Supplemental Materials and Methods

Generation of antibody to detect phosphorylation at Ser423 of CDK12

A thirteen-amino-acid peptide corresponding to Ser423 of human CDK12 (GenBank No. 51755), with both phosphorylated and non-phosphorylated forms, was synthesized with an added cysteine at the C-terminus by CosmoBio. The phosphorylated Ser423 peptide was conjugated to KLH via the C-terminal cysteine. Rabbits were intradermally immunized with the KLH-conjugated peptide. Lymph node lymphocytes were collected from the rabbits, and ELISA was performed using culture supernatants to select lymphocytes showing optimal titers.

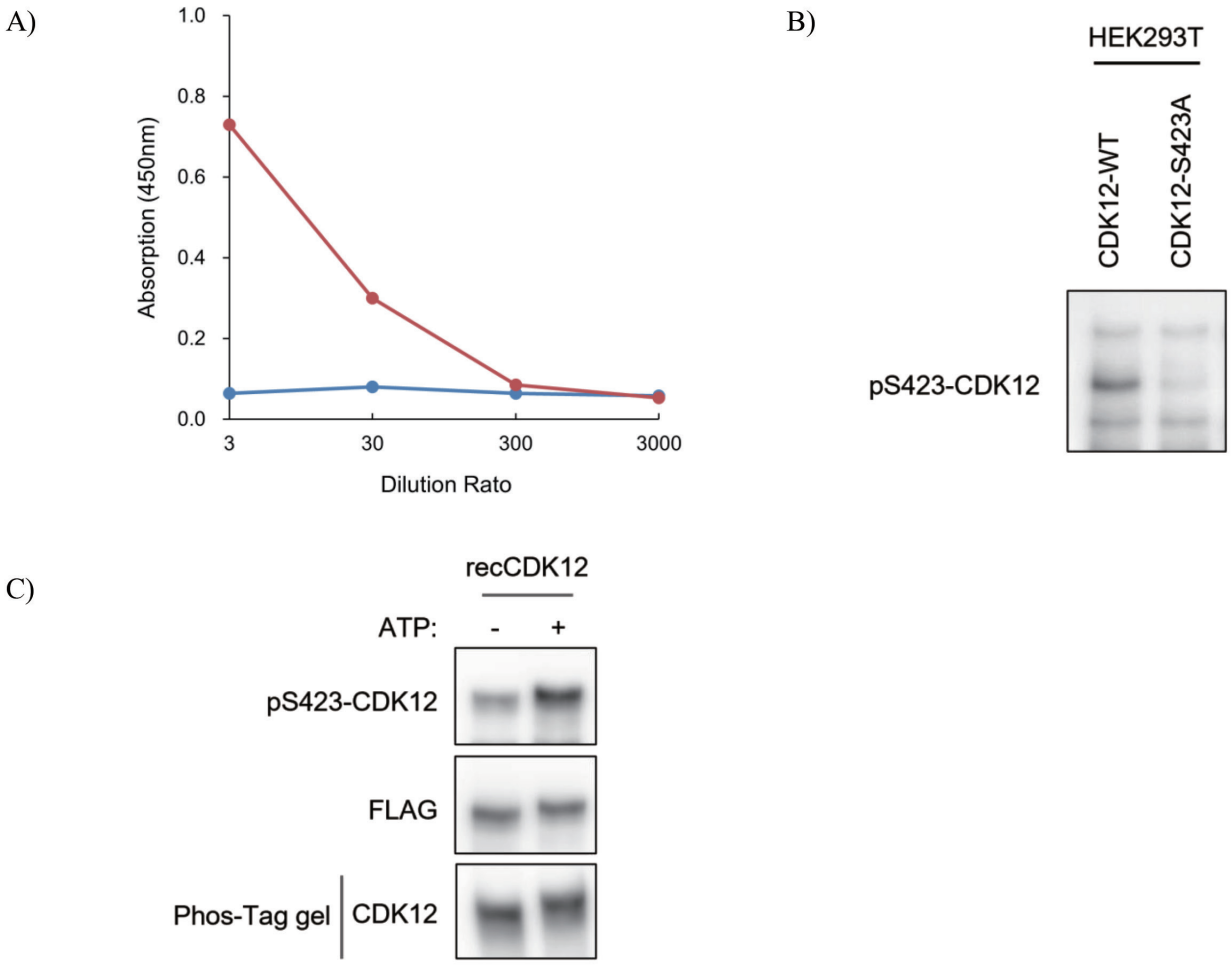
in vitro kinase assay for confirmation of phosphorylation of S423 of CDK12

For the CDK12 assay, recombinant CDK12 protein was solubilized with lysis buffer [0.2% NP40, 150 mmol/L sodium chloride, 50 mmol/L Tris-HCl (pH 7.6), 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L aprotinin]. The kinase activity of CDK12 was initiated by adding ATP. The reactions were electrophoresed and subjected to WB analysis. Phosphorylation status was detected using an anti-phospho Ser423 antibody and a Phos-tag gel (#193-16571, Fujifilm Wako).

Immunostaining

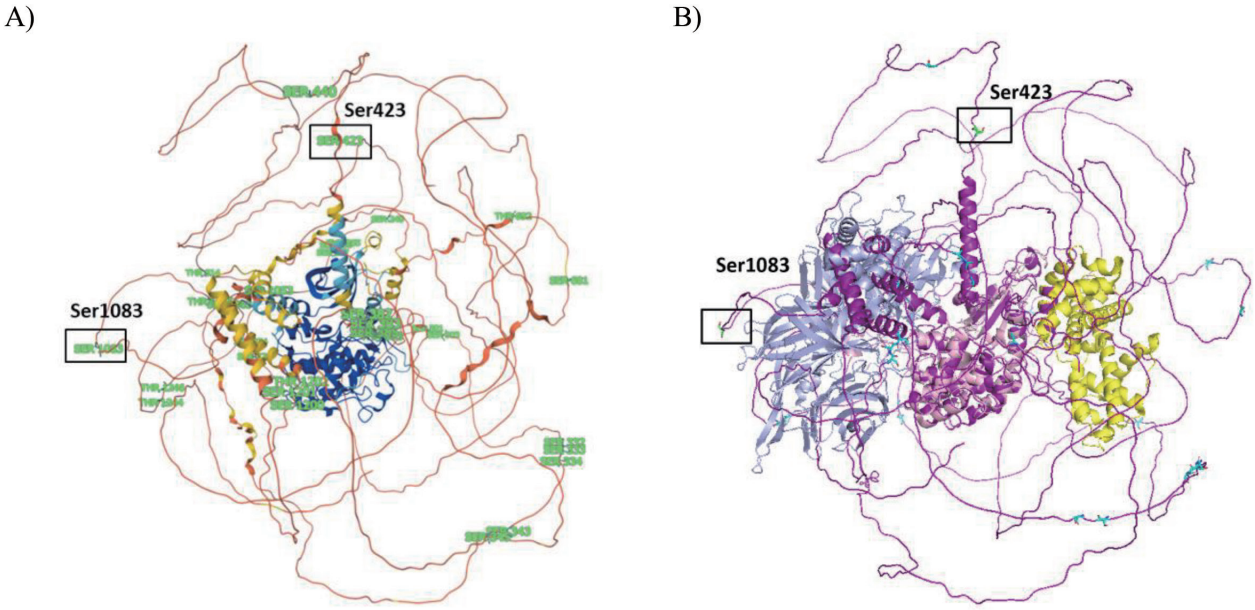
293T cells were transfected with the appropriate plasmids. After 5 days of transfection, cells were fixed in 4% formaldehyde in PBS. Following washing and permeabilization, GFP-tagged CDK12 WT, S423A, S1083A or S423/S1083A was detected using an immunofluorescence microscope (LSM700, Zeiss). Nuclei were stained with Hoechst 33342. Detailed immunostaining procedures have been described previously.[6)]

Cells were washed with 1× PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and permeabilized with 0.2% Triton X-100. Cells were incubated in blocking buffer (1× PBS, 5% normal goat serum, 0.1% Triton X-100) for 1 h at room temperature. For immunofluorescence staining, cells were incubated overnight at 4°C with antibodies diluted with Can Get Signal Solution B (TOYOBO). Cells were washed three times with 1× PBS and incubated with antibodies for 1.5 h at room temperature. After immunostaining, the cells were covered with ProLong Gold Antifade Reagent-containing DAPI (Cell Signaling Technology).



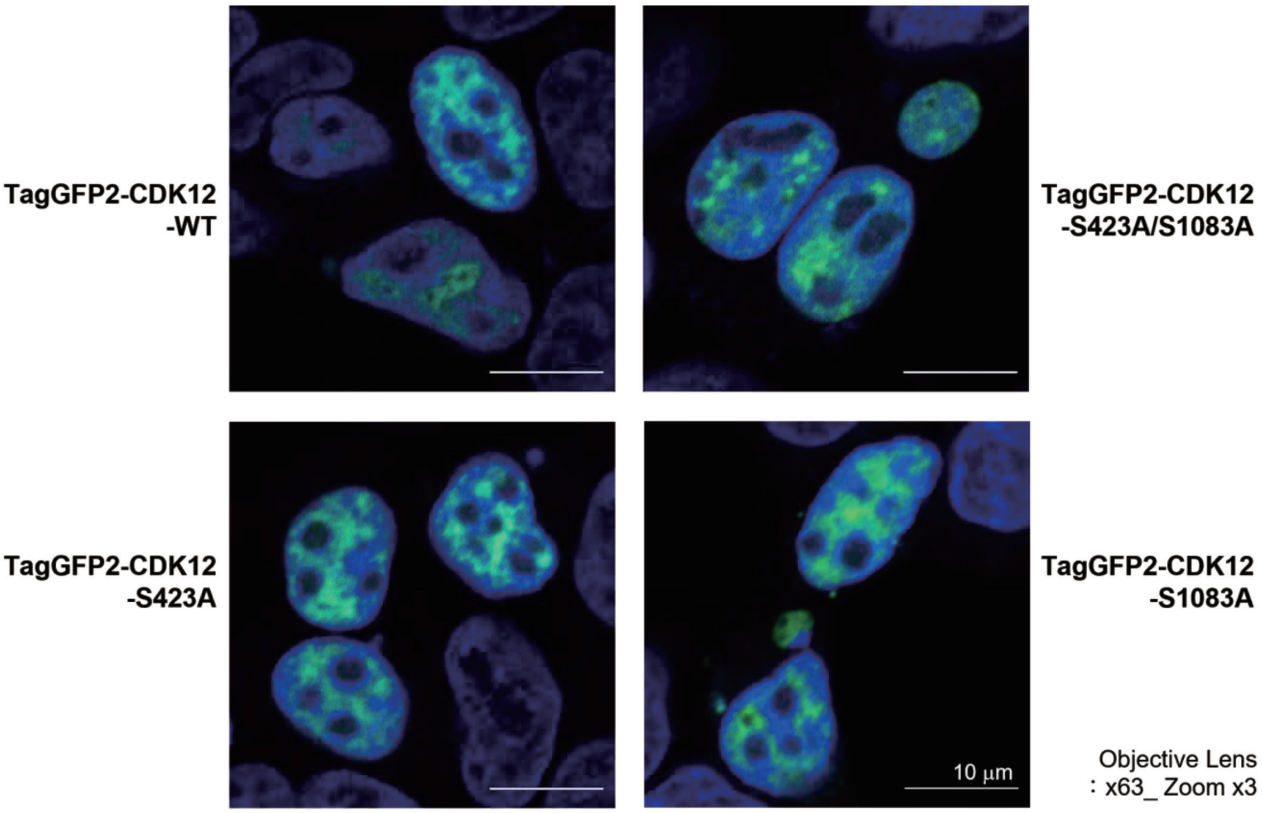
Supplemental Fig. 1. Generation of Phospho-Ser423 Antibody

(A) Purified monoclonal rabbit antibody was evaluated by ELISA assay using biotinylated phospho-CDK12 (Red) and non-phosphorylated CDK12 peptides (Blue). Specific binding was confirmed by measuring absorption at 450 nm. (B) Evaluation of the antibody for WB. 293T cell lysate transduced with either CDK12-WT or CDK12-S423A was subjected to WB. (C) CDK12 kinase activity was assessed by addition of ATP to recombinant CDK12 protein. Phosphorylation status was detected by WB using antiphospho-Ser423 antibody and Phos-tag gel.



Supplemental Fig. 2. 3D Structural Alignment of the Identified Amino Acids and the Location of Ser423 and Ser1083 on the Complex Structure of CDK12 with Cyclin K and DDB1

(A) Location of all autophosphorylation sites of serine and threonine residues described in this manuscript, mapped onto the CDK12 AlphaFold structural model. (B) Overlay of a full-length CDK12 AlphaFold structural model (AF-Q9NYV4-F1.pdb) and a crystal structure of the CR-8 bound DDB1-CDK12-cyclin K complex (PDB code: 6TD3). CDK12 AlphaFold structure is shown in magenta, and DDB1, CDK12, and cyclin K in the complex are depicted in light blue, pink, and yellow, respectively.



Supplemental Fig. 3. (A) Localization analysis of exogenous CDK12-wild type (WT), S423A mutant, S1083A mutant, and S423A/S1083A double mutant in 293 cells