-CATS FL(1–238), GST-CATS NT(1–143), GST-CATS NT(1–143)S131A, GST-CATS(136–187) and GST-CATS CT(188–248) fusion protein expression in *Escherichia coli* strain Rosetta™(DE3)pLysS (GST-CATS FL(1–238)) (Novagen, Merck, Darmstadt, Germany) or BL21 (DE3) pRARE (Novagen) were achieved with 1 mM IPTG for 2 h at 37 °C. Cells were lysed by sonicating for 30 s in GSB buffer (25 mM potassium Hepes pH 7.5, 0.3 m KCl, 1 mm EDTA, 0.1% NP-40, 10% glycerol) and 1× protease inhibitor cocktail (Roche). Recombinant proteins were purified with glutathione Sepharose 4B beads (GE Healthcare).

2.7. GST pull-down

About 200 μl of cellular extract of HEK293 cells, transfected with pcDNAHis-KIS, [27] was incubated with 25 μl of immobilized GST-CATS fusion proteins or GST for 4 h at 4 °C. After extensive washes with binding buffer, beads were resuspended in SDS sample buffer, boiled and separated on a 10% SDS-PAGE gel. Subsequent Western blot analysis was carried out with Penta-His (Qiagen, Hilden, Germany) and anti-GST (sc-459, Santa Cruz Biotechnology) antibodies.

2.8. Phosphorylation reactions

GST-KIS kinase or GST-KISK54R dead kinase control was used in phosphorylation reactions as previously described [23] with the indicated purified CATS substrates. Phosphopeptide mapping reactions were carried out with 500 μM of the indicated synthetic peptides (PSL GmbH, Heidelberg, Germany), and 0.1 mM [γ-^{32}P]ATP for 150 min at 30 °C. Phosphate incorporation was measured by spotting the reaction onto 2-cm^2 pieces of P81 phosphocellulose paper (Whatman®), which were washed with 75 nM phosphoric acid, then with water, dried and exposed to phosphorimaging.

2.9. Reporter gene assay

HEK293 cells were seeded in 24 well plates and co-transfected with 100 ng of the pGAL4$_5$tk-LUC reporter plasmid [30], 20 ng of pRL-null co-reporter plasmid (Promega, Madison, USA), 100 ng of the indicated pM1-CATS plasmid variants (expressing the wild type, phospho-defective and phospho-mimic mutant proteins, respectively), together with variable amounts of pcDNA-KIS (200–400 ng) or pcDNA-KISK54R [27].

For the CATS-CALM/AF10 transactivation experiments, the cells were co-transfected with 100 ng of the pGAL4$_5$tk-LUC reporter plasmid, 50 ng of pRL-null co-reporter plasmid (Promega), 100 ng of GAL4-DBD-CALM/AF10 [19] or GAL4-DBD expressing plasmid and variable amounts of pSfiExp-CATS-HA (100 ng, 200 ng, 400 ng) or constant amounts of pSfiExp-CATS-HA (200 ng) and pcDNA-KIS (400 ng) in the case of KIS–CATS-CALM/AF10 experiments. DNA amount was kept constant in each well by adding pcDNA empty vector (Invitrogen, Carlsbad, CA, USA). Cells were harvested and assayed for firefly and *Renilla* luciferase activities using a Dual-Luciferase Reporter Assay System (Promega).

2.10. Cell cycle synchronization

U2OS cells were synchronized by a double thymidine block as previously described [20]. After being released from cell cycle block cells were collected at the indicated time points for protein extraction. Synchronization was controlled by flow cytometry analysis of the DNA content and confirmed by Western blot analysis using the indicated cyclin antibodies (Santa Cruz Biotechnology).

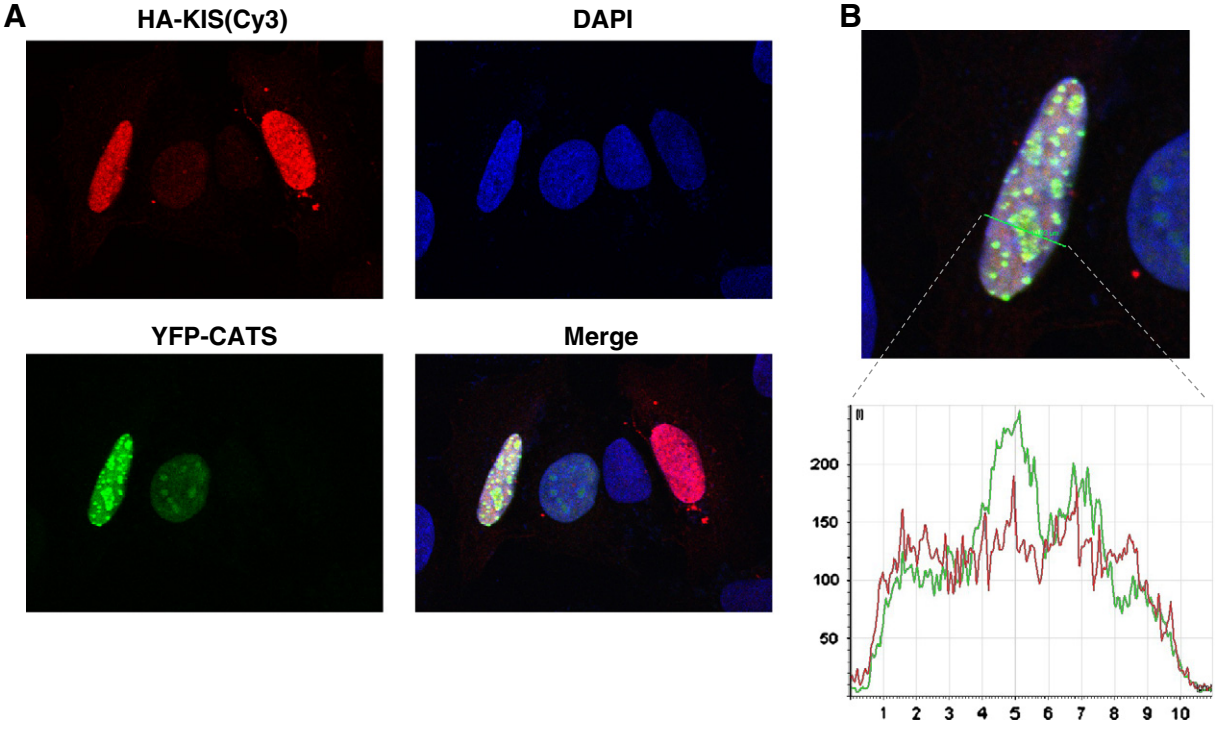


Fig. 2. Subcellular localization of CATS and KIS. Confocal images of U2OS cells transiently transfected with plasmids expressing YFP-CATS and HA-KIS. (A) CATS colocalize with KIS in the nucleus: Channels are indicated above the images (DAPI, YFP, Cy3 and merged). (B) Confocal line scan through the nucleus of the left cell from panel A. The image shows a cell with all three YFP (CATS), Cy3 (KIS) and DAPI channels merged. The graph shows YFP (CATS), Cy3 (KIS) signal intensities. Note that peak intensity of the CATS signal correlates with KIS staining.

3. Results

3.1. Identification of the kinase KIS as a CATS interacting protein

In order to identify interaction partners of CATS protein, we performed a Y2H screen. The bait encoded the coding region of CATS isoform 1 (residues 1–238) fused to the GAL4-DBD. Among different prey clones isolated we identified one coding for residues 33–419 of the Kinase Interacting Stathmin KIS (UHMK1) (Fig. 1A). The interaction between CATS and KIS was confirmed by co-transforming the KIS and additional 10 prey plasmids and the CATS bait plasmid into yeast strain AH109. Interaction was assayed by cell growth on selective plates (Fig. 1B).

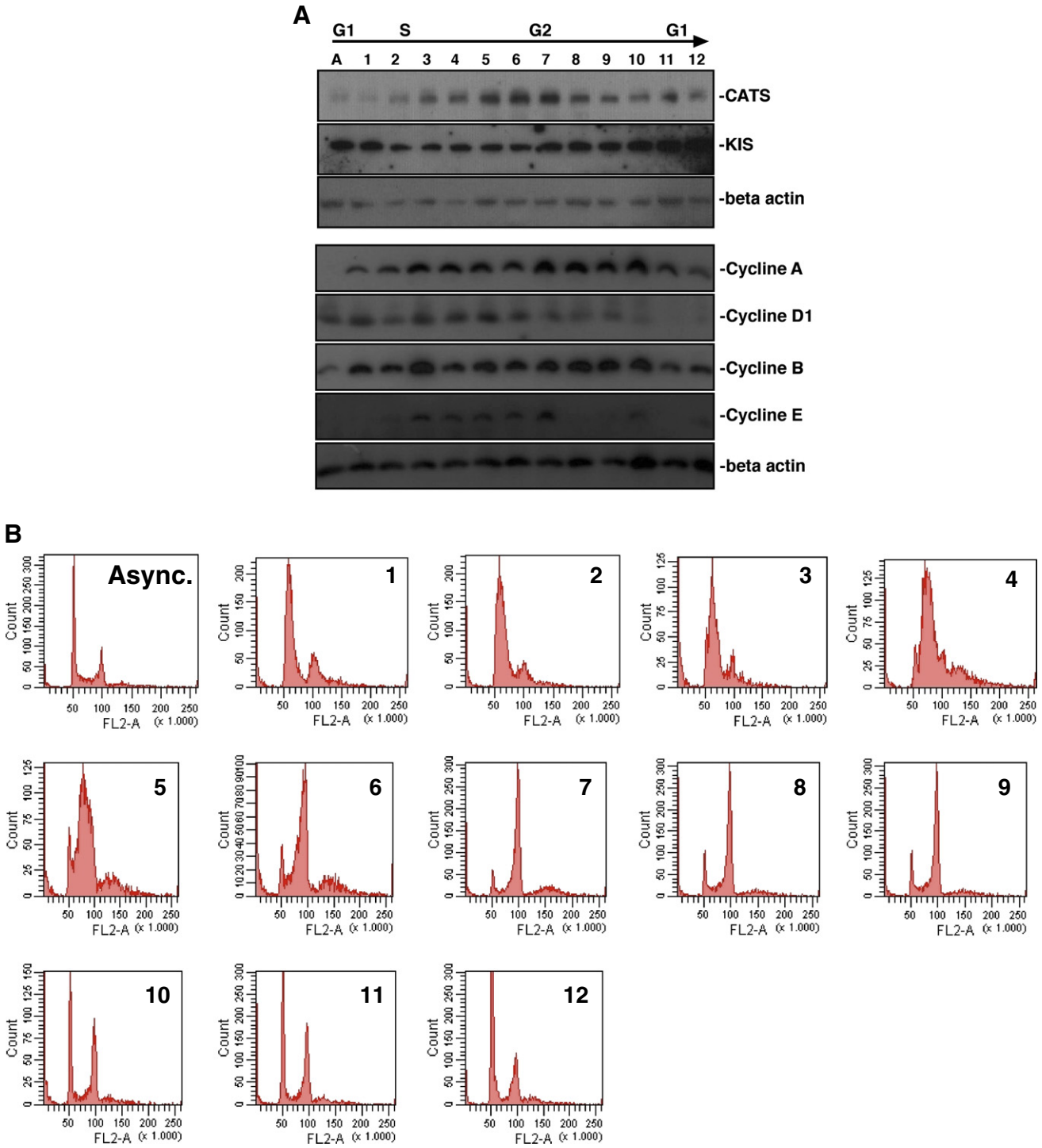


Fig. 3. Expression of KIS and CATS during the cell cycle. U2OS cells were synchronized by a double thymidine block. After thymidine release, cells were collected in 12 timepoints every 90 min (referred as samples 1–12). Synchronization was confirmed by FACS and Western blot using cycline antibodies. (A) Western blot with 50 μg extracts from the same cell samples (1–12) analyzed by FACS (3B). Sample A is from a non-synchronized cell population. Blots were stripped and reprobed with the indicated antibodies. (B) FACS analysis of the U2OS cells collected at the different timepoints; samples 1–12 (Async: non-synchronized sample).

3.2. CATS interacts with KIS in vitro and in vivo

To confirm that CATS and KIS interact and to determine which region of CATS is responsible for this interaction, a glutathione S-transferase (GST) pull-down assay was performed. To this end, CATS as well as its deletion mutants (amino acids 1–238, 1–143, 136–187 and 188–248; Fig. 1C) were expressed as GST fusion proteins in bacteria and immobilized on glutathione beads. The beads were incubated with

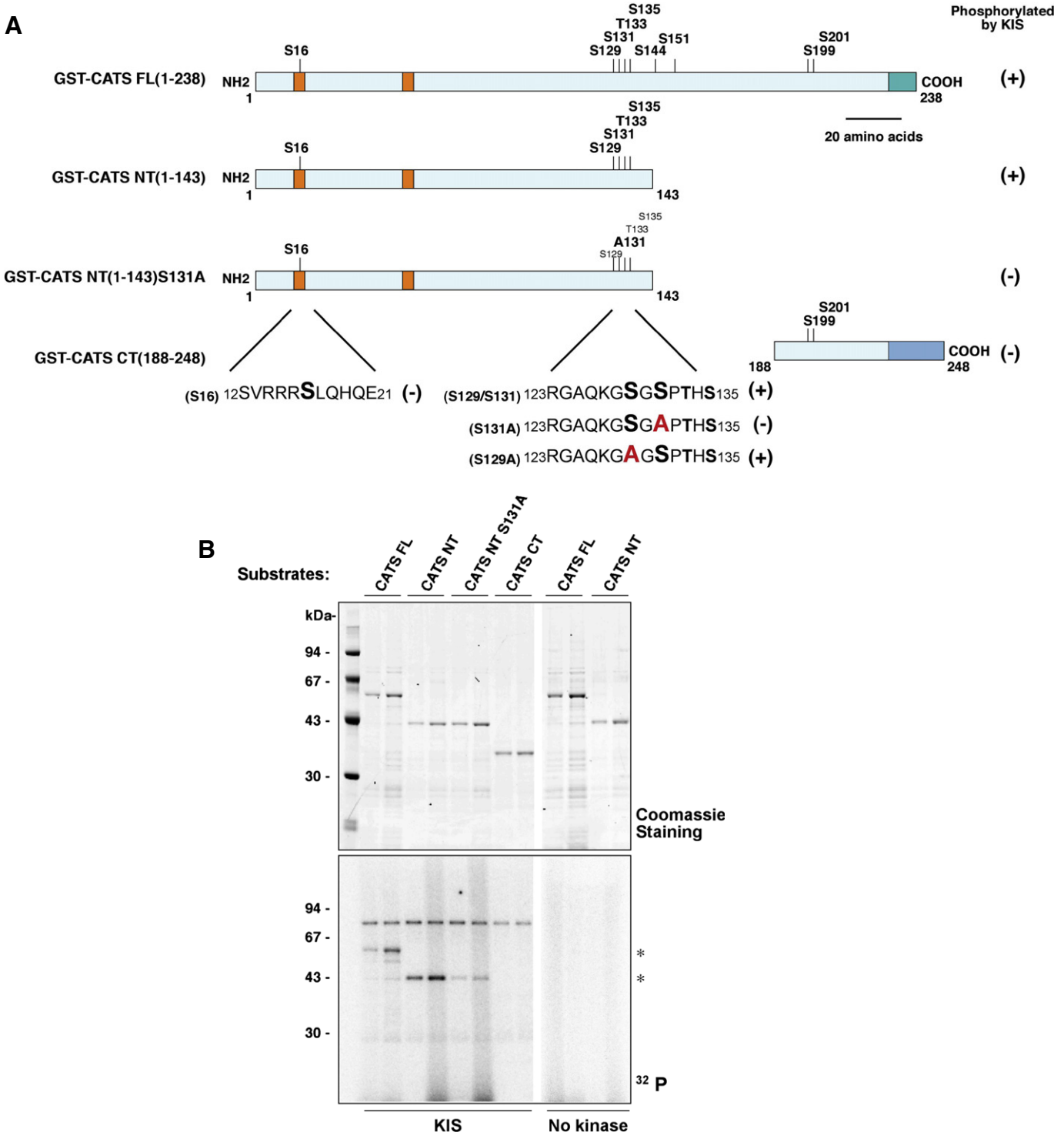


Fig. 4. CATS is a substrate for KIS at serine 131. (A) Diagram representing the protein structure of CATS and the phosphorylation sites within the protein as revealed by phosphoproteomic studies. DB: destruction box motifs are depicted as orange boxes. Phosphorylation sites within the recombinant proteins used as substrates in the phosphorylation assay. Peptide sequences are represented enlarged. Plus (+) and minus (−) signs mean phosphorylated or not phosphorylated by KIS, respectively. (B) Phosphorylation reactions were performed with constant amounts of kinase (recombinant GST-KIS) and two different concentrations of each substrate (20 and 40 pmol). Substrates were GST-fusions of CATS full length (CATS FL), CATS N-terminal 1–143 (CATS NT), CATS N-terminal phosphorylation deficient mutant (CATS NT S131A) and CATS C-terminal (188–248). No kinase: reactions without kinase used as negative control. (*) indicates the phosphorylated substrate. (C) Phosphorylation of synthetic CATS peptides or positive controls (pCP9 and SF1) by KIS. (D) The bars represent the average of normalized phosphate incorporation measurement from the reactions demonstrated in (C), a representative triplicate experiment is shown. Error bars show standard deviation.

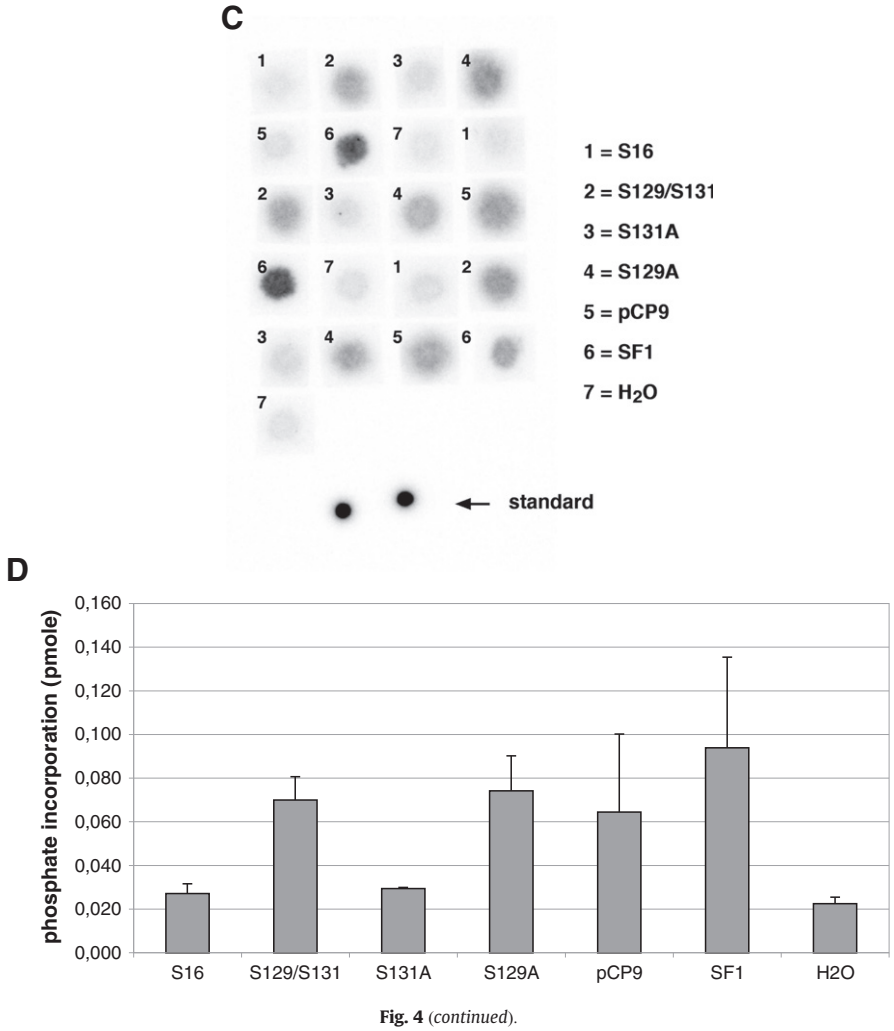


Fig. 4 (*continued*).

cellular extracts expressing His-tagged KIS. CATS full length and its central portion (CATS 136–187) clearly retained the His-KIS protein (Fig. 1D, upper panel lanes 3 and 5), while the N- and C-terminal portions of CATS as well as GST did not (Fig. 1D, upper panel lanes 2, 4 and 6). These results confirm the interaction between CATS and KIS and map the interaction region to CATS amino acids 136–187.

To establish whether CATS interacts with KIS *in vivo*, HA-CATS and GFP-KIS proteins were co-expressed in HEK293 cells and precipitated with anti-HA antibody, which detected the immunoprecipitated CATS-HA proteins as shown in Fig. 1E, left panel, lower lanes 1, 2 and 4 (isoforms 1 and 2, respectively). The anti-KIS 3B12 antibody identified the co-precipitated GFP-KIS in the same lanes with anti-HA immunoprecipitated proteins (Fig. 1E, left panel, upper lanes 1, 2 and 4), but not when isotype control was used (Fig. 1E, left panel, upper lanes 3 and 5). The same was observed in the reciprocal immunoprecipitation experiment, in which anti-GFP antibody efficiently precipitated GFP-KIS (Fig. 1E, right panel, lower lanes 1, 2 and 4) and the co-precipitated CATS-HA was detected with anti-HA (Fig. 1E, right panel, upper lanes 1, 2 and 4) but not with the IgG control (Fig. 1E, right panel, upper lanes 3 and 5).

To investigate whether the CATS–KIS interaction occurs between the endogenous proteins, we examined the CALM/AF10 positive monocytic cell line U937, as well as the lymphoblastic leukemia cell line Jurkat. In both cell lines the CATS 2C4 antibody identified the co-precipitated CATS in the lanes with anti-KIS immunoprecipitated proteins (Fig. 1F, lane 1, left and right panels), but not with isotype IgG immunoprecipitates (Fig. 1F, lane 2, left and right panels) demonstrating that endogenous CATS and KIS form a complex in leukemia cells.

3.3. CATS co-localizes with KIS in the nucleus

In order to learn more about the interaction between CATS and KIS, we performed *in vivo* sub-cellular co-localization experiment using overexpressed YFP-tagged CATS and HA-tagged KIS proteins. Immunofluorescence revealed co-localization of both proteins in the nucleus (Fig. 2A). This was also obvious in a confocal line scan through one of the nuclei where the intensity levels for YFP-CATS (green) and HA-KIS (red) showed a similar distribution, in particular at the nucleolus (Fig. 2B).

3.4. KIS and CATS expression during the cell cycle

We have previously shown that CATS is expressed in a cell cycle-dependent fashion with higher levels present during S-, G_2- and G_2/M-phases and lower levels of CATS in G_1 [20]. Therefore we also analyzed KIS expression during the cell cycle. Western blot analysis of synchronized U2OS cells, revealed here that KIS expression also varies throughout the cell cycle. However, different from CATS, KIS expression peaks in the G_1 phase and is lower during S phase (Fig. 3). Thus KIS and CATS expression levels show opposite expression patterns during cell cycle progression.

3.5. CATS is a phospho-protein

Since we identified the kinase KIS as CATS interacting partner, we searched the phosphoproteomic databases PhosphoSitePlus™ (http://www.phosphosite.org) and Phosida (http://www.phosida.com/) [31] for phosphorylated CATS peptides. Combining the information available in these databases we identified 9 residues within CATS with post-translational modification (Fig. 4A) [31–35]. Most of these residues except for S144 and S151 of CATS were described as phosphorylated in a cell cycle dependent manner [31,33,34]. Only serine 131 of CATS was located within the SGSP consensus sequence for KIS phosphorylation [36].

3.6. CATS serine 131 is the target of KIS phosphorylation

In order to investigate whether CATS is a substrate for KIS phosphorylation we performed an *in vitro* kinase assay. The substrate proteins and peptides used in this assay are shown in Fig. 4A. Recombinant KIS efficiently phosphorylated full length CATS and the N-terminal portion of the CATS protein (aa 1–143) but not the C-terminal quarter of the protein (aa 188–248) (Fig. 4B).

To map the KIS phosphorylation site in CATS, peptides containing all known phospho-sites of the N-terminal portion of CATS as well as two peptides containing mutations of the putative KIS target motif (S129A and S131A) were tested for phosphorylation by KIS (Fig. 4C). Since

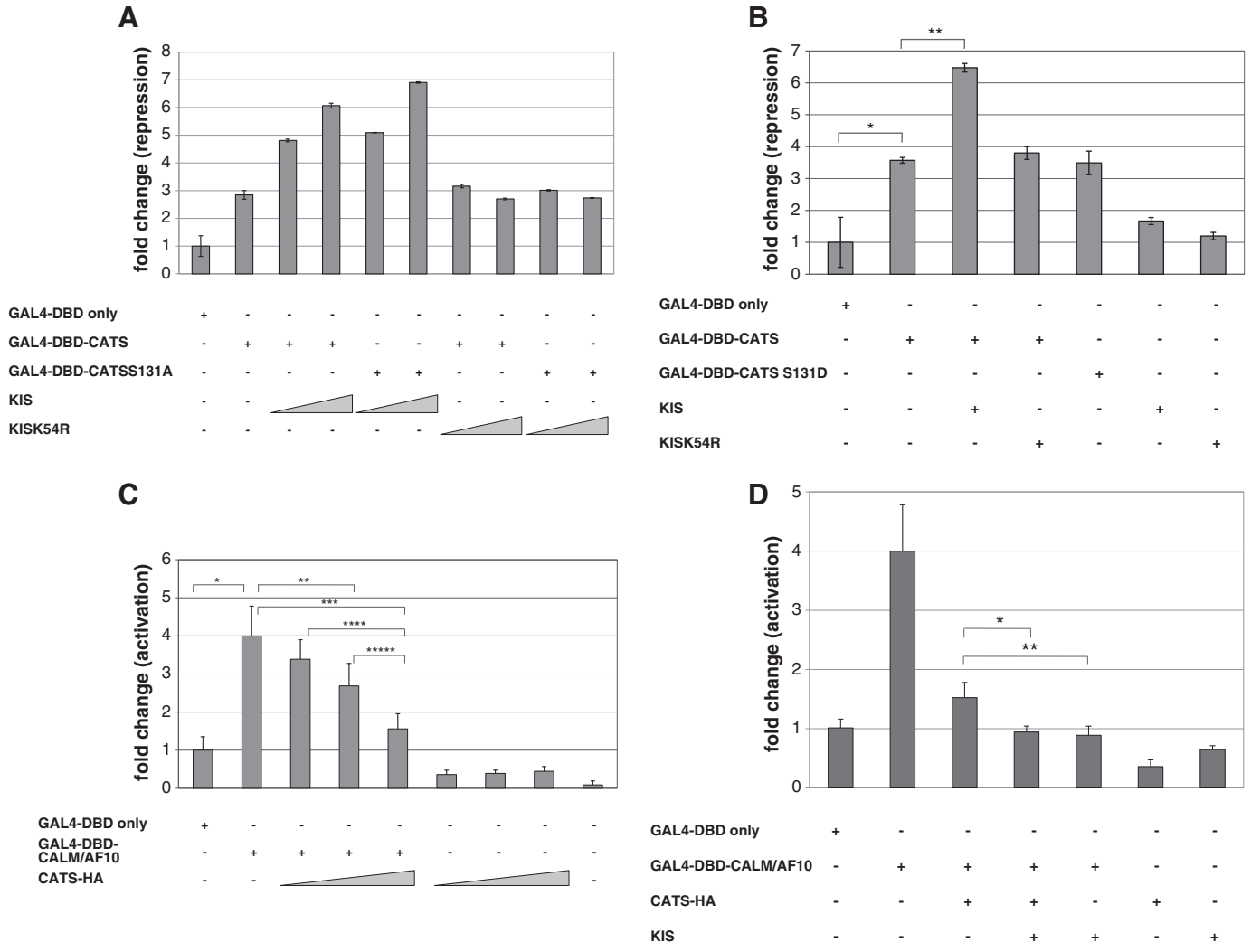


Fig. 5. KIS enhances the transcriptional inhibitory function of CATS. HEK293 cells were co-transfected with GAL4-tk-luciferase reporter plasmid and the constructs indicated. Fold change of the luciferase activity (normalized to the activity of the *Renilla* co-reporter) compared to the background level of GAL4-DBD set as 1 (fold repression: panels A and B; fold activation: panels C and D). (A) Increasing amounts of KIS (200 ng and 400 ng) enhanced CATS-mediated repression of transcription from 3- to 6-fold in a dose-dependent manner (third and fourth bars). The same effect is observed when KIS is co-expressed with the phosphorylation deficient CATS mutant (DBD-CATSS131A) (fifth and sixth bars). The data represent mean values of one representative out of two independent triplicate experiments. Error bars show standard deviation. (B) KIS approximately doubles the CATS-mediated repression of transcription (compare second and third bars). The kinase dead mutant (HKISK54R) has no effect on CATS function (fourth bar). Phosphomimetic CATS (DBD-CATSS131D) exerted the same transcriptional activity as the CATS wild type alone or co-transfected with the kinase dead mutant (compare fifth with fourth and second bars). The data represent mean values of one representative out of four independent triplicate experiments. Error bars show standard deviation. * $p = 0.0348$, ** $p = 0.0356$; Student's *t*-test. (C) CATS antagonize the transactivation capacity of CALM/AF10. GAL4-DBD-CALM/AF10 activates the reporter gene expression under the control of a GAL4-tk responsive promoter about 4.2 fold compared to GAL4-DBD alone (second and first bars). Increasing amounts of CATS (100 ng, 200 ng and 400 ng) reduced CALM/AF10-mediated transactivation from 4.2 to 1.7 fold in a dose-dependent manner. Increasing amounts of CATS in the absence of CALM/AF10 were used as a control for the specificity of CATS repression on CALM/AF10-mediated transactivation. The bars represent the average of normalized firefly luciferase values obtained from two independent triplicate experiments measured in duplicates. Error bars show standard deviation. * $p = 0.0002$, ** $p = 0.0361$, *** $p = 0.0074$, **** $p = 0.0179$ and ***** $p = 0.0018$; Student's *t*-test. (D) KIS enhances CATS effect over CALM/AF10-mediated transactivation (compare third and fourth bars). Individual constructs expressing DBD-CALM/AF10, CATS and KIS alone were used as a control for the specificity of the effect observed upon its combination. The bars represent the average of normalized firefly luciferase values obtained from five independent triplicate experiments. Error bars show standard deviation. * $p = 0.0091$, ** $p = 0.0096$; Student's *t*-test.

KIS did not phosphorylate the recombinant CATS C-terminal, residues S199 and S201 were not included in these assays. Recombinant KIS did not phosphorylate peptide-S16 but peptides S129/S131 and S129A (same amino acid sequence as peptide-S129/S131 but the serine at position 129 replaced by an alanine). The levels of phosphate incorporation were similar to the well-known KIS substrates pCP9 and SF1 [25] (Fig. 4D). Phosphorylation was clearly reduced for peptide S131A and the recombinant protein GST-CATS NT(1–143)S131A, in which the serine 131 was replaced by an alanine (Fig. 4B, C and D). These results demonstrate that KIS is a kinase for CATS and identify CATS S131 as the unique target site for KIS phosphorylation.

3.7. KIS enhances the transcriptional repressor activity of CATS in a GAL4-based transactivation assay

In our previous studies we have shown that CATS acts as a transcriptional inhibitor when fused to a GAL4-DBD [16]. Therefore we used a reporter gene assay in order to determine the role of the KIS kinase on the transcriptional inhibitory properties of CATS and to address whether KIS phosphorylation of CATS on serine 131 might modulate this function of CATS. Co-expression of constant amounts of GAL4-DBD-CATS and increasing amounts of KIS led to an enhanced inhibitory capacity of CATS on the transactivation of the GAL4-tk-luciferase reporter by up to 50%. This effect of KIS was observed for both wild-type CATS and the CATS phospho-defective mutant (CATS S131A) but not when a kinase dead mutant of KIS (KISK54R) was used (Fig. 5A). Moreover, the CATS phospho-mimetic clone (CATSS131D) exerted the same transcriptional activity like the wild type CATS (Fig. 5B). These results demonstrate that KIS enhances the transcriptional repressor activity of CATS, and this effect is independent of CATS phophorylation on S131 but dependent on the kinase activity of KIS.

3.8. CATS and KIS antagonize the transactivation capacity of the leukemic fusion protein CALM/AF10

We previously showed that CALM/AF10 can be retained in the nucleus upon interaction with CATS [16] and that CALM/AF10 strongly activates transcription when fused to a heterologous DNA-binding domain (GAL4-DBD) [19]. Thus, we wanted to investigate whether CATS would affect the transactivation function of CALM/AF10. Coexpression of constant amounts of GAL4-DBD-CALM/AF10 and increasing amounts of CATS led to a decrease of the transactivation capacity of the CALM/AF10. Higher amounts of CATS (400 ng) reduced CALM/AF10-mediated transactivation of the GAL4-tk-luciferase reporter from 4.2- to 1.7-fold (Fig. 5C).

We further investigated the effect of KIS on CATS-CALM/AF10 context by introducing KIS in the aforementioned assay. Coexpression of GAL4-DBD-CALM/AF10, CATS and KIS led to a lower CALM/AF10 transactivation, compared with when GAL4-DBD-CALM/AF10 was coexpressed with CATS only. Moreover, KIS was also able to decrease the transactivation capacity of the CALM/AF10 in the absence of CATS coexpression (Fig. 5D).

4. Discussion

Phosphorylation regulates protein function, subcellular localization, complex formation and protein stability. Therefore defining and understanding kinase-substrate pairs is a major step towards understanding cell signaling networks. Here we report the identification of the Kinase Interacting Stathmin KIS as a CATS interacting partner and identified CATS S131 as the unique target site for KIS phosphorylation. Interestingly, Olsen and colleagues have reported full phosphorylation of CATS S131 during mitosis [34]. These findings suggest that KIS might be the kinase phosphorylating CATS S131 during mitosis. Recently, CATS has been reported as a mitotic regulator, that controls the metaphase to anaphase transition. In this context CATS is a substrate of the APC/C complex degraded during mitotic exit [22].

Expression analysis on synchronized cells revealed increased levels of KIS protein during the G_1 and G_2/M phases. The pattern of KIS expression was a mirror image of the CATS levels, as KIS protein increased exactly at the time when CATS levels started to decrease in G_2/M phase cells. Thus, it is tempting to speculate that KIS regulates CATS stability during mitotic exit and consequently its role in metaphase–anaphase transition. Further studies will be necessary to address a possible role of KIS phosphorylation on CATS S131 on CATS stability and cell division.

We showed that CATS and KIS colocalize in the nucleus and in the nucleolus. We have previously reported the accumulation of CATS at the nucleoli of cells arrested at the G_1/S phase boundary [20]. Interestingly, this is the time point when the highest levels of KIS are observed. Since subcellular localization of proteins can be regulated through phosphorylation events, one possibility is that CATS phosphorylation on S131 controls CATS translocation to the nucleoli, and consequently its function in the nucleoplasm.

Using a GAL4-based reporter gene assay we demonstrate that KIS enhanced the repressor transcriptional activity of CATS. However, the effect of KIS on CATS does not rely on CATS phosphorylation on S131 but most likely on the protein–protein interaction between these proteins. Nonetheless, the fact that the inactive form of KIS was unable to enhance CATS transcriptional repression activity suggests that KIS kinase activity is also required for this effect. Additionally, we show that CATS interferes with the activation of transcription by CALM/AF10. Unexpectedly, KIS alone was also able to modulate CALM/AF10 transactivation. One possible explanation for this effect could be that it would be mediated through KIS interacting with endogenous CATS, known to be highly expressed in the cells used for the reporter assay (HEK293). The fact that CATS modulates the CALM/AF10 transactivation capacity suggests that the CATS-CALM/AF10 (and possibly CATS-KIS) interaction is important for leukemogenesis. However, it remains to be shown whether this effect of CATS and KIS on CALM/AF10 transcriptional activity does also play a role for direct cellular targets of CALM/AF10, which are unknown so far.

At the present time, the mechanism by which CATS exerts its repression effect on transcription is not known. Whether CATS simply disturbs transcriptional activation complexes formed or whether it is involved in the recruitment of transcriptional corepressors like histone deacetylases or chromatin remodeling complexes is presently unknown. There is some evidence that CATS might be able to recruit chromatin remodeling factors. Zhao and colleagues have recently described the interaction between CATS and components of the nucleosome remodeling and deacetylase (NuRD) complex [22]. The NuRD complex, implicated in transcriptional regulation, is thought to have a role in modifying the chromatin structure to initiate and maintain gene repression [37].

In addition, CATS may also play an important role in the regulation of KIS activity and function. KIS is the only kinase described to have a $U2AF^{65}$ homology motif (UHM). Through the UHM motif, KIS is capable of interacting with splicing factors such SF1 and SF3b155 [23]. Upon interaction, KIS phosphorylates splicing factor SF1, which in turn enhances SF1 binding to $U2AF^{65}$ and the 3′ splice site, an event known to take place during the early steps of spliceosome assembly [25]. Thus, the interaction between CATS and KIS suggests a possible role for CATS in RNA metabolism. This hypothesis is further supported by the identification of the RNA binding protein, PCBP1 (hnRNPE1) [38] as the most frequent CATS interacting protein in our Y2H screen (Fig. 1B and data not shown) and the recently described interaction between CATS (FAM64A) and ROD1, an RNA-binding protein involved in non-sense mediated RNA decay [39].

The CATS–KIS interaction might be especially important in neurons. Although KIS is ubiquitously expressed, higher levels are detected in the nervous system, suggesting a particularly significant role in this tissue

[27,40–42]. We demonstrated earlier that the murine *CATS* homologue is expressed at high levels in the central nervous system (CNV) of developing embryo [20]. Additionally, recent report by Satoh et al. has described CATS as a newly identified cellular prion protein (PrPC) interactor and suggest that CATS is as a neuronal protein that co-expresses with PrPC [21]. The fact that both CATS and KIS share similar expression pattern in the CNS implies a biological relevance of this interaction in the CNS and possibly relationship to PrPC. In this context it is noteworthy that PrPC interacts with synapsin I [43], a strong substrate of KIS *in vitro* [36].

5. Conclusions

In summary, we provide evidence for a novel interaction between CATS and the kinase KIS. Thus characterizing a novel kinase–substrate pair, which is part of a complex signaling network. We propose that KIS is a critical modulator of CATS function and that the CATS–KIS interaction is important in controlling gene expression and possibly leukemogenesis.

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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.bbamcr.2013.02.004.

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