博士論文

Genetic and biochemical analyses of mammalian Claspin, a factor required for genome replication

（動物細胞複製因子Claspinの機能の遺伝学的および生化学的解析）

楊 其駿 (Yang Chichum)

論文題目：**Genetic and biochemical analyses of mammalian Claspin, a factor required for genome replication**
（動物細胞複製因子 Claspin の機能の遺伝学的および生化学的解析）

研究分野：臨床医科学分野
氏名：楊其駿（**Yang Chichum**） 47-117346

CONTENTS

- **Conclusions**
- **Future perspectives**

Acknowledgements

References

Figure legends

Table

Figures

Summary

Claspin is an important upstream regulator of the checkpoint factor Chk1, originally discovered in *Xenopus*, and is conserved from yeast (Mrc1) to human. Claspin plays a critical role as a mediator molecule that transmits the replication stress signal from ATR to Chk1 effector kinase. Claspin binds to Chk1 via a region known as the Chk1-binding domain (CKBD) that contains multiple phosphopeptide motifs that are phosphorylated by an unknown kinase and are needed for ATR-dependent phosphorylation of Chk1. Cdc7-ASK has been reported to bind and phosphorylate Claspin. In addition to its well-known important role for initiation of DNA replication at each origin, Cdc7 plays a role also in replication stress checkpoint. However, the details on the roles of Cdc7-Claspin interaction during normal replication and replication stress response have been unclear. Therefore, I have conducted detailed analyses of human Claspin to clarify the biological significance of Claspin-Cdc7 interaction.

I identified a small segment on Claspin that is rich in acidic amino acids (glutamic acid and aspartic acid; AP, acidic patch) as being essential for binding to Cdc7 and other initiation factors. To functionally dissect AP, I then generated Claspin-deficient mice and established Claspin conditional knockout cell lines. The Claspin-deficient embryos are dead between E9.5 and E12.5. Conditional knockout of Claspin in MEF cells resulted in deficient replication checkpoint and reduced DNA replication. Using the Claspin-deficient MEF cells, I have demonstrated the critical role of AP during normal DNA replication as well as during checkpoint response. The alanine substitution of the acidic residues (DE/A) in the acidic patch led to loss of binding to Claspin of important initiation factors including Cdc7, Cdc45, DNA polymerase ε/δ, and TopBP1. Purified Cdc7 protein physically interacts with Claspin, but this binding is lost by the DE/A mutation. The DE/A mutant cannot be phosphorylated by Cdc7-ASK *in vitro*, as expected from its inability to interact with Cdc7. Importantly, phosphorylation of Mcm2 and 4, critical targets of Cdc7 and essential for replication, is greatly reduced by the DE/A mutation of Claspin, indicating that Claspin plays an important role in recruitment of Cdc7 to pre-replicative complexes. I also show that AP can interact with the N-terminal segment of Claspin. DE/A mutation or an internal deletion of the acidic patch resulted in much stronger DNA binding activity and increased PCNA interaction (through the newly identified PIP box present in the N-terminal segment), showing that AP is inhibitory for DNA and PCNA binding activity of the N-terminal segment. I further showed that Cdc7-mediated phosphorylation could counteract the inhibition. These results show a crucial and novel role of Claspin in initiation of DNA replication and checkpoint through recruiting an essential kinase. The results also point to an intramolecular interaction as a potentially conserved and important means for

regulation of protein functions.

After release from serum starvation, MEF cells lacking Claspin cannot progress cell cycle normally and cells are dead by 48 hours after the release. During release, p53 is induced but MCM2 is absent in the Claspin mutant. The DE/A mutant is defective in this process, but the PIP mutant Claspin (unable to bind to PCNA) is proficient. These results imply a critical role of Claspin during the recovery from the quiescent (resting) state.

Introduction

A key feature of the DNA replication mechanism is its strict regulation. In bacteria, the initiator protein DnaA recognizes and binds to a single replication origin, *oriC*, on the chromosome before DNA replication starts. Then, DnaB, a DNA helicase, is recruited to origin to start DNA synthesis. On the other hand, in eukaryotes, the origin recognition complex (ORC) assembles at multiple origins. Cdc6 and Cdt1 assemble pre-RC by recruiting Mcm proteins, components of replicative DNA helicase complex, during G1 phase. Cdc7, a serine/threonine-directed kinase with associated activation subunit Dbf4/ASK, and Cdk phosphorylate the Mcm subunits and recruit Cdc45, GINS and other factors including DNA polymerases for initiation. Then, DNA replication is initiated bi-directionally from the origins (Masai et al, 2006, Moyer et al, 2006, Aparicio et al, 2009, Im et al, 2009).

Claspin was first identified as Chk1-interacting protein in *Xenopus* (Kumagai and Dunphy, 2000). In response to replication stress, Claspin binds to Chk1 via a region known as the Chk1-binding domain (CKBD) that contains repeated phosphopeptide motifs that are phosphorylated and are needed for ATR-dependent phosphorylation of Chk1 both in *Xenopus* and human (Chini and Chen, 2003; Kumagai and Dunphy, 2003; Clarke and Clarke, 2005, Chini and Chen, 2006). However, Claspin phosphorylation is complex. Many kinases have been implicated in Claspin phosphorylation at multiple sites (Tanaka, 2010). The conserved phosphorylation sites, Thr916 and Ser945 of CKBD in humans, do not have ATM/ATR consensus phosphorylation motifs (SQ/TQ). They also are not directly phosphorylated by ATR (Kumagai and Dunphy, 2003; Lindseyr et al, 2009), either. Chk1 was reported to phosphorylate Thr 916 (Chini and Chen, 2006), but contradictory results have also been reported (Bennett et al., 2008). Recently, Casein kinase 1 gamma 1 (CK1γ1) was implicated in Claspin phosphorylation and Chk1 activation (Meng et al, 2011).

Claspin expression levels are regulated during the cell cycle. Claspin is expressed at a high level in S and G2 phases, and is significantly downregulated after the entry into mitosis until G1 phase (Mailand et al, 2006). In G1 phase, Claspin is degraded by E3 ubiquitin ligase Cdh1-APC/C (Bassermann et al, 2008; Gao et al, 2009; Faustrup et al, 2009). The deubiquitination enzyme USP28 was proposed to antagonize Cdh1-APC/C-dependent Claspin degradation (Bassermann et al, 2008). In G2 phase, Claspin is degraded by SCFβTrCP-mediated ubiquitination through Plk1-dependent phosphorylation of a phosphodegron during checkpoint recovery. (Mailand et al 2006 , Peschiaroli et al, 2006; Mamely et al) The deubiquitination enzyme USP7, was

proposed to prevent Claspin degradation by targeting the βTrCP1/2–SCF complex (Faustrup et al, 2009). Recently, it was reported that another deubiquitination enzyme USP29 down regulation caused Claspin destabilization, whereas its overexpression increased Claspin levels (Martín et al,2014).

During the normal course of DNA replication, Claspin is required for normal fork progression (Lin et al., 2004; Petermann et al., 2008; Scorah and McGowan, 2009). This feature appears to be conserved also in budding yeast Mrc1, yeast homologue of Claspin (Szyjka et al., 2005). In *Xenopus*, recruitment of Claspin to chromatin is dependent on replication factors MCM2-7 and Cdc45, but is independent of checkpoint-related proteins, such as ATR, RPA, 9-1-1 complex, and Rad17-RFC. (Lee et al, 2003; Lee et al, 2005; Tanaka et al, 2009). Furthermore, Tim-Tipin complex promotes the recruitment of Claspin to chromatin under replication stress. (Errico et al, 2007). Claspin interacts with various replication factors including ATR, Chk1, Cdc7 kinase, Cdc45, Tim, MCM4, MCM10, PCNA, DNA polymerases α, δ, ε and And-1 in human cells (Lee et al., 2005; Serçin and Kemp, 2010; Gold and Dunphy; 2010; Uno et al., 2011; Hao et al., 2015). Its was also reported that Claspin is needed for efficient cell growth and normal replication fork rates in human cells. (Lee et al, 2004; Petermann et al 2008). When human cells suffer from replication stress, proliferating cell nuclear antigen (PCNA), essential for DNA replication, was ubiquitinated in a manner dependent on Claspin (Moldovan et al. 2007; Yang et al, 2008). Furthermore, depletion of HERC2 alleviated the slow replication fork progression in Claspin-deficient cells, suppressed enhanced origin firing, and led to a decrease in MCM2 phosphorylation (Izawa et al, 2011).

In *Xenopus*, Cdc7-Drf1 is recruited to chromatin in a manner dependent on Claspin under DNA damage (Yanow et al, 2003). Drf1 interacts with Claspin through Chk1 binding domain. (Gold and Dunphy, 2010). Claspin was reported to be a substrate of Cdc7 kinase *in vitro* and *in vivo* for checkpoint function and influence MCM2 interaction. (Kim et al, 2008; Rainey et al,2013)

Although the mechanism of Claspin in replication checkpoint control has been studied a lot, roles of Claspin in normal replication have been mostly unclear. The functional significance of subdomains in Claspin molecule has not been well understood either. Under these circumstances, I have decided to dissect the functional domains of Claspin by first focusing on its interacting proteins. Then, I tried to define the functional domains of Claspin by utilizing Claspin KO cells. I next attempted to clarify the mechanism of how the defined domains are involved in replication and checkpoint regulation by Claspin. Finally, I made an attempt to discover novel roles of Claspin in cells under different physiological conditions.

In my dissertation, I first analyzed interacting proteins of Claspin and mapped its

interaciting domains on Claspin. I then generated Claspin knockout (KO) mice and cells and used them to evaluate the roles of the identified domains during the growth under normal and replication stress conditions. I have identified the C-terminal acidic patch sequence (AP) and a PIP motif (PCNA binding motif) and showed that they play important roles for DNA replication as well as for replication stress responses.

Cdc7 kinase is recruited to Claspin through AP and phosphorylates Claspin. AP also makes intramolecular interaction with the N-terminal segment containing DNA binding domain and PIP box. When Claspin is phosphorylated by Cdc7, the intramolecular interaction is weakened significantly. In the absence of AP, the intramolecular interaction is lost and DNA and PCNA bindings are increased. The DE/A mutant in which all the acidic residues were replaced by alanine did not interact with Cdc7 and with other replication factors including Cdc45 and TopBP1, showing the importance of acidic residues. This mutant could not restore the replication or checkpoint in Claspin KO MEF or Claspin-depleted normal human dermal fibroblast cells. It could not support phosphorylation of MCM, a main target of Cdc7. These results indicate that Claspin plays a crucial role in recruiting Cdc7 kinase for initiation of DNA replication.

Materials and Methods

Cell lines

293T, HeLa, U2OS, Normal Human Dermal Fibroblast (NHDF) and Mouse embryonic fibroblasts (*MEFs*) were cultured at 37°C in a 5% CO2 humidified incubator in Dulbecco's modified Eagle's medium (high glucose) supplemented with 15% fetal bovine serum (HANA-NESCO), 2mM L-glutamine, 1% sodium pyruvate, 100U/ml penicillin and 100μg/ml streptomycin.

Plasmid construction

The Claspin insert (*Xho*I/*Xba*I fragment) of CSII-EF MCS- mAG-6His-Claspin-3Flag, CSII-EF MCS-6His-Claspin-3Flag or CSII-EF MCS-6His-Claspin-HA plasmid DNA (Uno et al. 2012) was replaced by DNA fragments encoding parts of Claspin, amplified by PCR to express truncated forms of Claspin. To express Claspin mutant with internal deletion, two PCR-amplified fragments (*XhoI-Bam*HI and *Bam*HI –*Xba*I or *Xho*I-*Nhe*I and *Nhe*I–*Xba*I) were inserted at the *Xho*I-*Xba*I site of CSII-EF MCS-6His-Claspin-3Flag to replace the Claspin insert. The *Eco*RI-*Hpa*I fragment of wild-type or mutant Claspin DNA from mAG-TEV- 6His-Claspin-3xFlag was inserted at the *Eco*RI/*Sna*BI site of pMX-IP (Addgene) to construct retroviral expression vectors.

Southern blotting

Genomic DNA of ES cells was digested by *Eco*RV or *Eco*RI, run on 0.8% agarose gel at 16V for 20 hrs and then transferred to nylon membrane (Genesscreen plus, PerkinElmer). DNA was detected by Southern hybridization using the probes indicated in the figures.

Transient expression in 293T cells

Transfection into 293T cells was conducted as previously described (Uno et al., 2012). 1.6ug DNA mix with 7ul 1mg/ml PEI with 200ul NaCl for 30 min. The mixtures were added to 30% confluence cells cultured in 6 well plate. After 40 hrs the cell were harvested.

Plasmids and antibodies

Antibodies used in this paper are as follows. Anti-human Claspin was developed in rabbit against a recombinant protein containing aa896~1014 of human Claspin produced in *E. coli*. Anti-mouse Claspin was developed in rabbit against a polypeptide LKTNGSSPGPKRSIFRYLES (aa1296~1315 of mouse Claspin). Anti-PCNA(sc-56), anti-Pol. α(sc-5920), Lamin B(sc-6216), anti-ATR(sc-1887), anti-Chk1(sc-8408) and

anti-MCM2(sc-9839) were from Santa Cruz Biotechnology; anti-TopBP1(A300-111A) and anti-MCM2 S53(A300-756A) were from Bethyl; anti-Cdc7(K0070-3) and anti-Flag (M185-3L) were from MBL; anti-Chk1 S345(#2341) from Cell signaling; anti-tubulin(T5168) was from Sigma-Aldrich; anti-myc(04362-34) was from Nacalai Tesque; anti-HA(16B12) was from Abcam; Anti-MCM4, anti-Cdc45, anti-Tim and anti-MCM4 S6T7 were previously described (Masai et al., 2006; Yoshizawa-Sugata and Masai, 2007); anti-pol δ (8A5-E3; Shikata et al, 2001) and anti-pol ε (ATCC, CRL-2284) antibodies were prepared from their hybridoma cell lines.

Genotyping from mouse tail

About 1 cm mouse tails were incubated in 50mM NaOH (in 60 µl) and boiled for 1 min. After spin down of insoluble materials, the supernatant was neutralized by addition of 50µl 1M Tris-HCl (pH 8.0) and 1µl of the resulting solution was used for PCR reaction.

Genotyping from MEF

MEFs were lysed in 500µl lysis buffer (50mM Tris-HCl [pH8.0], 100mM NaCl, 20mM EDTA) and 150µg/ml Proteinase K for 20h at 55°C, followed by phenol/ chloroform (1:1) extraction, and DNA was recovered by ethanol precipitation. The final pellet was dissolved in 100µl TE buffer, and used for PCR reaction.

Knockdown Claspin by siRNA

siRNA targeting non-coding mRNA segment of Claspin (see **Supplemental Table 1**) was transfected into NHDF or U2OS using lipofectamine 3000 (Lifetechnologies) for 48h.

Western blotting

Cell extracts were run on 5~20% gradient SDS-PAGE (ATTO) or 7.5% SDS-PAGE and then transferred to Hybond ECL membranes (GE Healthcare) followed by western blot analysis with the indicated antibodies. Detection was conducted with Lumi-Light PLUS Western Blotting Substrate (Roche) or SuperSignal™ West Pico Chemiluminescent Substrate (Thermo) and images were obtained with LAS3000 (Fujifilm).

BrdU incorporation

To the cells in 6 wells plates, BrdU was added at 20mM for 20min. Cells were harvested and were fixed at -20°C by 75% ethanol. After wash with wash buffer (0.5% BSA in PBS), cells were treated with 2N HCl for 20min and then with 0.1M sodium Borate (pH8.5) for 2min, both at room temperature. Then, cells were treated with FITC-conjugated anti-BrdU antibody (BD biosciences, 51-23614l) for 20min at room

temperature in the dark, and further incubated with PI (25μg/ml) and RNaseA (100μg/ml) for 30min at room temperature, followed by analyses with FACS.

Cell fractionation and immunoprecipitation *in vivo*

Cells were lysed in CSK buffer (10mM PIPES-KOH [pH6.8], 100mM potassium glutamate, 300mM sucrose, 1mM $MgCl_2$, 1mM EGTA, 1mM DTT, 1mM Na_3VO_4, 50mM NaF, 0.1mM ATP, protease inhibitor (PI tablet [Roche] and 0.5mM PMSF), containing 0.1% TritonX-100 and 10units⁄ml Benzonase (Amersham plc.). After rotating for 60min in cold room, the supernatants were recovered and the pellets were washed two times and recovered as chromatin-enriched fractions. The supernatants were incubated with anti-Flag M2 affinity beads (SIGMA, A2220) for 60min at 4°C. The beads were washed with CSK buffer 3 times and proteins bound to the beads were analyzed by western blotting.

Growth curve

1.0×10^5 cells of stable clones or normal f/- MEFs treated with Ad-Cre for 48 hr or non-treated were passaged to new 6-well plates. The cells were harvested at 24 and 48 hr after the passage and cell numbers were counted.

Protein purification

293T cells (15 cm dish, 2 plates) incubated for 40h after transfection were harvested and lysed as above. For ED/A mutant, CSK buffer was supplemented with 500mM NaCl. The proteins bound to anti-Flag M2 affinity beads (Sigma-Aldrich) were recovered from the supernatants and washed by Flag wash buffer (50mM NaPi [pH7.5], 10% glycerol, 300mM NaCl, 0.2mM PMSF and PI tablet), and bound proteins were eluted with Flag elution buffer (50mM NaPi [pH7.5], 10% glycerol, 30mM NaCl, 200 μg⁄ml 3xFlag peptide [SIGMA], 0.1mM PMSF and PI tablet) (see Uno et al., 2012 for more details).

Preparation of labeled Y-fork

^{32}P-end labeled sense oligonucleotide (32mer-dT_{32}; 240ng) was mixed with antisense oligonucleotide (dT_{32}-32mer; 300ng) in 20mM Tris-HCl [pH7.4] and 10mM $MgCl_2$, and incubated at 96°C for 3min and then at 37°C for 60min (You et al., 2003). The generated ^{32}P-labeled Y-fork DNA was isolated from PAGE and dissolved in TE buffer.

Gel-shift assay

Twenty fmole of labeled Y-fork DNA was incubated with purified proteins of indicated amounts in gel-shift buffer (10mM Tris-HCl [pH7.5], 40μg/ml BSA, 1mM DTT, 50mM

EDTA, 20mM K-glutamate, and 8% glycerol), incubated at 30°C for 1h and the samples were applied onto 5% 29:1 PAGE (1x TBE and 5% glycerol). The gel was dried and autoradigraphed.

Annealing assay

Annealing assays contained ^{32}P-labeled oligonucleotide (32mer-dT32; 10fmole), unlabeled oligonucleotide (dT32-32mer; 15 fmole) and purified protein (0.5pmole and 1.0pmole) in 10 µl reaction buffer (10 mM Tris-HCl [pH7.5], 40 µg/ml BSA, 1 mM DTT, 50mM EDTA, 50 mM K-glutamate, 8% glycerol, and 5 mM Mg-Acetate) and were incubated at 30°C for 60 min, followed by analyses on 10% 29:1 PAGE (1x TBE, 5% glycerol). The gel was dried and autoradigraphed.

Filter binding assay

Binding reactions contained ^{32}P-labeled Y-fork DNA (80 fmole) and purified wild-type or mutant Claspin protein of indicated amount in 1x binding buffer (10mM Tris-HCl [pH7.5], 40µg/ml BSA. 1mM DTT, 0.5mM EDTA, 20mM K-glutamate, and 8% glycerol) and were incubated for 1h at 30°C. HA membranes (MFTM Membrane Filters [0.45µm HA], Millipore) were activated by soaking in 4M KOH solution for 10 min followed by wash in water twice for 10 min each. The processed HA membranes and untreated DE81membranes were washed in 1x binding buffer for 1h. On the filtration unit, a HA membrane (trapping proteins) was placed on top of a DE81 membrane (trapping all the ssDNA) and washed with 3 ml of 1x binding buffer. Next, a binding reaction mixture was passed through and further washed twice with 3ml of 1x binding buffer. After drying, radioactivity trapped on each membrane was counted by liquid scintillation counter. Radioactivity trapped on the HA membrane was divided by the sum of that on the HA and DE81 membranes and the values were presented as binding activity.

Pull down assay in vitro

For pull-down assays of PCNA and Cdc7-ASK, recombinant wild-type or mutant Claspin protein was incubated with PCNA (BioAcademia) or Cdc7-ASK, as indicated in each experiment, in the reaction buffer (40mM HEPES-KOH [pH 7.6], 100mM K-glutamate, 1mM $MgCl_2$, 1mM EGTA, 0.01% TritonX-100, 0.1mM ATP, and 0.5mM PMSF) at 4°C for 1h. Anti-Flag M2 affinity beads were added and beads were recovered by centrifugation, washed 2 times with the reaction buffer. Proteins attached to the beads were analyzed by SDS-PAGE and detected by western blotting.

Limited trypsin digestion

The wild-type and DE/A mutant Claspin (0.8μg) was digested with 0, 0.01, 0.1, 1 or 10 ng of trypsin at 24°C for 5 min. Digestion was terminated by boiling with sample buffer, and the samples were applied on 7.0% SDS-PAGE, followed by western blotting analyses with anti-Flag antibody.

Evaluation of Cdc7-mediated phosphorylation in the cells

Wild type or mutant Claspin was transfected into 293T cells alone or with pME18S-mycASK and pME18S-HACdc7 plasmid DNAs. After 40 hrs, cells were harvested, and extracts were prepared. Extracts, incubated with lambda phosphatase or non-treated, were analyzed by western blotting with indicated antibodies.

***In vitro* kinase assays with Cdc7-ASK**

0.3pmol wild-type or mutant Claspin protein was incubated in the presence or absence of 0.03pmol Cdc7-ASK complex in the kinase reaction buffer (50mM HEPES-KOH [pH7.9], 10mM $MgCl_2$, 2mM DTT, 10μM ATP and 1μCi of [γ-^{32}P] ATP) for 30 min at 37°C. 1/4 volume of 5x sample buffer was added, heated at 96°C for 3 min and analyzed by SDS-PAGE, followed by CBB staining and autoradiogram.

Analysis of the survival rate of cells released from quiescent state.

Flox/- MEF cells cultured in 0.5% serum medium with or without adeno virus for 48 hours. Added final concentration 15% serum and observed the cells by microscope at 0, 26 and 48 hours.

Analysis the cell released from quiescent state by western blotting

Stable clones of wild type, DE/A and PIP flox/- MEF cells and normal flox/- MEF cells cultured in 0.5% serum medium with or without adeno virus for 48 hours. Final concentration 15% serum were adding to the medium. The cells were harvested at indicated time and were lysed in CSK buffer (10mM PIPES-KOH [pH6.8], 100mM potassium glutamate, 300mM sucrose, 1mM $MgCl_2$, 1mM EGTA, 1mM DTT, 1mM Na_3VO_4, 50mM NaF, 0.1mM ATP, protease inhibitor (PI tablet [Roche] and 0.5mM PMSF), containing 0.1% TritonX-100 and 10units⁄ml Benzonase (Amersham plc.). After rotating for 60min in cold room, the supernatants were recovered and the pellets were washed two times and recovered as chromatin-enriched fractions then analyzed by western blotting.

Analysis the cell released from quiescent state by BrdU incorporation

Stable clones of wild type, DE/A and PIP flox/- MEF cells and normal flox/- MEF cells cultured in 0.5% serum medium with or without adeno virus for 48 hrs. Final

concentration 15% serum were adding to the medium. Before cells were harvested at indicated times, BrdU was added at 20mM for 20min and were fixed at -20°C by 75% ethanol. After wash with wash buffer (0.5% BSA in PBS), cells were treated with 2N HCl for 20 min and then with 0.1M sodium Borate (pH8.5) for 2 min, both at room temperature. Then, cells were treated with FITC-conjugated anti-BrdU antibody (BD biosciences, 51-23614l) for 20 min at room temperature in the dark, and further incubated with PI (25μg/ml) and RNaseA (100μg/ml) for 30 min at room temperature, followed by analyses with FACS.

Analysis the survival rate of p53 inhibited cells released from quiescent state

Flox/- MEF cells cultured in 0.5% serum medium with or without adeno virus for 48 hrs. Added final concentration 15% serum with 0, 5, 10and 50 μM Pifithrin-α and observed the cells by microscope at 12, 24 and 48 hrs.

RESULTS

Section I Proteins interacting with Claspin: Molecular dissection of Claspin

I-1. Claspin interacts with replication factors in a manner dependent on an acidic patch near its C terminus

Our group and others previously reported that Claspin interacts with many factors involved in checkpoint regulation and replication fork machinery, including ATR, ATM, Chk1, Tim, MCM4, MCM10, Cdc45, DNA polymerases α, δ, ε and Cdc7 kinase (Lee et al., 2005; Kim et al., 2008; Serçin and Kemp, 2010; Gold and Dunphy; 2010; Uno et al., 2011; Hao et al., 2015). I have generated a series of deletion derivatives of Claspin tagged with Flag at its C-terminus (**Figure 1A**), transiently expressed them in 293T cells and examined their interaction with these factors by immunoprecipitation using anti-Flag antibody (**Figure 1B**). I noted that interaction of Claspin with Cdc7, TopBP1, polδ and ATR depends on the presence of a C-terminal segment, 897~1100. Indeed, the 204aa segment was sufficient for binding to Cdc7 (**Figure 1B**, lane 24, #27). Similar segments were required for binding to polδ and TopBP1, and the 204aa segment alone bound to polδ and TopBP1. #21 containing aa986~1209 interacted with Cdc7, polδ and TopBP1 to a significant extent, suggesting that aa986~1100 is crucial for binding of these factors. Indeed, the internal deletion of 897~1209 or 897~1100 resulted in complete loss of binding to Cdc7 (**Figure 1C**). This segment (aa986-1100) is highly enriched in acidic residues (aspartic acid and glutamic acid; see **Figure 1D**). I noted that #18 (aa897-1209) and #24 (aa1-350+aa897-1209) barely bound to Cdc7 or other factors in spite of the fact they contain the acidic region. aa1210-1339 is highly enriched in basic amino acids (26 out of 130) and therefore this region may interfere with the binding of factors to the acidic patch segment. In contrast, the N-terminal segments were required for binding to PCNA and Tim as well as for DNA binding. In fact, aa1~350 was sufficient for binding to PCNA and Tim (**Figure 1B**, lane 15, #2 and data not shown).

I-2. PCNA interacts with Claspin through a PIP box present in the N-terminal segment.

Claspin was reported to interact with PCNA (Brondello et al., 2007). I have identified a potential PIP box at aa311~318 (NKTIHDFF) and mutated the conserved residues to alanine (AKTAHDAA, **Figure 2A and B**). Both full-length and the N-terminal polypeptide #2 (aa1~350) expressed in 239T cells bound to PCNA in immunoprecipitation (**Figure 2C**, lanes 6 and 7). I noted that the polypeptide #2 consistently bound significantly more PCNA compared to the full-length. In the same assay, the PIP box mutants generated on the full-length and #2 did not bind to PCNA

(**Figure 2C**, lanes 8 and 10). The PIP mutant is defective specifically in interaction with PCNA and could interact with other replication factors (**Figure 2D**). I also examined the interactions using purified proteins. The purified full-length Claspin or #2 polypeptide were mixed with PCNA and immunoprecipitation by anti-Flag antibody was conducted. The immunoprecipitate with the wild-type form of Claspin contained PCNA but that with the PIP mutant did not (**Figure 2E**), showing that Claspin directly binds to PCNA through PIP.

Section II Generation of Claspin knockout mutant mice and MEF cells.

In order to genetically dissect the functions of Claspin in development and in cell proliferation, I have generated conditional knockout mice. LoxP sequences were introduced in the introns before and after the 2nd exon (**Figure 3A**). The expression of Cre recombinase results in deletion of the 2nd exon containing the initiation codon, leading to inactivation of Claspin. Whereas crossing of +/- and +/- mice generated -/- embryos at E9.5, albeit at a rate lower than expected, no -/- embryos were detected at E12.5, suggesting that Claspin knockout mice is non-viable by E12.5 (**Figure 3B**). I have generated flox/- MEF cells, and infected them with recombinant adenovirus encoding Cre recombinase (Ad-Cre), which resulted in loss of Claspin expression (**Figure 3C**). I noted that growth was retarded and DNA synthesis, as measured by BrdU incorporation, was also reduced upon Ad-Cre infection (**Figure 3D**). This is consistent with previous report on cancer cells depleted of Claspin by siRNA (Lin et al., 2004; Petermann et al., 2008). HU-induced Chk1 activation (phosphorylation of S345) was also lost in Ad-Cre infected Claspin flox/- MEF cells, as was previously reported for Claspin-depleted cancer cells (Lin et al., 2004; data not shown; see **Figure 5C**).

Section III Identification of a novel domain on Claspin that is critical for both replication and checkpoint functions.

III-1. The acidic patch is crucial for Claspin functions.

In order to understand the physiological functions of the acidic patch, I constructed a DE/A mutant in which all the aspartic acids and glutamic acids in the acidic patch were substituted with alanine (aa988-1086; **Figure 1D**). I examined the interaction of the DE/A mutant with various proteins after its overexpression in 293T cells, as above. The DE/A mutant did not interact with Cdc7 or TopBP1, while it interacted with PCNA, ATR, DNA polymerase α and Tim, although interaction with ATR and Tim slightly decreased and that with polε significantly reduced compared to the wild-type (**Figure 2D,** lane 7). Purified full-length Claspin interacted with purified Cdc7-ASK, while the

mutant DE/A Claspin did not (**Figure 4A**), showing that Claspin directly interacts with Cdc7-ASK. It also did not interact with Chk1 (**Figure 4B,** lane 9). These results support the idea that the acidic amino acids play crucial roles in binding of these factors to Claspin.

I then examined the physiological functions of the DE/A mutant using Claspin knockout MEF cells. I have generated retroviruses encoding mutant Claspin which had been fused with mAG at the N-terminus. The Claspin flox/- MEF cells were transfected with the retroviruses and mAG-positive stable cell lines were isolated. Upon Ad-Cre infection, endogenous Claspin was knocked out, and growth or checkpoint activation was examined. The DE/A mutant could not restore the growth, while the wild-type Claspin could fully recover the growth of Claspin knockout MEF cells (**Figure 5A**). The DE/A mutant could not correct the defect of DNA synthesis, either (**Figure 5B**). This indicates that the acidic patch is required for Claspin to support normal DNA replication. Chk1 activation (phosphorylation of Chk1 S345) after HU treatment was restored by the wild-type Claspin, but not by the DE/A mutant (**Figure 5C,** lanes 12 and 14). Thus, the acidic patch is required also for checkpoint activation.

III-2. PIP mutant is defective in normal growth and checkpoint activation.

Using Claspin knockout MEF cells, I examined the functions of the PIP mutant. The PIP mutant could not restore the growth and DNA synthesis of Claspin-/- cells, while the wild-type Claspin could fully recover them (**Figure 5A** and **Figure B**). This indicates that PCNA binding is required for Claspin to support normal DNA replication. Chk1 activation after HU treatment was also not restored completely by the PIP mutant (**Figure 5C**). Thus, PCNA binding is required also for checkpoint function of Claspin.

Section IV Mechanism of regulation of Claspin functions

IV-1. The acidic patch is required for phosphorylation of Mcm proteins by Cdc7 kinase.

Mcms are known to be critical targets of Cdc7 kinase in initiation of DNA replication. Therefore, we examined whether Mcm phosphorylation is affected in Claspin knockout MEF cells. I monitored phosphorylation of Mcm2 and Mmc4, using phospho-specific antibodies (Mcm2S53 and Mcm4S6T7) that recognize residues known to be phosphorylated by Cdc7 (Masai et al., 2006; Montagnol et al., 2006). In Claspin-/- MEF cells, both Mcm2 S53 and Mcm4 S6T7 signals were significantly reduced (**Figure 6A**, lane 4). Similar reduction of Mcm phosphorylation was observed also in NHDF (normal human dermal fibroblast cells) depleted of Claspin by siRNA (**Figure 6B**). Interestingly, the Mcm phosphorylation was not affected in cancer cells (U2OS and HeLa cells)

similarly depleted of Claspin (**Figure 6B**), consistent with previous report (Izawa et al., 2011). I then examined whether the acidic patch of Claspin is involved in Mcm phosphorylation. Phosphorylation of both Mcm2 and Mcm4 was fully restored by ectopic expression of the wild-type full-length Claspin fused with mAG (**Figure 6A**, lane 5). In contrast, the DE/A mutant could not restore the phosphorylation of Mcms (**Figure 6A**, lane 6). These results strongly suggest that Claspin plays a crucial role in recruiting Cdc7 kinase for phosphorylation of pre-RC through its acidic patch. Interestingly, this requirement for Claspin is specific to normal cells, and cancer cells appears to have acquired mechanisms that bypass this pathway.

IV-2. DNA binding activity of Claspin is regulated by the C-terminal acidic patch.
It was previously reported that Claspin can bind to DNA. It binds to dsDNA but prefers fork-like DNA (Sar et al., 2004). I generated various deletion derivatives of Claspin (**Figure 7A**), purified them (**Figure 7B**) and assayed their DNA binding activity in gel shift assays using Y-fork DNA as a substrate (**Figure 7C**). DNA binding activity is lost in mutants lacking N-terminal segments, confirming that DNA binding activity lies in the N-terminal segment (Sar et al., 2004; Zhao et al., 2004; Lee et al., 2005; Uno et al, 2011). I noted that derivatives lacking C-terminal domains bind to DNA with much higher affinity than that of the full-length Claspin. The segment responsible for the suppression of DNA binding was mapped to the acidic patch (aa986 to aa1101) discussed above (**Figure 7C, Cdel1, Cdel6, Cdel7, Cdel6N-1, and Cdel6N-2**). Cdel2, lacking 1101~1209, showed higher binding activity than the full-length, suggesting that this segment is also involved in the suppression.

I then examined the DNA binding activity of the DE/A mutant. The DE/A mutant showed significantly higher affinity to DNA than the wild-type protein did (filter binding assays; **Figure 7D**). In gel shift assays, the DNA-protein complexes appeared as multiple bands (**Figure 7E**). This appears to be due to the aberrant migration of DE/A mutant protein on the native gel (**Figure 7F**), which may reflect unusual morphology of the mutant protein in comparison with the full-length protein. Furthermore, the DE/A mutant protein, expressed in 293T cells, was highly enriched in the Triton-insoluble fractions (**Figure 7G**). Washing the triton-insoluble pellet with increasing salt indicated that the full-length Claspin dissociated from the pellet at 100 mM NaCl, whereas the DE/A mutant Claspin stayed in the pellet even at 400 mM NaCl (**Figure 7H**). This result indicates higher affinity of the DE/A mutant to chromatin. These results are consistent with the idea that the DE/A mutant binds to DNA with higher affinity and suggest that the acidic residues in the acidic patch somehow inhibit the DNA binding activity of the N-terminal DNA binding segment.

IV-3. Interaction between the N-terminal Claspin and DNA or PCNA is inhibited by the C-terminal acidic patch.

Above results suggest a possibility that the C-terminal segment regulates the interaction of Claspin with DNA and PCNA. To test this idea, C-terminal polypeptides (#13, aa897~1209; #18, aa897~1339; #27, aa897~1100; HA-tagged) were expressed and purified and mixed with the purified N-terminal polypeptide (#25, aa1~896; Flag-tagged). Immunoprecipitates with anti-Flag antibody contained HA-tagged C-terminal polypeptides (**Figure 8A**), indicating that N-terminal and C-terminal polypeptides of Claspin interact with each other. I noted that the binding of #18 to the N-terminal polypeptide was less efficient than that of #13 or #27, a situation similar to the interaction with Cdc7 (**Figure 1C**, lanes 9-11). The presence of basic residues in aa1210-1339 may interfere with the association of AP with the N-terminal segment. On the other hand, the fragment (aa897~1209; same as #13) containing DE/A substitution did not bind to the N-terminal fragment (**Figure 8B**). Furthermore, addition of the C-terminal polypeptide (#13, aa897~1209) inhibited the DNA binding activity of the N-terminal polypeptide (**Figure 8C**). These results directly indicate that the intramolecular interaction between the C-terminal acidic patch and the N-terminal segments containing the DNA binding domain regulates the DNA binding activity of Claspin.

I conducted limited trypsin digestion of the wild-type and DE/A mutant Claspin to investigate the effect of the mutation on conformation of the protein. The purified proteins were digested by varied concentrations of trypsin, and were analyzed on SDS-PAGE. The polypeptides were detected by western blotting with anti-Flag antibody (detect C-terminus derived polypeptides) to simplify the interpretation of the results. On the wild-type protein, a band of 120 kDa (arrow #1) was detected, whereas two bands of 130 kDa (arrowhead #2) and 110 kDa (arrowhead #3) were detected on DE/A (**Figure 8E**). The 110 KD band on DE/A and the 120 kDa band on the wild-type are probably derived from the same digestion. The reduced DE residue content in the DE/A mutant causes the DE/A-derived polypeptides to migrate faster. The 130 kDa fragment on DE/A would be generated by digestion at a further N-terminus proximal location. The lack of this digestion in the wild-type may be consistent with the presence of the intramolecular interaction which may inhibit the access of trypsin to the interacting segment (NTAPI [N-terminal AP interacting domain]; **Figure 8F**).

Apparent higher affinity of the N-terminal polypeptide to PCNA (**Figure 2C**) suggests a possibility that the C-terminal segment may interfere also with the PCNA binding. Consistent with this speculation, expression of the C-terminal polypeptide (aa 897~1100) decreased the binding of PCNA to the N-terminal polypeptide (**Figure 8D**; compare lane 6 with lanes 7 and 8). Thus, the C-terminal acidic patch inhibits DNA

binding and PCNA binding activities of Claspin through directly interacting with the N-terminal segment.

IV-4. Phosphorylation of Claspin with Cdc7 kinase is important for its functions and inhibits interaction between the C-terminal and N-terminal segments of Claspin.

I previously reported that Cdc7 is required for HU-mediated checkpoint responses and Claspin is phosphorylated in a manner dependent on Cdc7 kinase (Kim et al., 2008). Later, use of Cdc7 inhibitors confirmed this finding (Rainey et al., 2013).

In order to examine the roles of Cdc7-mediated phosphorylation of Claspin, I tried to localize the phosphorylation sites on Claspin. Since the target sequences of Cdc7 kinase are rather promiscuous except that it favors acidic environment near the phosphorylation sites (Masai et al. 2000, 2006; Sheu and Stillman, 2006; Sasanuma et al., 2008; Wan et al., 2008), they are generally hard to predict. It is also often the case that multiple residues are phosphorylated by Cdc7 and the phosphorylation of these residues redundantly contributes to the physiological effect (Masai et al. 2006). Indeed, 9 potential Cdc7-mediated phosphorylation sites *in vitro* have been reported on Claspin (Rainey et al., 2013). Thus, I have mutated clusters of serine/threonine residues in the C-terminal segment near the acidic patch. ST19A, ST5A and ST27A mutants carry alanine substitutions of all the serine and threonine residues within the segments aa1219~1337, aa1121~1218 and aa903~1120, respectively (**Figure 9A**). These phosphorylation site mutant Claspin proteins were purified and were used as substrates for *in vitro* phosphorylation assays with Cdc7 kinase. Although ST27A mutant was less efficiently phosphorylated by Cdc7 than the wild-type was, ST5A or AT19A was phosphorylated to similar extent as the wild-type. These mutant Claspin were also analyzed for mobility-shift induced by coexpression with Cdc7-ASK (**Figure 9B**). Mobility-shift, observed in the wild-type Claspin after overexpression of Cdc7-ASK, was reduced with ST27A mutant, but was not appreciably affected with ST5A or AT19A. These results show that aa903~1120 contain major phosphorylation sites, but that other segments are also phosphorylated by Cdc7.

In contrast, Cdc7 did not phosphorylate DE/A mutant or the internal deletion mutants lacking the acidic patch (Cdel1 and Cdel7; **Figure 9A**, lanes 3, 4, 13, 14, 19 and 20), and no mobility-shift was observed on the FE/A mutant after coexpression with Cdc7-ASK (**Figure 9B**, lanes 4 and 5). These results indicate that the interaction with Cdc7-ASK through the acidic patch is essential for phosphorylation of Claspin by this kinase, and multiple sites are phosphorylated.

The ST27A and ST27E mutants were expressed in 293T cells and pull-down assays were conducted, as above. They interacted with TopBP1, Cdc45 and Cdc7.

ST27E interacted with Cdc7 more strongly than the wild-type Claspin did (**Figure 9C,** lanes 9 and 10). On the other hand, interaction with Chk1 was significantly reduced in ST27A but not in ST27E or in ST5A or AT19A (**Figure 4B**, lane 8 and data not shown), suggesting that phosphorylation of aa903~1120 by Cdc7 is important for interaction with Chk1. This is expected since the mutations include Chk1BDs, which contain the serine/ threonine residues whose phosphorylation is known to be required for Chlk1 binding (Kumagai and Dunphy, 2003; Chini and Chen, 2006).

I then examined the effect of Cdc7-mediated phosphorylation on the interaction between the N-terminal and C-terminal segments of Cdc7. The C-terminal polypeptides (#13, aa897~1209) and the N-terminal polypeptide (#25, aa1~896) were coexpressed in 293T cells with or without Cdc7-ASK, and pulled down with Flag-tag attached to #25. The level of the coimmunoprecipitated #13 polypeptide was significantly reduced in the presence of Cdc7-ASK (**Figure 9D**; compare lanes 9 and 12). I speculate that Cdc7-ASK is recruited to #13(C)-#25(N) complex, phosphorylates N-terminal segments, resulting in the destabilization of the complex. These results indicate that Cdc7 phosphorylates Claspin and inhibits interaction between its N-terminal and C-terminal segments, which may result in increased DNA and PCNA binding.

Section V. Claspin plays essential role during the recovery from the quiescent (resting) state.

In order to examine to role of Claspin in other phases of the cell cycle, I synchronized the flox/- MEF cells at G0 phase by culturing the cells in 0.1% serum medium (low serum medium) and released the cells into growth by addition of the serum to 15%. Amazingly, almost all of the cells died within 48 hrs after the release (**Figure 10A**). Next I analyzed the cell cycle of those cells released from G0 phase for 0, 14, 18, 22, 26 and 48 hr by flow cytometry. In Claspin deficient MEFs, S phase entry was delayed and the level of DNA synthesis was low (**Figure 10B and 10C)**. I then analyzed Claspin KO MEF cells stably expressing DE/A or PIP mutant under the same condition. DE/A mutant gave results similar to Claspin KO MEF cells, not being able to enter the first S phase, while PIP mutants could at least proceed through the first S phase, although they appeared to halt the cell cycle at G2/M phase (**Figure 10D)**. Both mutant cells died in 48 hrs after release.

It has been reported that the mTOR signaling pathway plays important roles in cells released from the quiescent (resting) state (Zoncu, 2011). To examine the effect of Claspin on the mTOR signaling pathway, I checked factors involved in the mTOR signaling pathway in Claspin KO or control MEF cells released form quiescent state. The expression of phosphorylated Akt(S473) and 4EBP1(T37/46), downstream of Akt,

and PDK1(S241), upstream of Akt, in Claspin deficient MEF cells decreased at 22 hrs compared to the wild-type MEF cells (**Figure 10E)**. Phosphorylation of the p70 S6k(T389), downstream of Akt, also decreased in 14 hrs after release (**Figure 10E)**. These factors promote cell growth and proliferation in a manner dependent on the nutrition. Furthermore, p53, a factor promoting apoptosis, increased after releasing from quiescent state (**Figure 10F)**. To examine the role of Claspin in release from the quiescent state in detail, p53, an mTOR signaling pathway protein, was inhibited by pifithrin-α, an inhibitor of p53. In the presence of pifithrin-α, the survival rate increased after release from quiescent state in Claspin deficient MEF cells. These results suggest that Claspin plays an important role in cell cycle entry from the quiescent state by modulating the mTOR pathway.

DISCUSSION

Claspin/ Mrc1 is a conserved factor that plays crucial roles in replication stress checkpoint signaling as a signal-mediator protein. Recent findings indicate that it plays roles also during unperturbed DNA replication process as well. Claspin has been reported to interact with many checkpoint- and replication-related factors (Lee et al., 2005; Serçin and Kemp, 2010; Gold and Dunphy; 2010; Uno et al., 2011; Hao et al., 2015). It was also reported that Mrc1/ Claspin is required for efficient replication fork progression (Lee et al, 2004; Petermann et al 2008). However, its precise role during normal DNA replication process has been largely elusive.

In my dissertation, I set my goals as follows. 1) Define the functional domains of human Claspin, and clarify the domains that interact with various replication/ checkpoint factors, 2) Evaluate the functions of these domains by generating mutants and assaying their functionalities in vivo, 3) Biochemically characterize each mutant Caspin protein and clarify the mechanisms of Claspin actions during DNA replication under both normal and stressed conditions, 4) Search for novel functions of Claspin under varied physiological conditions.

Toward these goals, I generated Claspin KO mice and cells for the use of functional assays of various mutant proteins. I also took advantage of highly efficient transient expression system utilizing 293T (human embryonic kidney) cells for the identification of interacting molecules as well as for rapid purification of various mutant forms of Claspin.

Acidic patch of Claspin recruits Cdc7 to chromatin

One of the novel and important findings in my dissertation is the discovery of a crucial role of AP (acidic patch) segment of Claspin for efficient initiation of DNA replication. I have shown that AP is crucial for Claspin functions under both normal and replication-stress conditions. I have discovered that AP binds to Cdc7 kinase both in vivo and in vitro. Previously, a docking sequence for Cdc7 kinase (DDD; aa175-333) was identified on budding yeast Mcm4, which is a crucial substrate of Cdc7 kinase *in vivo* (Sheu and Stillman, 2006). The human Mcm4 sequence corresponding to DDD has about 30% identity. The human counterpart sequence did not interact with Cdc7 (Yang and Masai, unpublished data). It was also recently reported in budding yeast that Tof1 and Csm3 (Tim-Tipin in higher eukaryotes) recruit Cdc7 to the replisome during the course of premieotic DNA replication, enabling it to phosphorylate a key substrate for DSB formation (Murakami and Keeney, 2014). Thus, Cdc7 may be recruited to its substrates through varied “recruiter” molecules for efficient and timely phosphorylation events. During the initiation of DNA replication in higher eukaryotes, Claspin may

assume this role. Additionally, recruitment of Cdc7 by Claspin may add another layer of complexity to regulation of origin regulation.

AP of Claspin as a recruiter of Cdc7 kinase

I demonstrate here that the Claspin plays crucial role in recruiting Cdc7 kinase through AP, and that this interaction of Cdc7 with AP is required for efficient phosphorylation of Mcm proteins.

Cdc7 is a conserved kinase and it is well established that Cdc7-mediated phosphorylation of Mcm is a crucial event for initiation of DNA replication (Francis et al., 2009; Labib, 2010; Masai et al., 2000, 2002, 2006; Sheu and Stillman, 2006 and 2010). Cdc7-mediated phosphorylation triggers the assembly of initiation complex; namely "fires" the replication origin. Thus, selective recruitment of Cdc7 kinase to preformed pre-RCs could be a major determinant for replication timing regulation. Here, I show that AP is required and sufficient for binding to Cdc7 kinase. AP is rich in acidic residues to which Cdc7 kinase generally has affinity. I further showed that the DE/A mutant of Claspin which cannot bind to Cdc7 is defective in phosphorylation of not only Claspin itself but also crucial substrates of Cdc7, Mcm subunits.

It was previously reported that Cdc7-Drf1 (a second subunit of ASK; ASKL1) binds to Claspin in *Xenopus* egg extracts (Yanow et al., 2003). This binding was reported to be mediated by a segment encompassing one of the Chk1 binding boxes previously identified (Gold and Dunphy, 2010). AP that was identified in this work does not overlap with this sequence, although the critical segment that may be phosphorylated by Cdc7 overlaps with it (aa903~1120; **Figure 9A**). It is of interest that Claspin may be present at origins so that Cdc7 could be recruited and the pre-RCs are converted to initiation complex and eventually to active replication forks. Indeed, it was reported before in fission yeast that Mrc1 may mark the early-firing origins by binding to them in the pre-initiation stage (Hayano et al., 2010).

The DE/A mutant is defective in interaction with Cdc45, TopBP1 and other replication factors. This could be secondary effect of loss of Cdc7-mediated critical phosphorylation of the Mcm complex, which would be required for subsequent recruitment of Cdc45 and other replisome factors. Among the replisome components, PCNA and Tim/Tipin are distinct in that they interact with the N-terminal segment of Claspin, and their interactions are not affected by the DE/A mutation.

AP of Claspin regulates DNA and PCNA bindings of Claspin through intramolecular interaction with the N-terminal segment of Claspin

The second role of AP is mediated by its interaction with the N-terminal segment of Claspin. I have found that DNA binding activity of Claspin is significantly increased

upon truncation of the C-terminal segment. Further analyses showed that a mutant Claspin carrying the internal deletion of the AP segment bound to DNA with high affinity. Similarly, the DE/A mutant also bound to DNA with much higher affinity than the wild-type Claspin did. The significantly different gel shift patterns with the DE/A mutant may be due to its morphological versatility.

Binding of PCNA to Claspin was also suppressed by the C-terminal segment containing AP. Thus, AP regulates the DNA and PCNA binding activities through intramolecular interaction with an N-terminal segment. This interaction is probably mediated by ionic interaction between the acidic segment and the basic segment found in the N-terminal segment of Claspin (see **Figure 1C**). This speculation is supported by the loss of interaction and suppression by DE/A mutation in the AP segment.

Cdc7-mediated phosphorylation may regulate DNA and PCNA bindings of Claspin

It has been reported that Claspin is one of the substrates of Cdc7 kinase (Kim et al., 2008; Rainey et al., 2013). *In vitro* Cdc7 kinase assays with various polypeptides derived from Claspin indicated that AP is required for efficient phosphorylation of Claspin (**Figure 9A**). This is due to loss of recruitment of Cdc7 kinase, since DE/A mutation similarly led to complete loss of phosphorylation, whereas ST/A substitutions in selective segments surrounding AP resulted in only partial loss of phosphorylation. *In vivo*, Cdc7-induced mobility-shift (caused by phosphorylation) of Claspin is also almost completely lost in the DE/A mutant (**Figure 9B**).

Claspin recruits Cdc7 at the onset of S phase through AP and the recruited Cdc7 phosphorylates Mcm as well as Claspin. This phosphorylation may trigger the dissociation of the N-terminal segment of Claspin from the AP. Indeed, Cdc7-ASK downreguated the N-C interaction *in vivo*. Thus, Cdc7-ASK may activate DNA and PCNA bindings of Claspin through AP-mediated phosphorylation.

Differential mechanism of origin activation in cancer and normal cells.

Requirement of AP of Claspin for efficient phosphorylation of Mcm was observed not only in mouse embryonic fibroblast cells but also in Claspin-depleted normal human fibroblast cells. In contrast, loss of Cdc7-mediated Mcm phosphorylation was not observed in Claspin-depleted cancer cells, including HeLa and U2OS cells, consistent with previous reports (Izawa et al., 2011). This could be related to the overproduction of Cdc7-ASK in cancer cells (Kim et al., 2008; Ito et al., 2008; Swords et al., 2010; Cheng et al., 2013), which may overcome the requirement of Claspin for recruitment of Cdc7 kinase to critical substrates. Alternatively, there may be intrinsic differences in assembly and activation of pre-RCs between normal and cancer cells. It is interesting

to speculate that Claspin serves as a safeguard for regulated initiation of DNA replication in normal cells. In cancer cells, this protection is somehow overrided, which would contribute to their unregulated growth.

Role of AP and Cdc7 in replication stress checkpoint

The DE/A mutant is defective in checkpoint activation as indicated by loss of induced phosphorylation of Chk1 upon addition of HU. Indeed, it failed to interact with Chk1. This is intriguing since the CKBDs (Chk1-binding domain) are intact in the DE/A mutant. It has been suggested that phosphorylation of CKBD is required for interaction between Chk1 and Claspin (Kumagai and Dunphy, 2003; Chini and Chen, 2006), but the kinase responsible for this phosphorylation has not been clear (Bennett et al., 2008). Our results strongly suggest that Cdc7 is responsible for the phosphorylation of CKBD required for interaction between Claspin and Chk1 kinase. This is consistent with previous report that Claspin is phosphorylated in a manner dependent on Cdc7 upon exposure to replication stress and that Cdc7 is required for replication stress induced checkpoint activation (Kim et al, 2008). Cdc7 phosphorylates not only the C-terminal segment but also the basic N-terminal segment of Claspin (Raoney et al, 2013) and this phosphorylation may decrease the ionic N-C interaction.

Identification of the PIP motif on Claspin essential for interaction with PCNA

Although the interaction between Claspin and PCNA has been known (Brondello et al., 2007), the PIP motif responsible for this interaction has not been known. I have identified a PIP motif that is required for Claspin-PCNA interaction. The sequence is slightly deviated from the consensus sequence but amino acid replacements at the conserved residues resulted in complete loss of PCNA binding both *in vivo* and *in vitro*.

Binding of PCNA to Claspin is required for both normal S phase progression and replication checkpoint activation. It is of interest to note that Claspin associates with Tim-Tipin through its N-terminal segment. Claspin-Tim was reported to be required for HU/ UV-induced PCNA ubiquitination (Yang et al., 2008). Thus, Claspin may be a platform on which PCNA is ubiquitinated. During the normal course of replication, PCNA clamp may provide a link between the replisome and replication stress surveillance machinery. A similar role of PCNA was suggested for APE2, an adaptor molecule which links oxidative stress to Chk1 activation (Willis et al., 2013).

Claspin plays novel and essential roles during the release from the quiescent state into cell cycle.

I found that Claspin KO MEF cells could not enter S phase from the serum starvation induced quiescent state and eventually died. The DE/A mutant could not rescue S phase

entry under the same condition, suggesting that acidic patch, possibly recruitment of Cdc7 kinase is essential for S phase entry from the resting state. In contrast, the PIP mutant could rescue the S phase entry, although it appeared to be arrested G2/M phase. This suggests that Cdc7 recruitment through AP is crucial for initiation of DNA replication in release from the quiescence. On the other hand, PIP mutant showed normal entry into S phase, while it apparently stopped the cell cycle at G2/M phase.

The regulation mTOR activity by growth factors is mediated by the PI3K/Akt signaling pathway (Hay and Sonenberg, 2004). I found that many mTOR signaling pathway-related factors (P-p70 S6K T389; p-PDK1 S241 etc.) are expressed at al low level in Claspin KO cells. Notably, Mcm is expressed at a much reduced level in Claspin KO cells during the release. It is of interest that Claspin may function in a novel stress response pathway, which may be triggered by serum starvation. Claspin, as a signal mediator, may play a role in induction of PI3K/Akt signaling pathway for entry into S phase and subsequent growth stimulation. AP may be required for this pathway, possibly by recruiting some other factors.

Conclusions

In summary, I report here a novel role of Claspin as a recruiter of Cdc7 kinase and a crucial role of its C-terminal acidic patch for the recruitment. AP serves as a binding pad for Cdc7 kinase and also regulates its DNA and PCNA bindings through intramolecular interaction with the N-terminal segment. The recruited Cdc7 not only phosphorylates critical substrates for initiation (Mcm) but also phosphorylates Claspin to disrupt the intramolecular interaction which would activate DNA and PCNA bindings. Recruitment of Cdc7 though AP for efficient phosphorylation of Mcm may be bypassed in cancer cells. Thus, Claspin may provide additional safeguard for initiation of DNA replication specifically in normal cells. Claspin may play unknown, essential functions during cell cycle entry from the quiescent state, and this process appears to require AP, but not PIP.

It was recently reported that TopBP1 is regulated by intramolecular interaction, which is disrupted by acetylation, causing its BRCT to be recognized by other phosphorylated proteins (Rad9 or Treslin; Liu et al., 2014). Intramolecular interaction in Rad9 was also reported to regulate its binding to DNA and TopBP1 (Takeishi et al., 2015). Thus, regulation by intramolecular interaction may be a common mechanism to switch on/off functions of proteins.

Future perspectives

On the basis of the new findings on the functions of Claspin described in this dissertation, I would like to propose the following experiments to further clarify the mechanisms of Claspin actions and its novel functions.

1. Generate tissue-specific Claspin knockout mice to explore novel in vivo roles of Claspin
2. Generate Claspin(DE/A)-Cdc7 fusion protein to see if AP functions can be bypassed by fusing Cdc7.
3. Construct YFP-Claspin-CFP sensor molecule to analyze the AP-N-terminal segment interactions by the use of FRET assays.
4. Construct CKBD SE (phosphor-mimetic) mutant in the DE/A background to see if checkpoint defect can be specifically restored by the SE mutation.
5. Overexpression of Cdc7-ASK in normal cells and downregulation of Cdc7-ASK in cancer cells; to determine if the level of Cdc7-ASK determines the requirement of AP for Cdc7-mediated phosphorylation of Mcm.
6. Search for novel AP-specific interactors of Claspin during the release from the quiescence to clarify the exact role of Claspin in this process
7. Effect of various stress in Claspin KO cells: search for novel roles of Claspin in other stress response reactions.

ACKNOWLEDGEMENTS

First of all, I would like to thank my advisor Dr. Masai continued to support my research, because of his enthusiasm and wealth of knowledge, by his guidance to help me complete the research and writing of this paper.

I would also like to thank all the partners in the laboratory. They assist me in the experiment as well as difficulties in life.

Finally, I want to thank my family. Parents gave me the economic and moral support. And my wife supported and accompanied me through my PhD life.

REFERENCES

Aparicio, T., Guillou, E., Coloma, J., Montoya, G., Méndez, J., 2009. The human GINS complex associates with Cdc45 and MCM and is essential for DNA replication. Nucleic Acids Res. 37, 2087–2095.

Bassermann, F., Frescas, D., Guardavaccaro, D., Busino, L., Peschiaroli, A., Pagano, M., 2008. The Cdc14B-Cdh1-Plk1 Axis Controls the G2 DNA-Damage-Response Checkpoint. Cell 134, 256–267.

Bennett, L.N., Larkin, C., Gillespie, D.A., and Clarke, P.R. (2008). Claspin is phosphorylated in the Chk1-binding domain by a kinase distinct from Chk1. Biochem. Biophys. Res. Commun. *369*, 973–976.

Brondello, J.M., Ducommun, B., Fernandez, A., and Lamb, N.J. (2007). Linking PCNA-dependent replication and ATR by human Claspin. Biochem. Biophys. Res. Commun. *354*, 1028–1033.

Cheng, A.N., Jiang, S.S., Fan, C.C., Lo, Y.K., Kuo, C.Y., Chen, C.H., Liu, Y.L., Lee, C.C., Chen, W.S., Huang, T.S., et al. (2013). Increased Cdc7 expression is a marker of oral squamous cell carcinoma and overexpression of Cdc7 contributes to the resistance to DNA-damaging agents. Cancer Lett. *337*, 218–225.

Chini, C.C.S., and Chen, J. (2006). Repeated phosphopeptide motifs in human claspin are phosphorylated by Chk1 and mediate claspin function. J. Biol. Chem. *281*, 33276–33282.

Errico, A., Costanzo, V., Hunt, T., 2007. Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in Xenopus egg extracts. Proc Natl Acad Sci U S A 104, 14929–14934. doi:10.1073/pnas.0706347104

Francis, L.I., Randell, J.C., Takara, T.J., Uchima, L., and Bell, S.P. (2009). Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. Genes Dev. *23*, 643-654.

Gao, D., Inuzuka, H., Korenjak, M., Tseng, A., Wu, T., Wan, L., Kirschner, M., Dyson, N., Wei, W., 2009. Cdh1 regulates cell cycle through modulating the claspin/Chk1 and the Rb/E2F1 pathways. Mol. Biol. Cell 20, 3305–16.

Gambus, A., Jones, R.C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R.D., and Labib, K. (2006). GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nat. Cell Biol. *8*, 358–366.Gambus, A., Jones, R.C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R.D., and Labib, K. (2006). GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nat. Cell Biol. *8*, 358–366.

Faustrup, H., Bekker-Jensen, S., Bartek, J., Lukas, J., Mailand, N., 2009. USP7 counteracts SCFbetaTrCP- but not APCCdh1-mediated proteolysis of Claspin. J. Cell Biol. 184, 13–19.

Francis, L.I., Randell, J.C.W., Takara, T.J., Uchima, L., Bell, S.P., 2009. Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. Genes Dev. 23, 643–654.

Gold, D.A., and Dunphy, W.G. (2010). Drf1-dependent kinase interacts with claspin through a conserved protein motif. J. Biol. Chem. *285*, 12638–12646.

Hae, Y.Y., Jeong, S.Y., and Dunphy, W.G. (2006). Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures. Genes Dev. *20*, 772–783.

Hao, J., de Renty, C., Li, Y., Xiao, H., Kemp, M.G., Han, Z., DePamphilis, M.L., and Zhu, W. (2015). And-1 coordinates with Claspin for efficient Chk1 activation in response to replication stress. EMBO J. *34*, 1845 – 1985.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. 18, 1926–1945.

Im, J.-S., Ki, S.-H., Farina, A., Jung, D.-S., Hurwitz, J., Lee, J.-K., 2009. Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. Proc. Natl. Acad. Sci. U. S. A. 106, 15628–15632.

Ito, S., Taniyami, C., Arai, N., and Masai, H. (2008). Cdc7 as a potential new target for cancer therapy. Drug News Perspect *21*, 481–488.

Izawa, N., Wu, W., Sato, K., Nishikawa, H., Kato, A., Boku, N., Itoh, F., and Ohta, T. (2011). HERC2 interacts with claspin and regulates DNA origin firing and replication fork progression. Cancer Res. *71*, 5621–5625.

Jiang, W., McDonald, D., Hope, T.J., Hunter, T., 1999. Mammalian Cdc7-Dbf4 protein kinase complex is essential for initiation of DNA replication. EMBO J. 18, 5703–5713.

Kim, J.M., Kakusho, N., Yamada, M., Kanoh, Y., Takemoto, N., and Masai, H. (2008). Cdc7 kinase mediates Claspin phosphorylation in DNA replication checkpoint. Oncogene *27*, 3475–3482.

Kumagai, A., and Dunphy, W.G. (2000). Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. Mol. Cell *6*, 839–849.

Kumagai, A., and Dunphy, W.G. (2003). Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1. Nat. Cell Biol. *5*, 161–165.

Kumagai, H., Sato, N., Yamada, M., Mahony, D., Seghezzi, W., Lees, E., Arai, K., Masai, H., 1999. A novel growth- and cell cycle-regulated protein, ASK, activates human Cdc7-related kinase and is essential for G1/S transition in mammalian cells. Mol Cell Biol 19, 5083–5095.

Labib, K. (2010). How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? Genes Dev. *24*,1208-1219.

Lee, J., Gold, D.A., Shevchenko, A., Shevchenko, A., and Dunphy, W.G. (2005). Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response. Mol. Biol. Cell *16*, 5269–5282.

Lee, J., Kumagai, A., and Dunphy, W.G. (2003). Claspin, a Chk1-regulatory protein, monitors DNA replication on chromatin independently of RPA, ATR, and Rad17. Mol. Cell *11*, 329–340.

Lin, S.-Y., Li, K., Stewart, G.S., and Elledge, S.J. (2004). Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation. Proc. Natl. Acad. Sci. U. S. A. *101*, 6484–6489.

Liu, T., Lin, Y.-H., Leng, W., Jung, S.Y., Zhang, H., Deng, M., Evans, D., Li, Y., Luo, K., Qin, B., et al. (2014). A divergent role of the SIRT1-TopBP1 axis in regulating metabolic checkpoint and DNA damage checkpoint. Mol. Cell *56*, 681–695.

Lindsey-Boltz, L.A., Serçin, Ö., Choi, J.H., and Sancar, A. (2009). Reconstitution of human claspin-mediated phosphorylation of Chk1 by the ATR (Ataxia Telangiectasia-mutated and Rad3-related) checkpoint kinase. J. Biol. Chem. *284*, 33107–33114.

Martín, Y., Cabrera, E., Amoedo, H., Hernández-Pérez, S., Domínguez-Kelly, R., Freire, R., 2014. USP29 controls the stability of checkpoint adaptor Claspin by deubiquitination. Oncogene 28, 1–6.

Montagnoli, a., Bosotti, R., Villa, F., Rialland, M., Brotherton, D., Mercurio, C., Berthelsen, J., Santocanale, C., (2002). Drf1, a novel regulatory subunit for human Cdc7 kinase. EMBO J. 21, 3171–3181.

Montagnoli, A., Valsasina, B., Brotherton, D., Troiani, S., Rainoldi, S., Tenca, P., Molinari, A., and Santocanale, C. (2006). Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J. Biol. Chem. *281*, 10281–10290.

Montagnoli, A., Valsasina, B., Croci, V., Menichincheri, M., Rainoldi, S., Marchesi, V., Tibolla, M., Tenca, P., Brotherton, D., Albanese, C., Patton, V., Alzani, R., Ciavolella, A., Sola, F., Molinari, A., Volpi, D., Avanzi, N., Fiorentini, F., Cattoni, M., Healy, S., Ballinari, D., Pesenti, E., Isacchi, A., Moll, J., Bensimon, A., Vanotti, E., Santocanale, C., 2008. A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. Nat. Chem. Biol. 4, 357–365.

Lou, H., Komata, M., Katou, Y., Guan, Z., Reis, C.C., Budd, M., Shirahige, K., and Campbell, J.L. (2008). Mrc1 and DNA Polymerase ε Function Together in Linking DNA Replication and the S Phase Checkpoint. Mol. Cell *32*, 106–117.

Mailand, N., Bekker-Jensen, S., Bartek, J., Lukas, J., 2006. Destruction of Claspin by SCFbetaTrCP Restrains Chk1 Activation and Facilitates Recovery from Genotoxic Stress. Mol. Cell 23, 307–318.

Mamely, I., van Vugt, M.A., Smits, V.A., Semple, J.I., Lemmens, B., Perrakis, A., Medema, R.H., Freire, R., 2006. Polo-like Kinase-1 Controls Proteasome-Dependent Degradation of Claspin during Checkpoint Recovery. Curr. Biol. 16, 1950–1955.

Masai, H., Matsui, E., You, Z., Ishimi, Y., Tamai, K., and Arai, K.I. (2000). Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a critical threonine residue of Cdc7 by Cdks. J. Biol. Chem. *275*, 29042–29052.

Masai, H., and Arai, K.-I. (2002). Cdc7 kinase complex: a key regulator in the initiation of DNA replication. J. Cell. Physiol. *190*, 287–296.

Masai, H., Taniyama, C., Ogino, K., Matsui, E., Kakusho, N., Matsumoto, S., Kim, J.M., Ishii, A., Tanaka, T., Kobayashi, T., et al. (2006). Phosphorylation of MCM4 by Cdc7 kinase facilitates its interaction with Cdc45 on the chromatin. J. Biol. Chem. *281*, 39249–39261.

Meng, Z., Capalbo, L., Glover, D.M., Dunphy, W.G., 2011. Role for casein kinase 1 in the phosphorylation of Claspin on critical residues necessary for the activation of Chk1. Mol. Biol. Cell 22, 2834–2847.

Moyer, S.E., Lewis, P.W., Botchan, M.R., 2006. Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc. Natl. Acad. Sci. U. S. A. 103, 10236–10241.

Peschiaroli, A., Dorrello, N.V., Guardavaccaro, D., Venere, M., Halazonetis, T., Sherman, N.E., Pagano, M., 2006. SCF??TrCP-Mediated Degradation of Claspin Regulates Recovery from the DNA Replication Checkpoint Response. Mol. Cell 23, 319–329.

Petermann, E., Helleday, T., and Caldecott, K.W. (2008). Claspin promotes normal replication fork rates in human cells. Mol. Biol. Cell *19*, 2373–2378.

Rainey, M.D., Harhen, B., Wang, G.N., Murphy, P. V., and Santocanale, C. (2013). Cdc7-dependent and -independent phosphorylation of Claspin in the induction of the DNA replication checkpoint. Cell Cycle *12*, 1560–1568.

Sar, F., Lindsey-Boltz, L.A., Subramanian, D., Croteau, D.L., Hutsell, S.Q., Griffith, J.D., and Sancar, A. (2004). Human claspin is a ring-shaped DNA-binding protein with high affinity to branched DNA structures. J. Biol. Chem. *279*, 39289–39295.

Sasanuma, H., Hirota, K., Fukuda, T., Kakusho, N., Kugou, K., Kawasaki, Y., Shibata, T., Masai, H., and Ohta, K. (2008). Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination. Genes Dev. *22*, 398–410.

Scorah, J., and McGowan, C.H. (2009). Claspin and Chk1 regulate replication fork stability by different mechanisms. Cell Cycle *8*, 1036–1043.

Serçin, O., and Kemp, M.G. (2011). Characterization of functional domains in human Claspin. Cell Cycle *10*, 1599–1606.

Shikata, K., Ohta, S., Yamada, K., Obuse, C., Yoshikawa, H., and Tsurimoto, T. (2001). The human homologue of fission Yeast cdc27, p66, is a component of active human DNA polymerase delta. J. Biochem. *129*, 699–708.

Sheu, Y.J., and Stillman, B. (2006) Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell. *24*,101-113.

Sheu, Y.J., and Stillman, B. (2010). The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4. Nature *463*, 113-117.

Shimmoto, M., Matsumoto, S., Odagiri, Y., Noguchi, E., Russell, P., and Masai, H. (2009). Interactions between Swi1-Swi3, Mrc1 and S phase kinase, Hsk1 may regulate cellular responses to stalled replication forks in fission yeast. Genes to Cells *14*, 669–682.

Swords, R., Mahalingam, D., O'Dwyer, M., Santocanale, C., Kelly, K., Carew, J., and Giles, F. (2010). Cdc7 kinase - a new target for drug development. Eur. J. Cancer *46*, 33–40.

Szyjka, S.J., Viggiani, C.J., and Aparicio, O.M. (2005). Mrc1 is required for normal progression of replication forks throughout chromatin in S. cerevisiae. Mol. Cell *19*, 691–697.

Takeishi, Y., Iwaya-Omi, R., Ohashi, E., and Tsurimoto, T. (2015). Intramolecular Binding of the Rad9 C Terminus in the Checkpoint Clamp Rad9-Hus1-Rad1 Is Closely Linked with Its DNA Binding. J. Biol. Chem. 290, 19923–19932.

Tanaka, H., Kubota, Y., Tsujimura, T., Kumano, M., Masai, H., Takisawa, H., 2009. Replisome progression complex links DNA replication to sister chromatid cohesion in Xenopus egg extracts. Genes to Cells 14, 949–963. doi:10.1111/j.1365-2443.2009.01322.x

Tanaka, K., 2010. Multiple functions of the S-phase checkpoint mediator. Biosci. Biotechnol. Biochem. 74, 2367–2373. doi:10.1271/bbb.100583

Uno, S., and Masai, H. (2011). Efficient expression and purification of human replication fork-stabilizing factor, Claspin, from mammalian cells: DNA-binding activity and novel protein interactions. Genes Cells *16*, 842–856.

Uno, S., You, Z., and Masai, H. (2012) Purification of replication factors using insect and mammalian cell expression systems. Methods. *57*, 214-221.

Wan, L., Niu, H., Futcher, B., Zhang, C., Shokat, K.M., Boulton, S.J., and Hollingsworth, N.M. (2008). Cdc28-Clb5 (CDK-S) and Cdc7-Dbf4 (DDK) collaborate to initiate meiotic recombination in yeast. Genes Dev. *22*, 386–397.

Yang, X.H., Shiotani, B., Classon, M., and Zou, L. (2008). Chk1 and claspin potentiate PCNA ubiquitination. Genes Dev. *22*, 1147–1152.

Yanow, S.K., Gold, D.A., Yoo, H.Y., and Dunphy, W.G. (2003). Xenopus Drf1, a

Regulator of Cdc7, Displays Checkpoint-dependent Accumulation on Chromatin during an S-phase Arrest. J. Biol. Chem. *278*, 41083–41092.

Yoshizawa-Sugata, N., and Masai, H. 2007. Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. J. Biol. Chem. *282*, 2729–2740.

You, Z., Ishimi, Y., Mizuno, T., Sugasawa, K., Hanaoka, F., and Masai, H. 2003. Thymine-rich single-stranded DNA activates Mcm4/6/7 helicase on Y-fork and bubble-like substrates. EMBO J. *22*, 6148–6160.

Zhang, D., Zaugg, K., Mak, T.W., Elledge, S.J., 2006. A Role for the Deubiquitinating Enzyme USP28 in Control of the DNA-Damage Response. Cell 126, 529–542.

Zhao, H., and Russell, P. (2004). DNA binding domain in the replication checkpoint protein Mrc1 of Schizosaccharomyces pombe. J. Biol. Chem. *279*, 53023–53027.

Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol. 12(1), 21–35. - See more at: http://www.cellsignal.com/contents/science-pathway-research-pi3k-akt-signaling-resources/mtor-signaling-pathway/pathways-mtor-signaling#sthash.2L6WWGtS.dpuf

LEGENDS TO FIGURES

Figure 1. Interaction of various replication factors with truncated or mutant forms of Claspin. (**A**) Schematic diagram of various truncated or mutant derivatives of *Claspin* generated. BP1 and BP2: Basic patch1 and 2. CKBD: Chk1 binding domain. (**B**) Interaction was examined as in (A). Mutant *Claspin* polypeptides tagged with 3x Flag at the C-termini were expressed in 293T cells and pulled down with M2 Flag beads (Flag IP). Co-pulled down proteins were analyzed by western blotting with indicated antibodies. Asterisk, IgG; black arrowheads, full length or mutant *Claspin* polypeptides pulled down by Flag antibody. (**C**) Upper: Schematic diagram of truncated *Claspin* generated. Lower: Loss of interaction with Cdc7 in Cdel1 and Cdel6 mutants. Mutant *Claspin* polypeptides tagged with 3x Flag at the C-termini were expressed in 293T cells and pulled down with M2 Flag beads (Flag IP). Co-pulled down proteins were analyzed by western blotting with indicated antibodies. (**D**) Amino acid sequences of aa988~1086 (acidic patch) of Claspin. Acidic amino acids are underlined. In DE/A mutant, all these residues were replaced with alanine.

Figure 2. PIP-dependent binding of PCNA to Claspin.
(**A**) Comparison of the putative PIP box sequences of Claspin homologues from various species. Conserved amino acids are in bold. (**B**) Schematic diagram of the full length and #2 fragment of Claspin (left). Gray and black boxes indicate the wild-type and mutant PIP, respectively. (**C**)The wild-type (WT) and PIP mutant (PIP) proteins expressed in 293T cells were pulled down with M2 Flag beads, and analyzed by western blotting using anti-Flag or anti-PCNA antibody (right). Arrowheads indicate the Claspin full-length and #2 polypeptides. (**D**) Wild-type (WT) and mutant Claspin indicated were expressed in 293T cells and treated with or without 2mM HU for 2 hours and were pulled down with M2 Flag beads. Associated proteins were analyzed by western blotting with indicated antibodies. (**E**) Interaction between purified Claspin polypeptides and PCNA. PCNA, 1.2pmole or 3.0pmol; full length or #2 Claspin polypeptide, 1.2pmole each. The materials pulled down by Dynabeads-conjugated anti-Flag antibody were analyzed by western blotting using anti-Flag or anti-PCNA antibody. Asterisk, IgG.

Figure 3. Generation of *Claspin*-deficient mice. (**A**) Upper, Schematic representation of wild type, flox and knock-out alleles of mouse *Claspin.* RI: *Eco*RI site. EV: *Eco*RV site. The numbered vertical bars indicate the exons. Lower, Southern blot analysis of genomic DNA from the wild-type ES clone and G418-resistant *Claspin*+/- heterozygous ES clone. Neo, neomycin-resistance gene. (**B**) Characterization of

Claspin knockout embryos. Upper, locations of primers used for genotyping of mutant mice. Lower, the table showing the numbers of embryos of each genotype obtained from crosses of heterozygous mice. The right panel shows the genotyping by PCR on genomic DNA isolated from heterozygous, knock-out (KO) or wild type (WT) cells. (**C**) Generation of *Claspin* knockout MEF cells. Upper: Cre-loxP induced knock-out of *Claspin*. PCR analysis of genomic DNA of MEF cells (f/w and f/-) non-treated or infected with Ad-Cre for 48h. Lower: MEF cells with indicated genotype was infected with Ad-GFP or Ad-Cre or non-treated for 48h, and whole cells extracts were analyzed by western blotting. (**D**) BrdU incorporation in *Claspin* knockout MEF cells. Left: MEF cells *(w/w, w/f* and *f/-*) were infected with Ad-Cre and incubated for 0, 24 and 48h. Before harvest, BrdU was added for 20 min. BrdU incorporation was analyzed by FACS. Right: Quantification of the result. FITC intensity of the each cell population is shown.

Figure 4. The C-terminal acidic patch is crucial for Claspin functions. (**A**) Purified wild-type or DE/A mutant Claspin was mixed with purified Cdc7-ASK complex and pulled down by Dynabeads conjugated with anti-Claspin antibody. Immunoprecipated materials were analyzed by western blotting, as shown. (**B**) Upper: Schematic diagram of mutant *Claspin* generated. BP1 and BP2: Basic patch1 and 2. CKBD: Chk1 binding domain. Black boxes show the mutated segments. Lower: Wild-type (WT) and mutant Claspin indicated were expressed in 293T cells and were pulled down with M2 Flag beads. Associated proteins were analyzed by western blotting with indicated antibodies.

Figure 5. The C-terminal acidic patch and N-terminal PIP of Claspin play roles in replication and checkpoint functions.

(**A**) Stable clones of WT, DE/A and PIP-expressing f/- MEFs were infected with Ad-Cre or non-treated for 48h and 1.0 x 10^5 cells were passaged to new plates. The cells were then harvested at 24h (Day 3 from Ad-Cre infection) and 48h (Day 4 from Ad-Cre infection) after the passage and cell numbers were counted. (**B**) Claspin (f/-) MEF cells stably expressing the wild-type, DE/A or PIP mutant Claspin were treated with Ad-Cre for 2 days and then passaged. At 3 days after passage, BrdU was added for 20 min before harvest, and cells were analyzed for BrdU incorporation by FACS. (**C**) Proteins indicated were examined by western blotting in the whole cell extracts of the cells used in (**A**). 2mM HU was added during the last 3h before the harvest (right) or non-treated (left).

Figure 6. The acidic patch is required for phosphorylation of Mcm proteins, a target of Cdc7 kinase

(**A**) Chromatin-enriched fractions of Ad-Cre treated or non-treated MEF (Claspin f/-)

stably expressing the wild-type or DE/A mutant Claspin were analyzed by western blotting with indicated antibodies. (**B**) NHDF and U2OS cells were mock-transfected or transfected with Claspin siRNA and Triton-soluble and -insoluble fractions were analyzed by western blotting using the indicated antibodies.

Figure 7. DNA binding activity of Claspin is regulated by the C-terminal acidic patch. (**A**) Schematic diagram of mutant *Claspin* containing internal deletions within the C-terminal segment. + marks indicate the relative strength of DNA binding. (**B**) Each protein was expressed in 293T cells and purified. 2.5μg of the purified protein fractions were run on 5-20% gradient gel, and stained with CBB. (**C**) Gel shift assays of mutant Claspin proteins on Y-fork DNA. Y-fork DNA, 20fmole; protein added, 0.5pmole and 1.0pmole. The reason why Cdel4 lacks DNA binding activity is not clear. (**D**) Filter binding assay of Cdel1 and DE/A mutant Claspin proteins on Y-fork DNA. Y-fork DNA, 80fmole; protein added, 0.5 and 1.0pmole. **(E)** Gel-shift assay of DE/A mutant. Y-fork DNA, 20fmole; protein added, 1.0pmole. (**F**) 800ng purified WT and DE/A proteins applied to 5~20% native gradient PAGE and stained with CBB. (**G**) **2**93T cells were transfected with expression plasmid DNAs for the wild-type, DE/A, PIP, ST27A, ST5A and ST19A Claspin. Triton-soluble and –insoluble fractions were analyzed by western blotting using the indicated antibodies. **(H)** Claspin (*f*/-) MEF cells stably expressing the Flag-tagged wild-type or DE/A mutant Claspin were fractionated into Triton-soluble and –insoluble fractions at different concentrations of NaCl and were analyzed by western blotting using the indicated antibodies.

Figure 8. The C-terminal acidic patch interacts with the N-terminal segments of Claspin and inhibits its binding to DNA or PCNA. (**A**) Schematic diagram of the Claspin polypeptides expressed and attached tags (upper). The Flag-tagged N-terminal segment (#25) was coexpressed with the C-terminal polypeptides and pulled down with M2 Flag beads, and analyzed by western blotting using anti-Flag or anti-HA antibody (lower). (**B**) The Flag-tagged N-terminal segment (#25) was coexpressed with the wild-type or DE/A mutant C-terminal polypeptide #13 and pulled down with M2 Flag beads, and analyzed by western blotting using anti-HA antibody. Note that the wild-type #13 polypeptide migrates anomalously on PAGE due to the presence of acidic residues. (**C**) The C-terminal acidic patch polypeptide (#13; 1.5pmole and 3pmole) was added to the gel shift reaction containing labeled Y-fork and the N-terminal polypeptide #2 (1.5pmole). The graph shows the quantification of the binding by calculating the ratio of bound radioactivity to the total radioactivity (bound + unbound). (**D**) The Flag-tagged N-terminal segment (#25) was coexpressed with the C-terminal polypeptides, pulled down with M2 Flag beads, and associated PCNA was analyzed by western

blotting. Arrowheads indicate the expressed proteins. *, IgG. (**E**) Wild type or DE/A mutant of Claspin was digested by trypsin and analyzed by western blotting with anti-Flag antibody. Different exposures are shown. Arrows indicate specifically cleaved polypeptides. (**F**) The schematic drawing of the predicted digestion patterns of the wild-type and DE/A Claspin. Boxes represent acidic patch (AP) and its interacting partner, N-terminal AP-interacting domain (NTAPI). Arrowheads represent the putative cleavage sites on the Claspin polypeptides. The cleavage shown by a red arrowhead on DE/A does not occur on the wild-type due to AP-NTAPI interaction.

Figure 9. Cdc7 phosphorylates Claspin in a manner dependent on AP and inhibits N-C interaction. (**A**) *In vitro* Cdc7-ASK kinase assays with wild-type and mutant Claspin proteins as substrates. Upper, schematic diagram of DE/A mutant and other mutant Claspin proteins in which serine/ threonine residues were replaced with alanine or glutamic acids; middle, autoradiogram; lower, CBB staining. (B) The wild-type or mutant Claspin proteins, as indicated, were coexpressed with HA-tagged Cdc7 and myc-tagged ASK in 293T cells. The whole cell extracts were analyzed by western blotting with anti-Flag antibody. Lanes 12-16, the samples were pretreated with lambda phosphatase before loading. (**C**) Wild-type (WT) and mutant Claspin proteins, as indicated, were expressed in 293T cells and were pulled down with M2 Flag beads. Associated proteins were analyzed by western blotting with indicated antibodies. (**D**) The Flag-tagged N-terminal segment (#25) was coexpressed with the HA-tagged C-terminal polypeptide (#13) along with HA-tagged Cdc7 and myc-tagged ASK and pulled down with M2 Flag beads, and association of #13 was analyzed by western blotting using anti-Flag or anti-HA antibody. Arrowheads indicate the pulled down #13 polypeptide. *, IgG; #, non-specifically reacting Cdc7 polypeptide.

Figure 10. Claspin plays essential role during the recovery from the quiescent state. (**A**) Quiescent state *f/-* MEF cells with or without adeno virus released by adding final concentration 15% serum and observed the cells by microscope at indicated times. Investigate cell cycle (**B**) and DNA synthesis ability (**C**) *f/-* MEF cells releasing from quiescent state. Treat MEF cells like (A) and before harvest the cell treats BrdU at 20mM for 20min and analysis by FACS. Wild-type (WT) and mutant Claspin proteins, as indicated, were expressed in 293T cells and were pulled down with M2 Flag beads. Associated proteins were analyzed by western blotting with indicated antibodies. (**D**) Analysis cell cycle of stable clones of WT, DE/A and PIP mutants *f/-* MEF cells. The procedure is the same with (B). (**E**) Treat MEF cells like (A) and separate Chromatin from cells by triton. Samples were analyzed by western blotting with indicated antibodies. (**F**) Add different concentration pifithrin-α in quiescent state *f/-* MEF cells

with adeno virus and released by adding final concentration 15% serum at the same time. The cells were observed by microscope at the indicated times.

Table

Sequences of the oligonucleotides used in this study

Name	Sequence (5’-3’)
Primers	
FL-F	ccgctcgagactagtatgacaggcgaggtgggttctg
FL-R	ctagtctagagctctccaaatatttgaagatgc
#2-R	ctagtctagaagtgtctatgatttctttgtg
#9-F	ccgctcgaggcaaatactactgaaatgaa
#9-R	ctagtctagacaatggcaatcgaggcttcaaag
#13-F	ccgctcgagactagtgccagtatggatgagaatgcc
#13-R	ctagtctagacagtatcataaactgactgtcc
#14-R	ctagtctagaaaaagagcctgagcaaagagcaag
#27-R	acctctagactttcttgatttgactctgcagttcc
#27N-F	accggctagcgccagtatggatgagaatgcc
#27N-R	accggctagctttcttgatttgactctgcagttcc
Cdel1-1-R	ccgggatccaaaagagcctgagcaaagag
Cdel1-2-F	ccgggatccgccaagaaagttacagccaaa
Cdel2-1-R	ccgggatcctttcttgatttgactctgcagttcc
Cdel2-2-F	ccgggatccgccaagaaagttacagccaaa
Cdel4-1-R	ccgggatccaaaagagcctgagcaaagag
Cdel4-2-F	ccgggatcccccacagacaaggaagagga
C-del6-1-R	ccggctagcaaaagagcctgagcaaagag
Cdel6-2-F	ccggctagcatacacatgaaaactatgttggatg
Cdel7-1-R	ccgggatccaaaagagcctgagcaaagagcaag
Cdel7-2-F	ccgggatccatacacatgaaaactatgttggatg
Cdel6N-1-1-R	accggctagccttcctgggtagatgtttttca
Cdel6N-2-1-R	accggctagcctctgatgaggctggagtgga
Cdel6N-3in-F	accggctagccaggctgaaaaacatctacc
Cdel6N-4in-F	accggctagccaggatgcctccactccag
Cdel6N-5in-F	accggctagcgacaaggaagaggaagacga
#2 PIP-1-F	gctaaaaccgctcatgatgccgccaaacgtaaac
#2 PIP-2-R	ggcggcatcatgagcggttttagcctcaggcata
Genotyping primers	
Cg 1F	aaacccgaaaaaccaagcgaatctg
Cg 2R	aaaacccgaaaaaccaagcgaatc
Cg 1Mr	agtgtggggacatcagctgca

Claspin knockdown

Sense	uuggccacugauuucaauutt
anti-sense	aauugaaaucaguggccaatt

Y-fork oligonucleotides

dT32-32mer

ttttttttttttttttttttttttttttttttggttggccgatcaagtgcccagtcacgacgtt

32mer-dT32

aacgtcgtgactgggcacttgatcggccaacctttttttttttttttttttttttttttttt

Southern probe

5’ probe

ttcctctcctcttcctctttacctatttgaacgtttctcactgacatttaacaaacttgccccccacccacccaccaaatgccaacc
acatgtgcatcatgatgacagccatgagaaatctcttgatttactagttgatactctagtaaaaccatcctctgggctgacggtg
ggcacagcgctagtctgtccagtactatctgtcccttgtcagctgtaactttcctgatagactttagctatctcacatatctgaaa
gggctcagacattaatcaacctataaagtaggtgctaagattgaacaggatgtagaaatgg

Neo probe

ctgcagacttacagcggatcccctcagaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcg
gcgataccgtaaagcacgaggaagcggtcagcccattcgccgccaagctcttcagcaatatcacgggtagccaacgctat
gtcctgatagcggtccgccacacccagccggccacagtcgatgaatccagaaaagcggccattttccaccatgatattcgg
caagcaggcatcgccatgggtcacgacgagatcctcgccgtcgggcatgcgcgccttgagcctggcgaacagttcggct
ggcgcgagcccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccgagtacgtgctcgctcgatgc
gatgtttcgcttggtggtcgaatgggcaggtagccggatcaagcgtatgcagccgccgcattgcatcagccatgatggata
ctttctcggcaggagcaaggtgagatgacaggagatcctgccccggcacttcgcccaatagcagccagtcccttcccgctt
cagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgctgcctcgtcctgca
g

3’ probe

ttccacctcttgtcccttctaggtcatgtaagtatcagtcgggccattacaaggaaaccgtaaaccctgcagatgcagctgga
atgggtgctgaggactccagcagaggttctgagcagaggacaggggctggaattgcagcggaaactaatgttctctctga
ggtctcagaagaagccgggatcactgctggatcagatgaggcttgtgggaaggatccggtaagacgaggagagctgga
aattgaggagactgagaagcacagtgatgacagaccttattctcctggggacagatccatgtcacagcaggaatcctccat
ccccaggatcgaagacaatgaggggcatcaggctggagacctcaccgaatctgaccctcctgccctggagggagaaga
actgaaaacagtagaaaaaacagacgcaaaagagg

Figure 1A

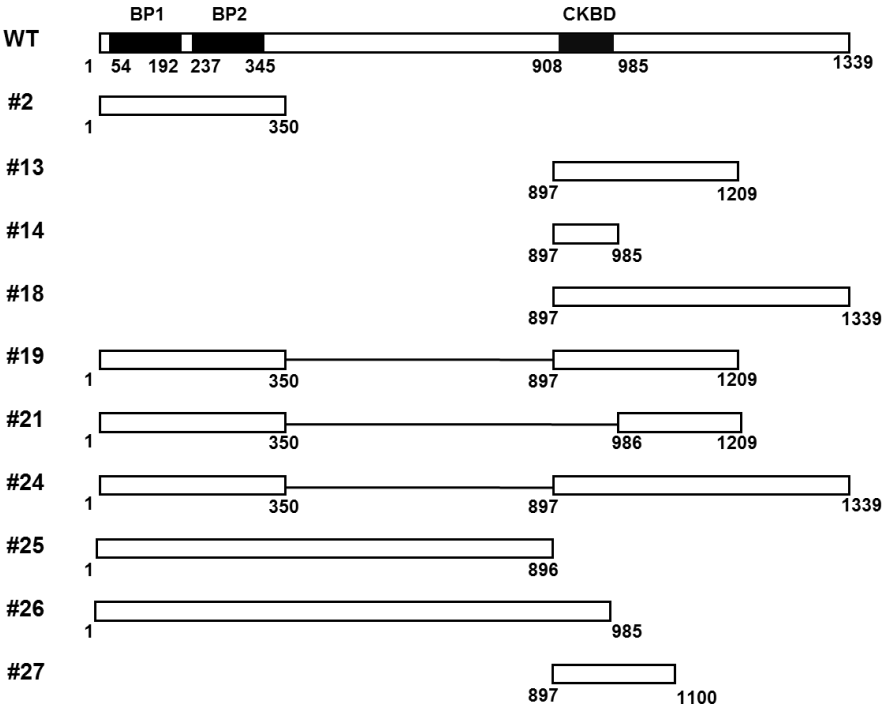
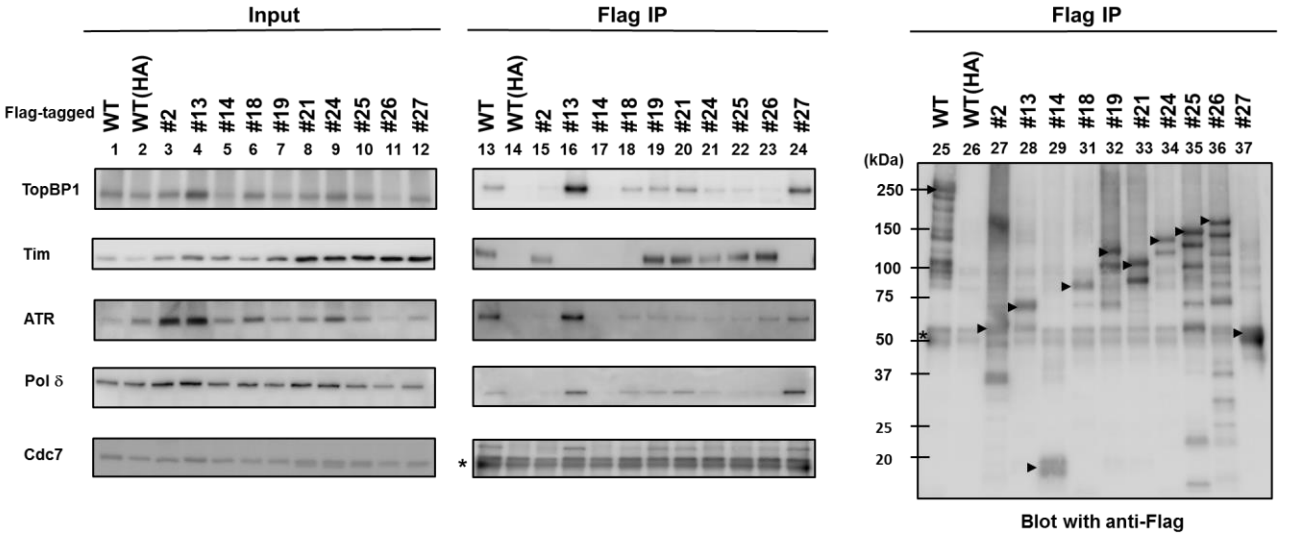
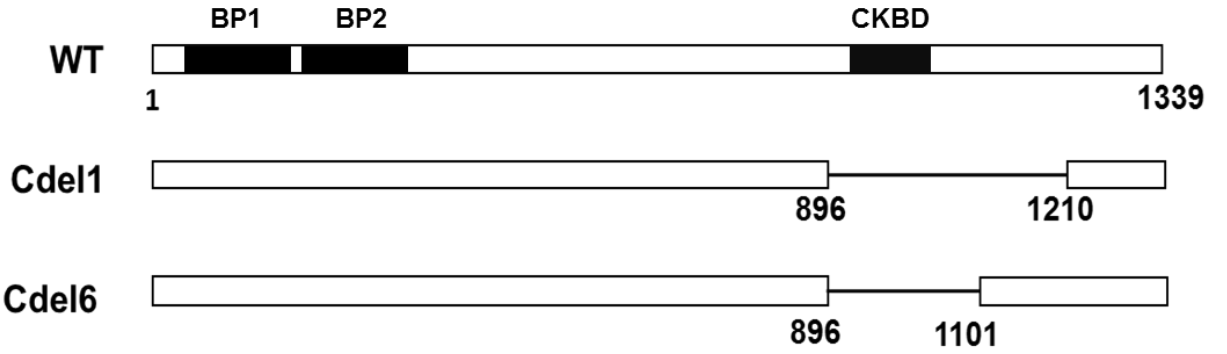


Figure 1B



Expressed in 293T cells

Figure 1C



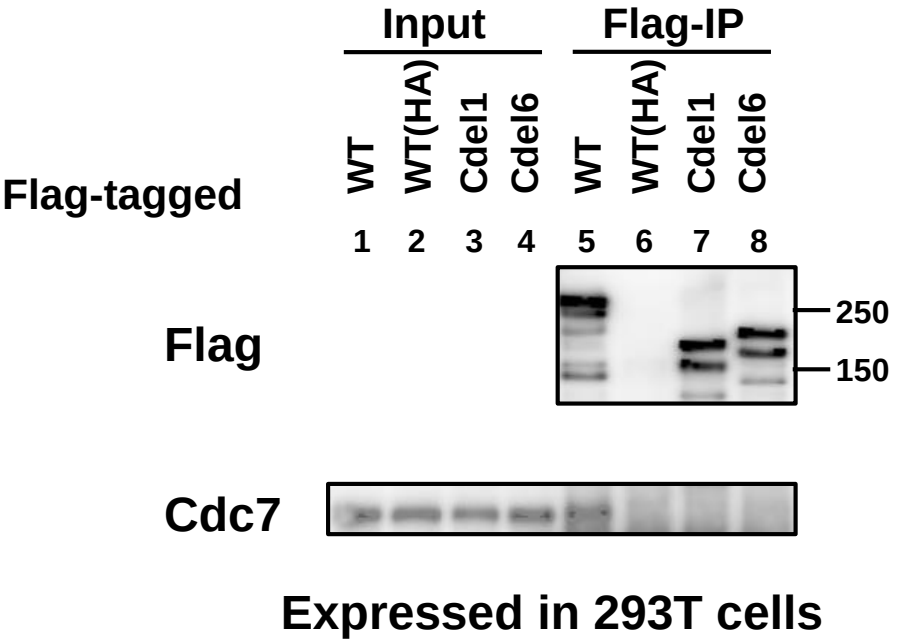


Figure 1D

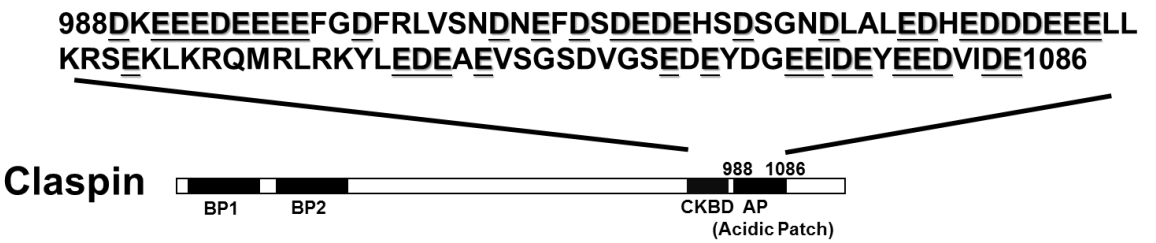
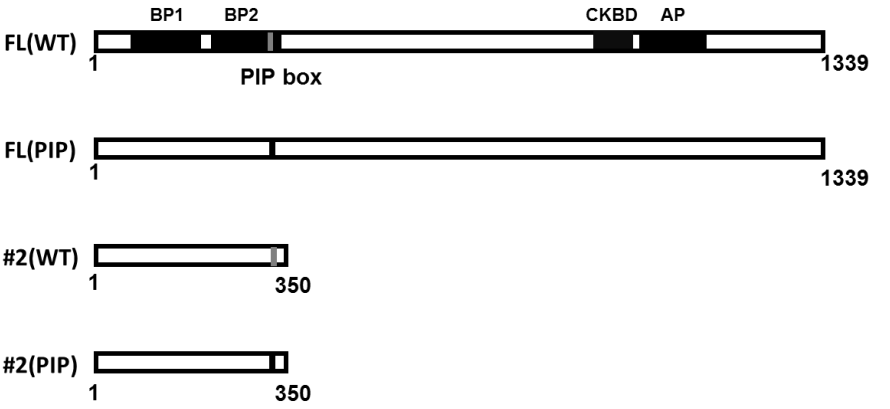


Figure 2A

PIP BOX		**Q X X L/I or X X F F**	
Claspin[Homo sapiens]	**311-**	N K T **I** H D **F F**	**-318**
Claspin[Hetercephalus glaber]	**310-**	N K T **I** H D **F F**	**-317**
Claspin[Cricetulus griseus]	**308-**	N K T **I** H D **F F**	**-315**
Claspin[Mus musculus]	**306-**	S K T **I** H D **F F**	**-313**
Claspin[Rattus norvegicus]	**295-**	S K T **I** H D **F F**	**-302**
Claspin[Gallus gallus]	**328-**	A K S V H E **F F**	**-335**
Claspin[Xenopus laevis]	**304-**	P K T **I** H D **F F**	**-311**
Claspin[Camponotus floridanus]	**60-**	**Q** R T **L** Q E **F** L	**-67**
Claspin[Acromyrmex echinatior]	**199-**	**Q** R T **L** Q E **F** L	**-206**
Claspin[Harpegnathos saltator]	**302-**	**Q** R T **L** Q E **F** L	**-309**
Mrc1[Schizosaccharomyces pombe]	**236-**	R V T **L** R E **F F**	**-243**

Figure 2B



PIP box : Qxx(ILM)xx(FY)(FY)

Original: 311-NKTIHDFF-318
Mutation: 311-AKTAHDAA-318

Figure 2C

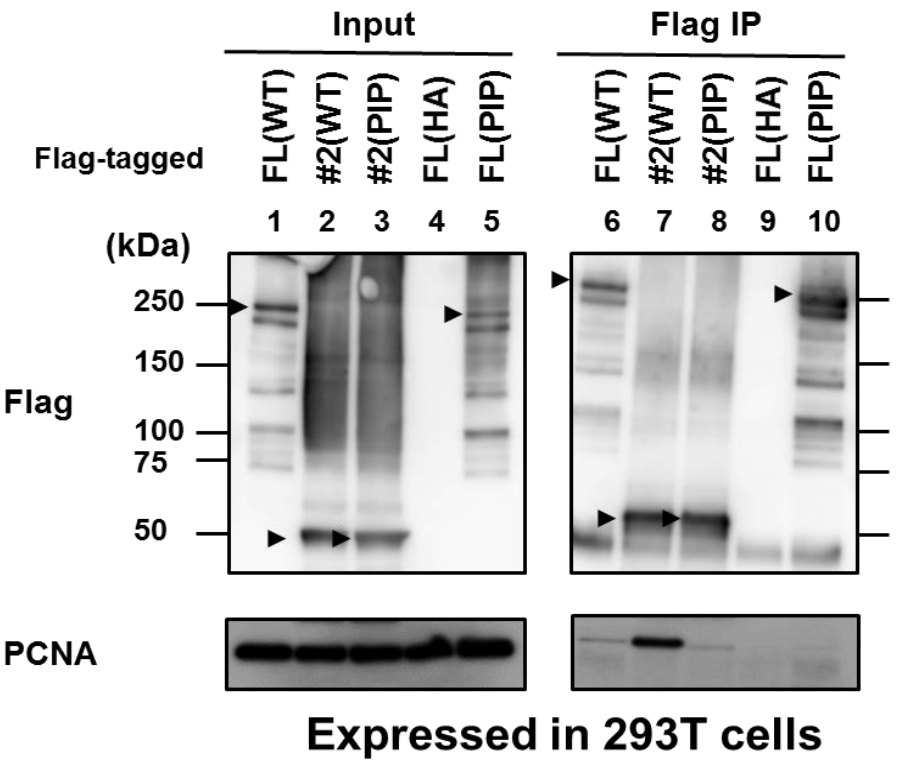


Figure 2D

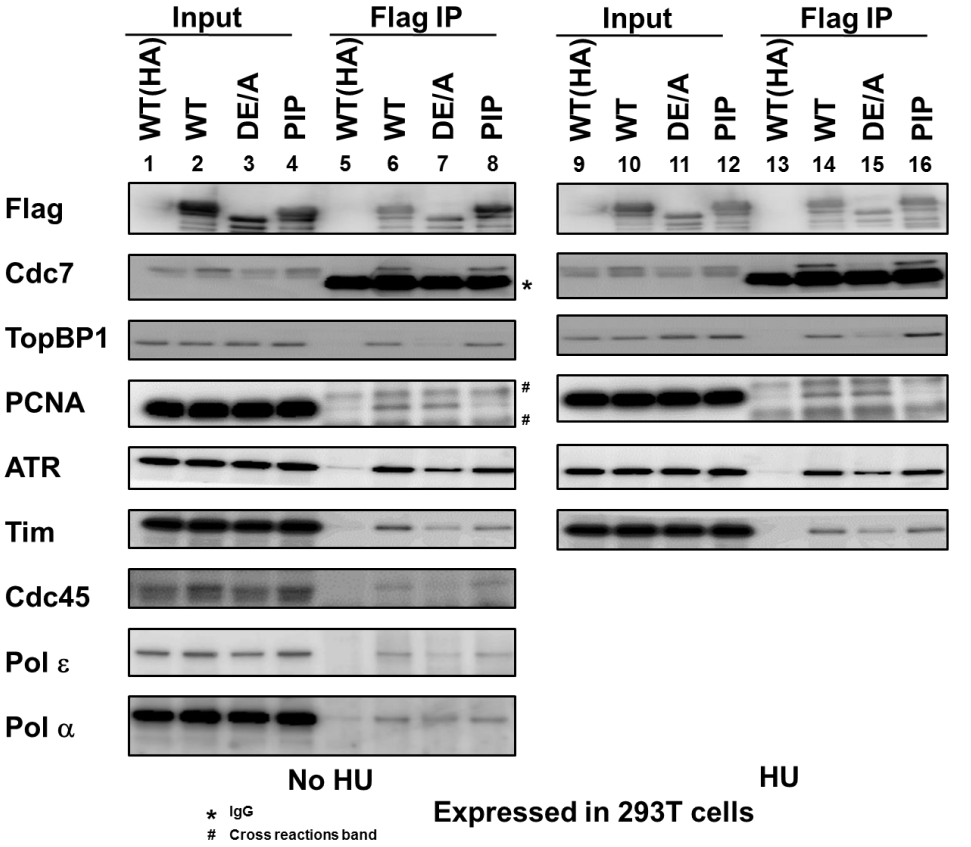


Figure 2E

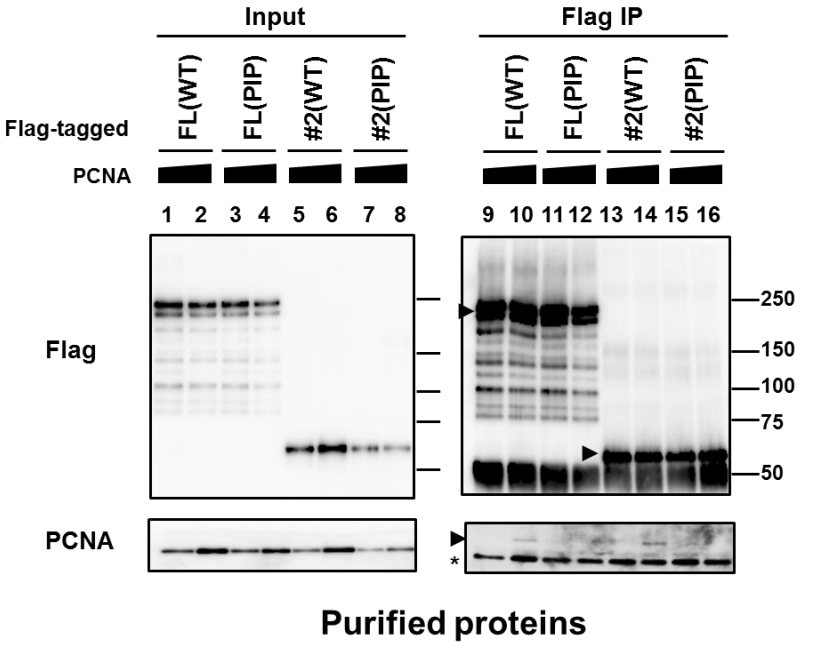


Figure 3A

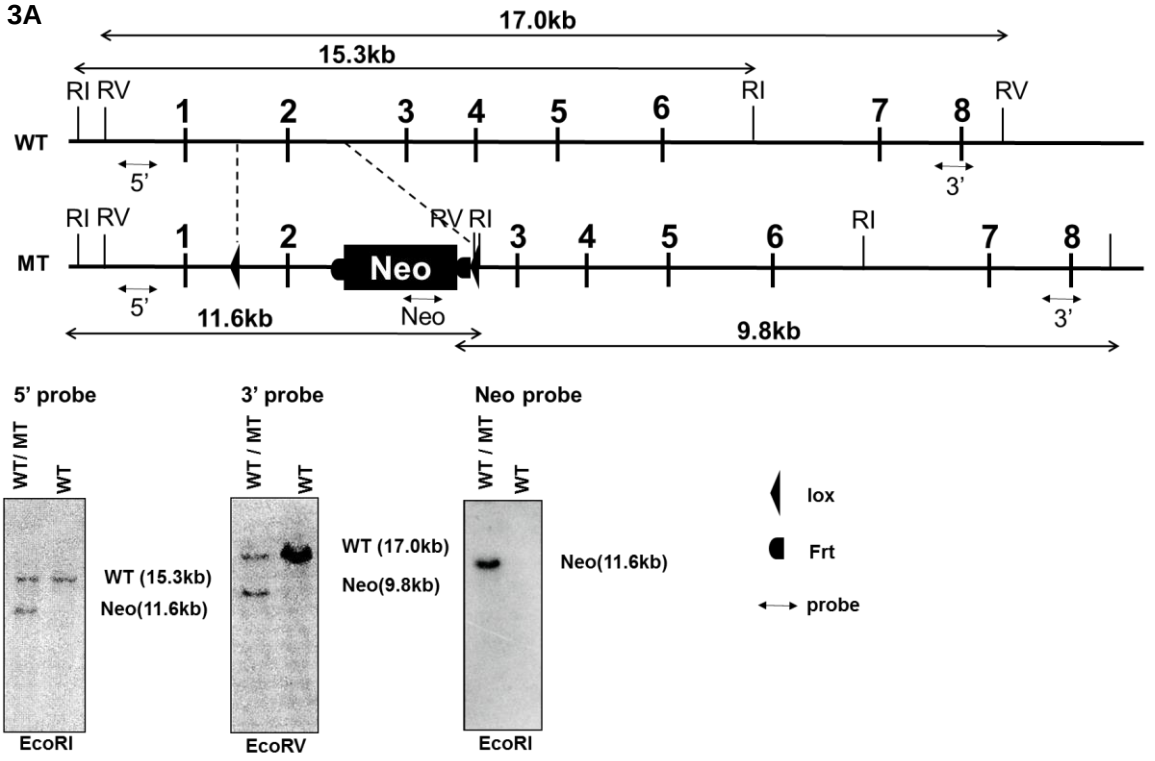
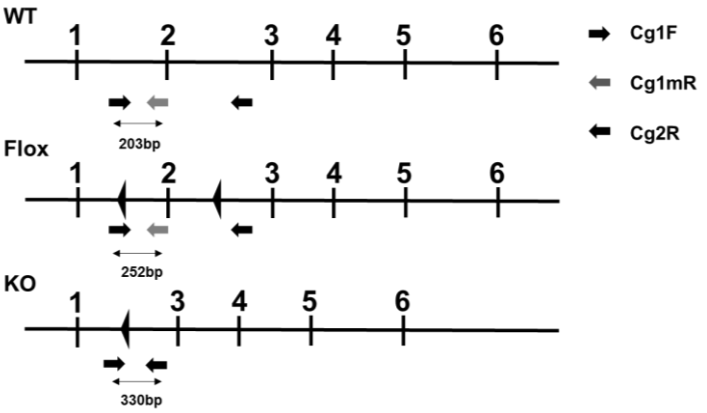


Figure 3B



w/- x w/-

Embryo	w/w	w/-	-/-	Ratio of -/-
9.5 days	3	3	1	1/7
12.5 days	5	12	0	0/17

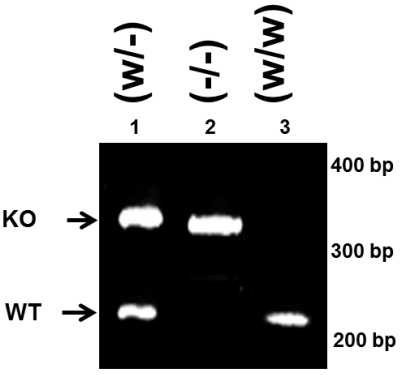


Figure 3C

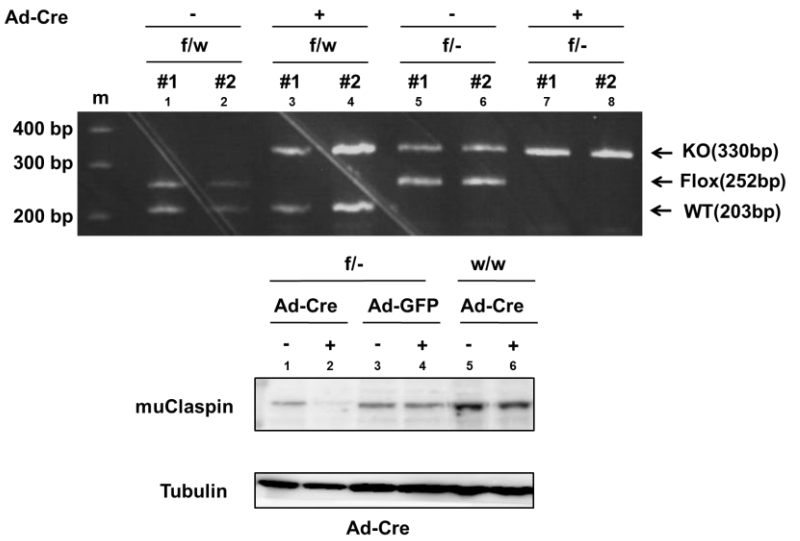


Figure 3D

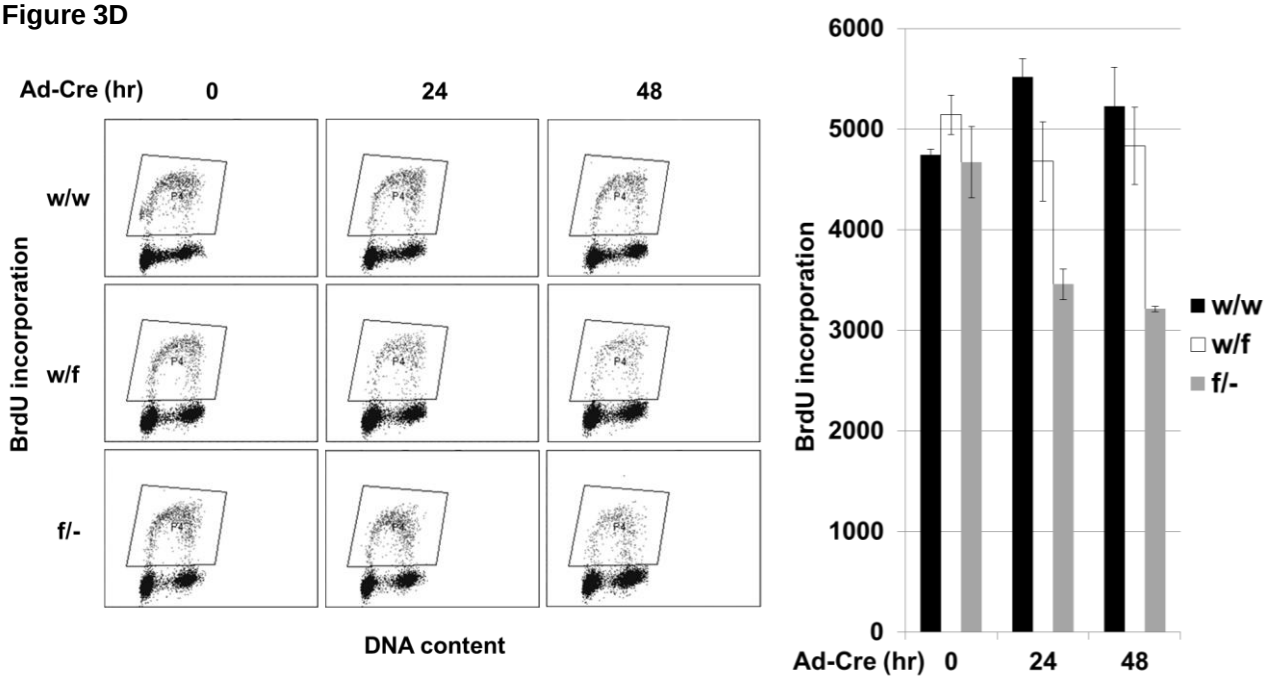


Figure 4A

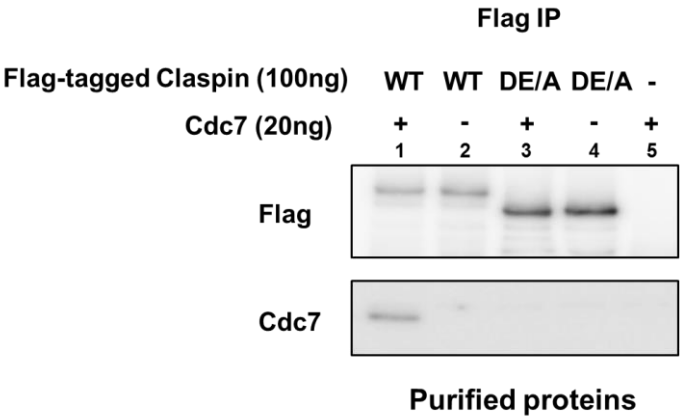


Figure 4B

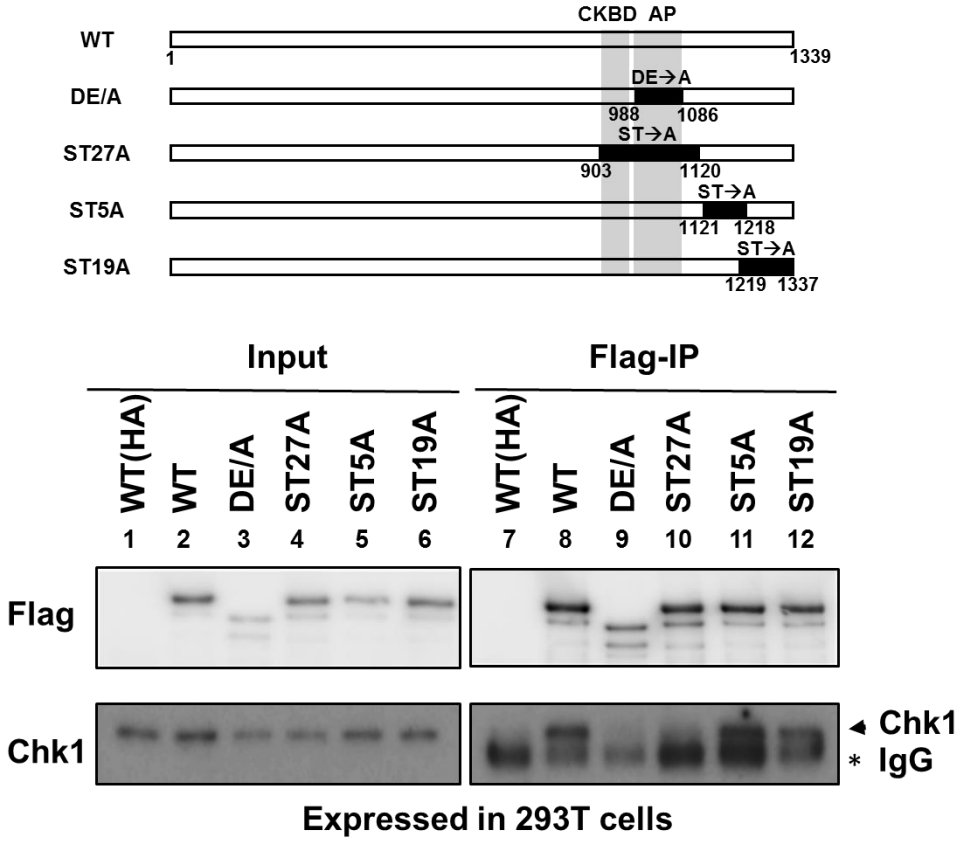


Figure 5A

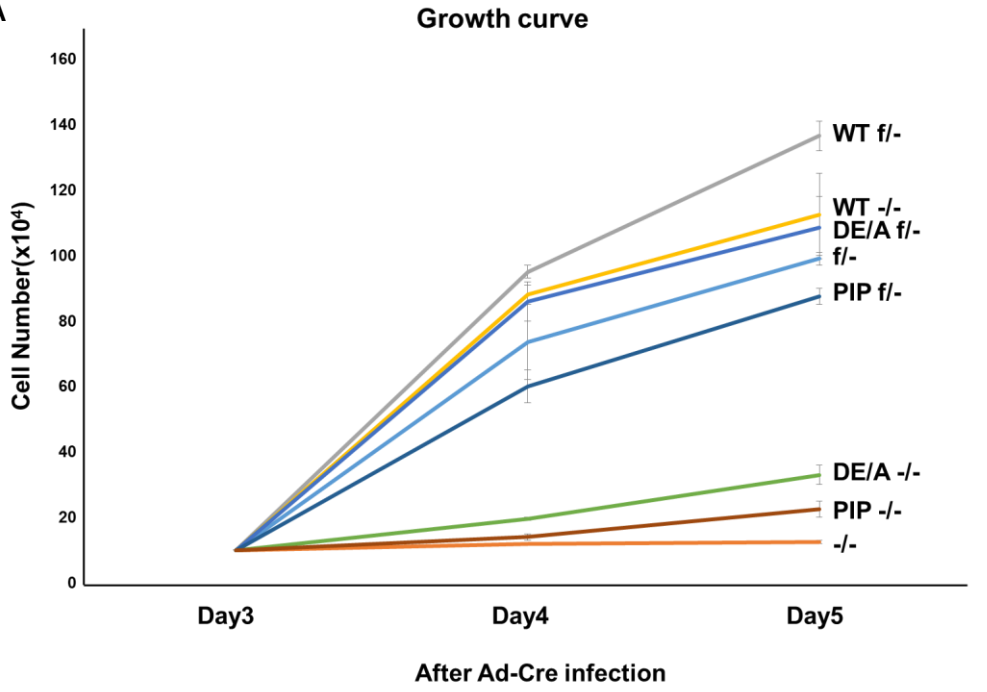


Figure 5B

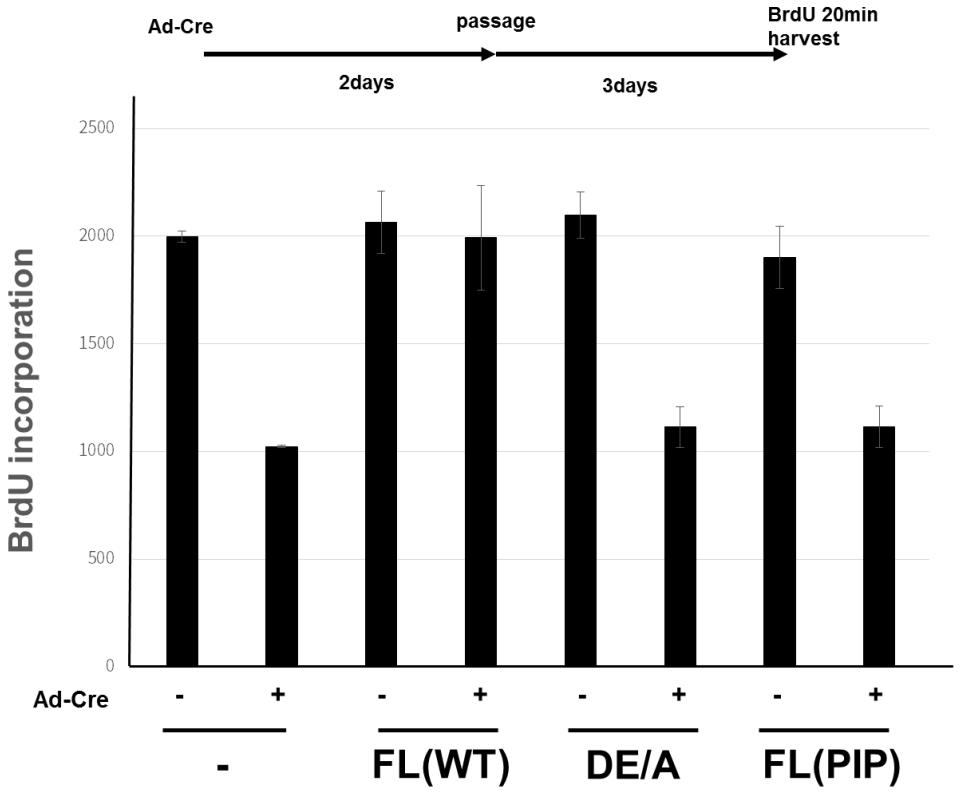


Figure 5C

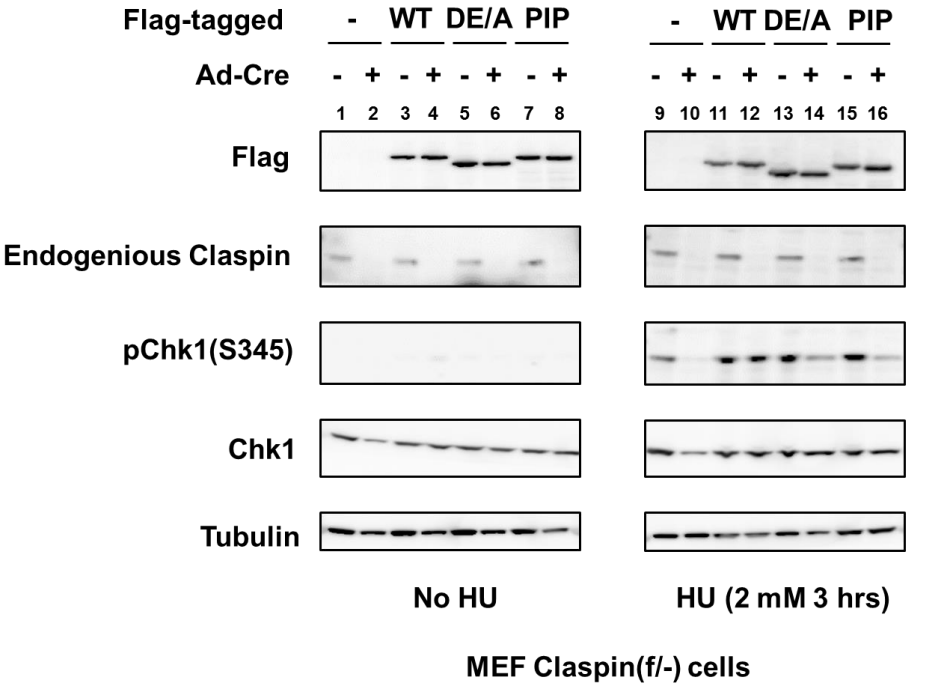

Flag-tagged
-
WT
DE/A
PIP
-
WT
DE/A
PIP
Ad-Cre
- + - + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Flag
Endogenious Claspin
pChk1(S345)
Chk1
Tubulin
No HU
HU (2 mM 3 hrs)
MEF Claspin(f/-) cells

Figure 6A

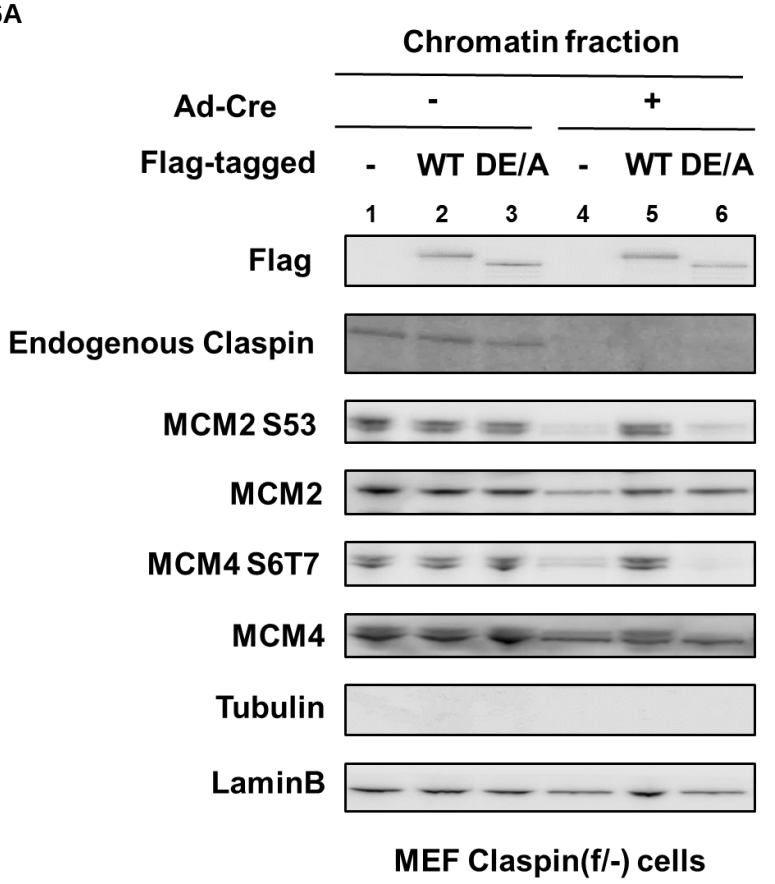


Figure 6B

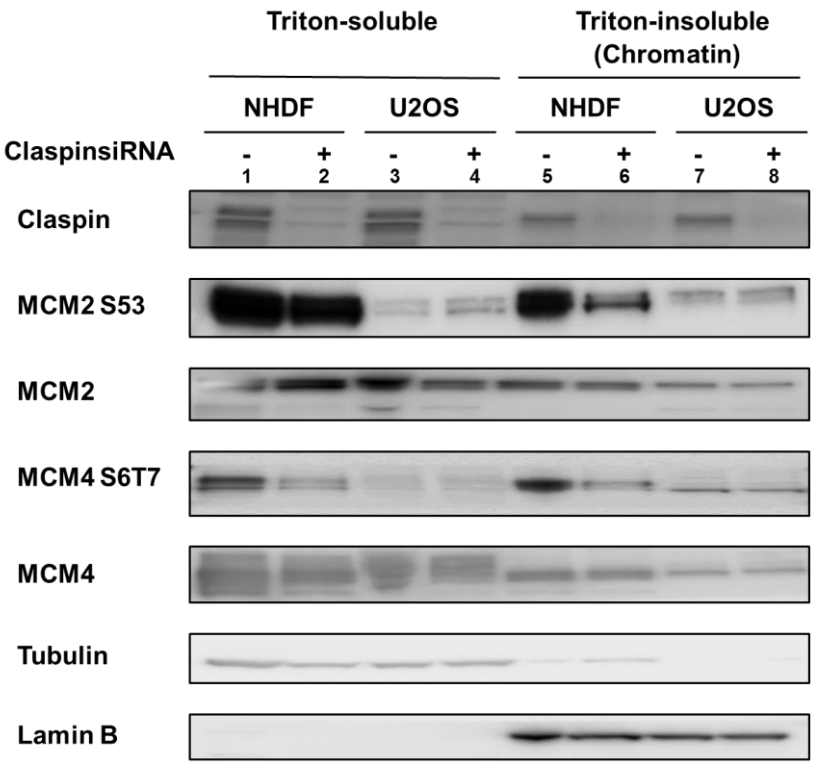


Figure 7A

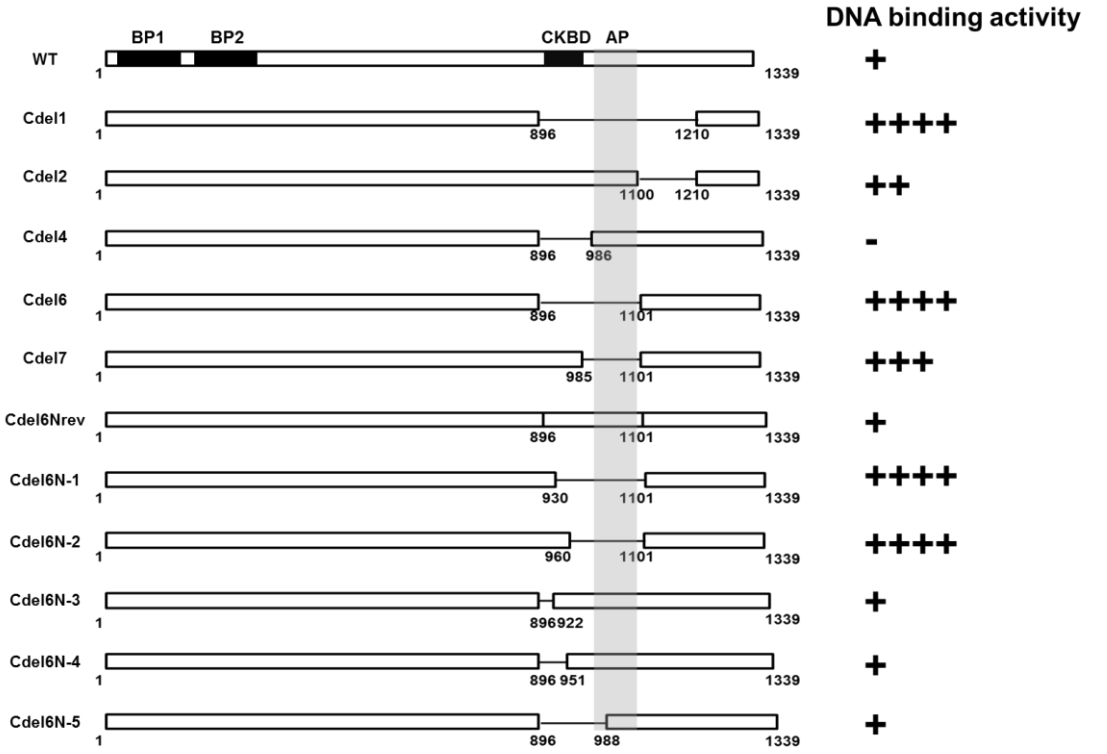
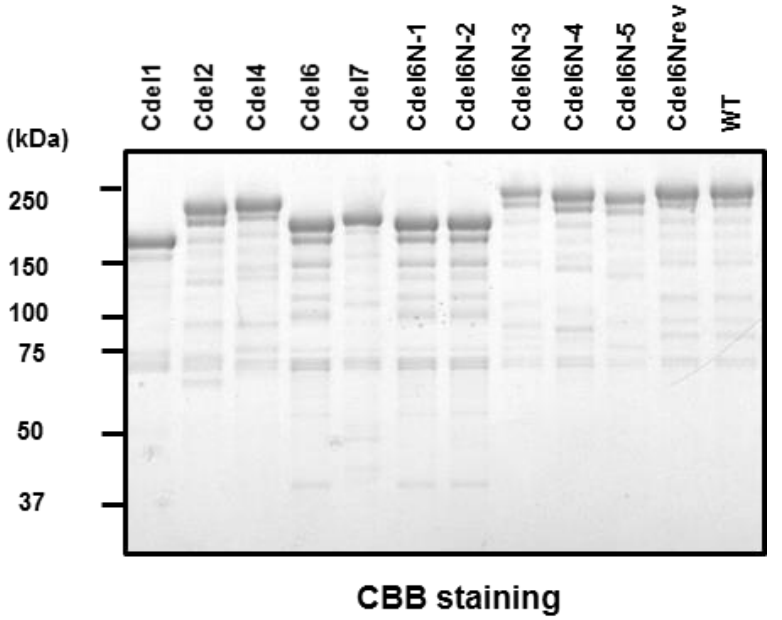
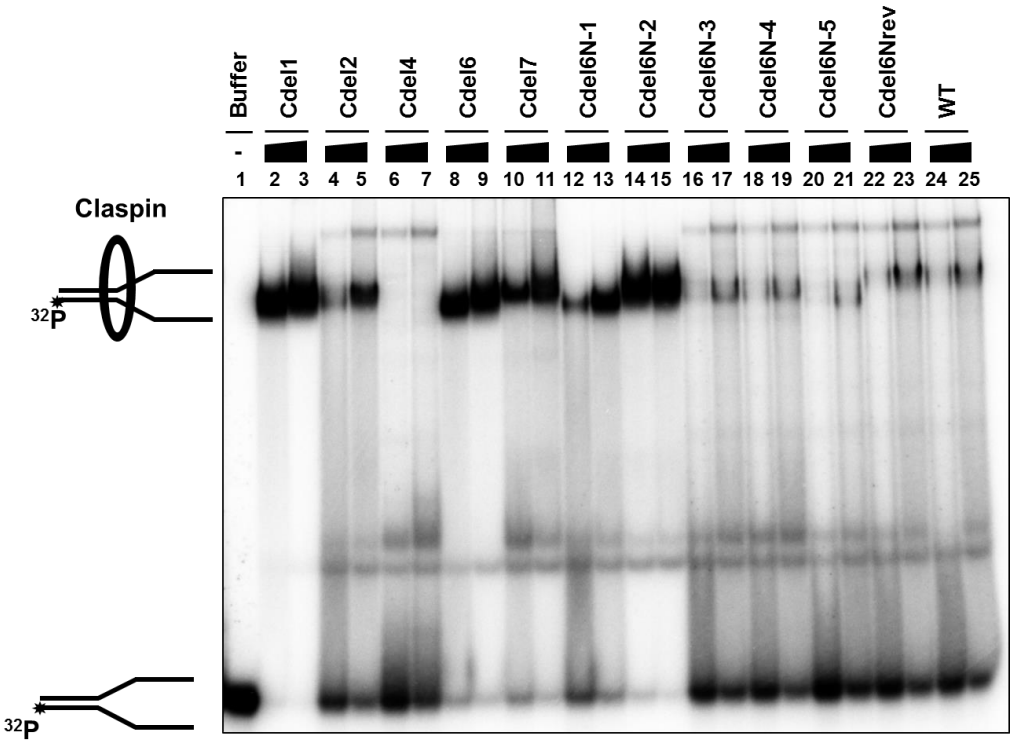


Figure 7B



CBB staining

Figure 7C



Gel shift assay

Figure 7D

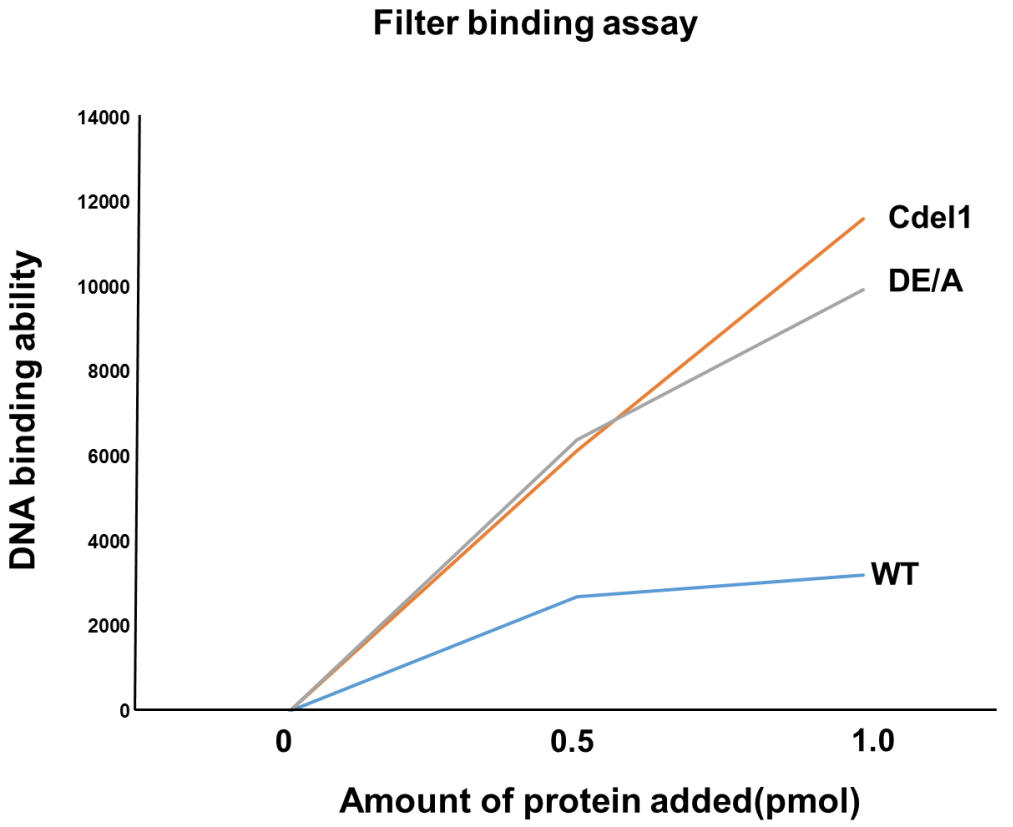


Figure 7E

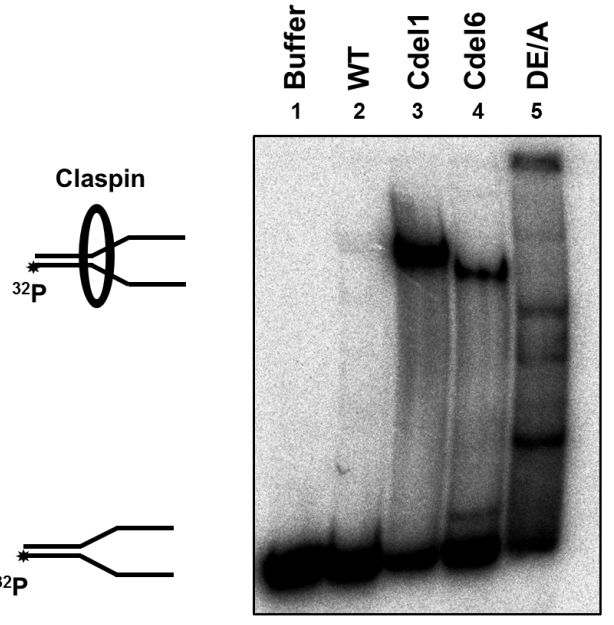


Figure 7F

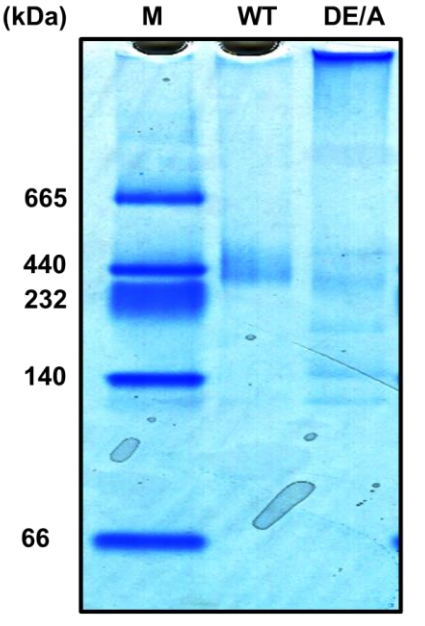


Figure 7G

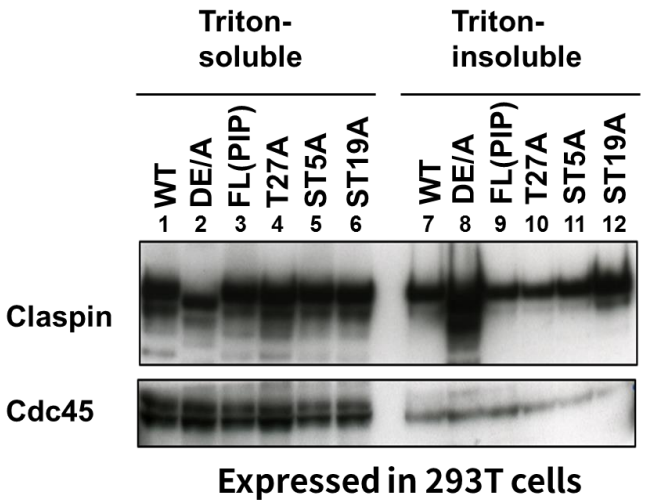


Figure 7H

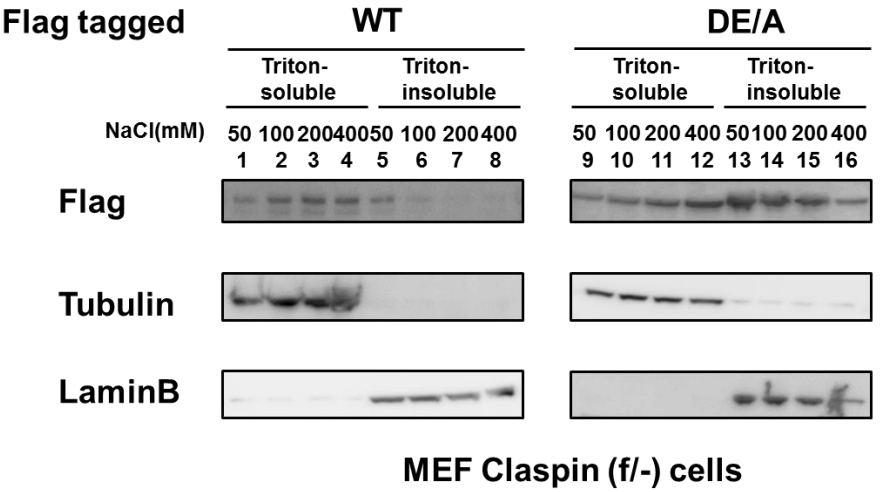


Figure 8A

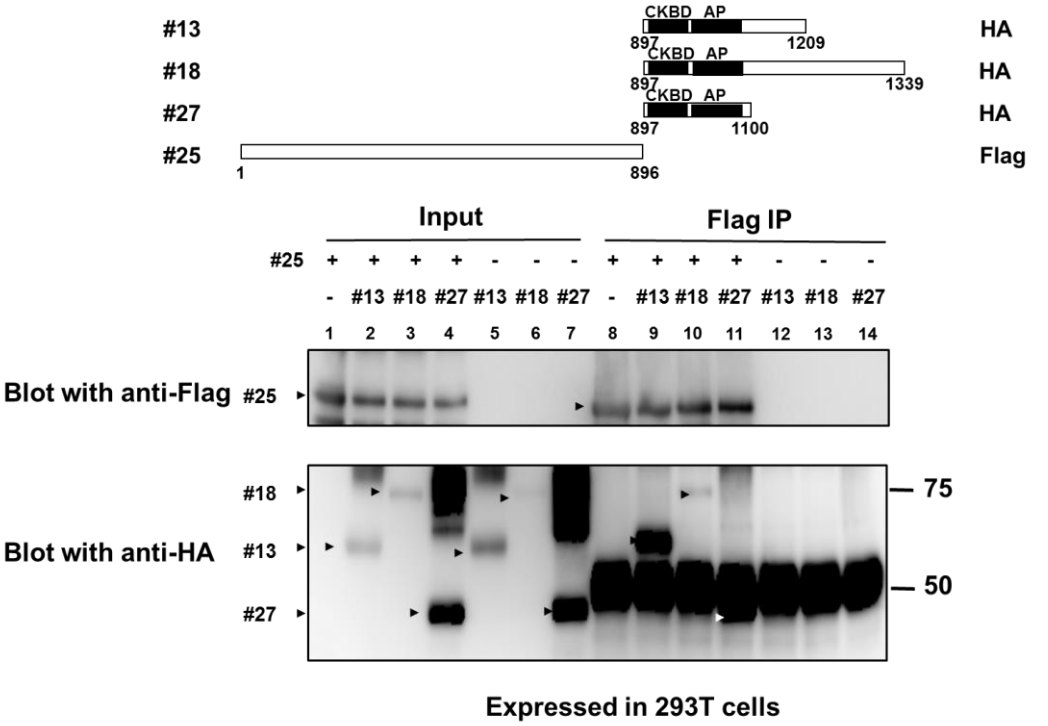


Figure 8B

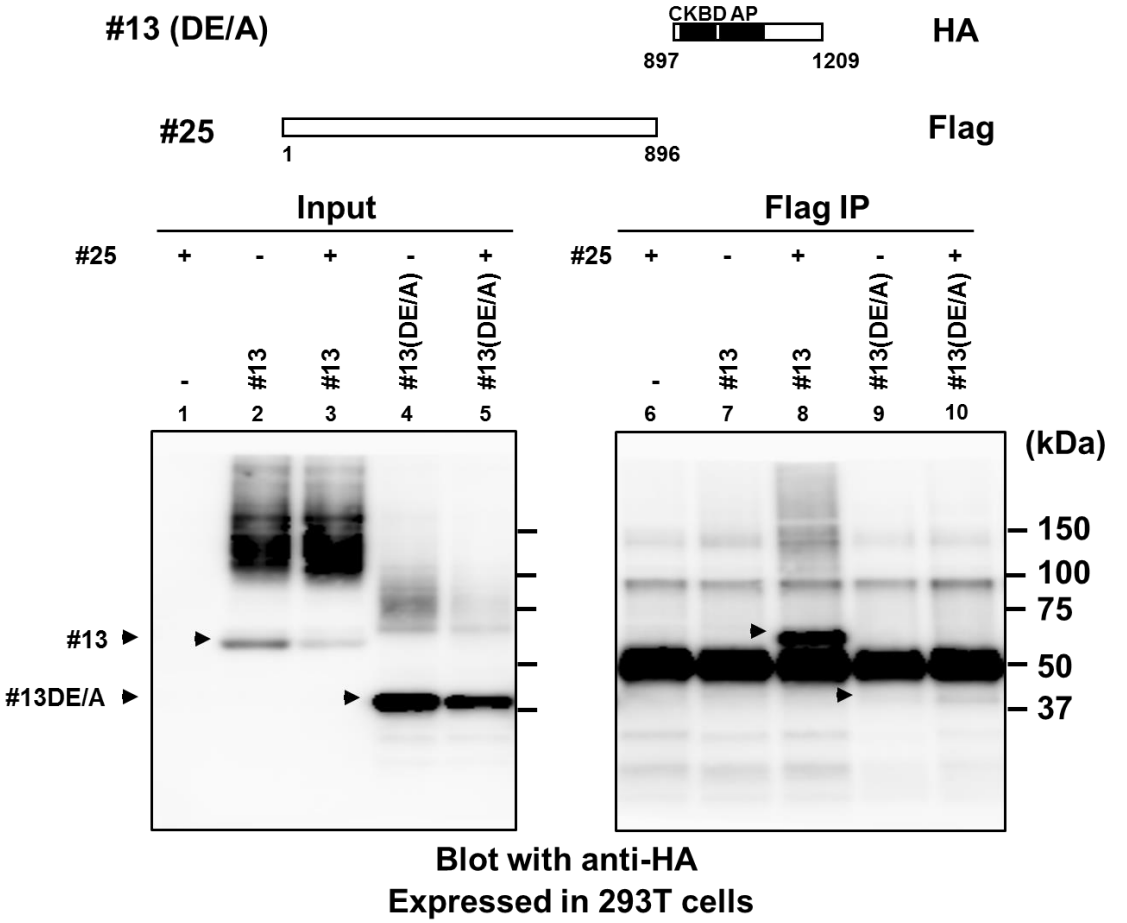
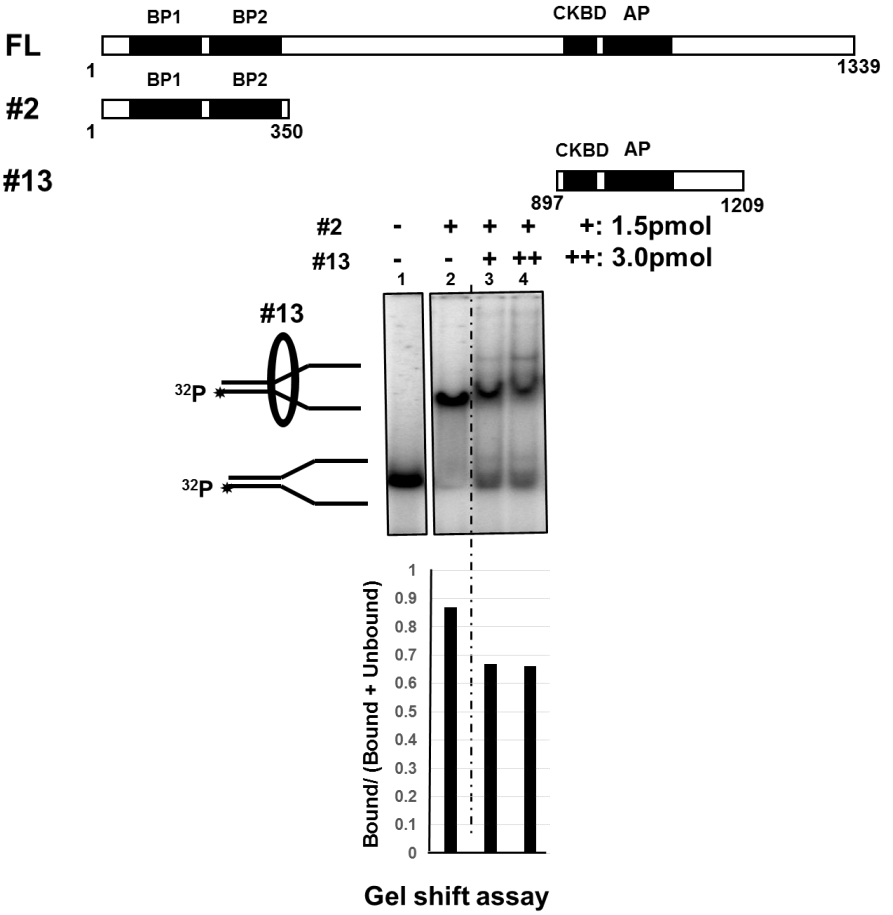
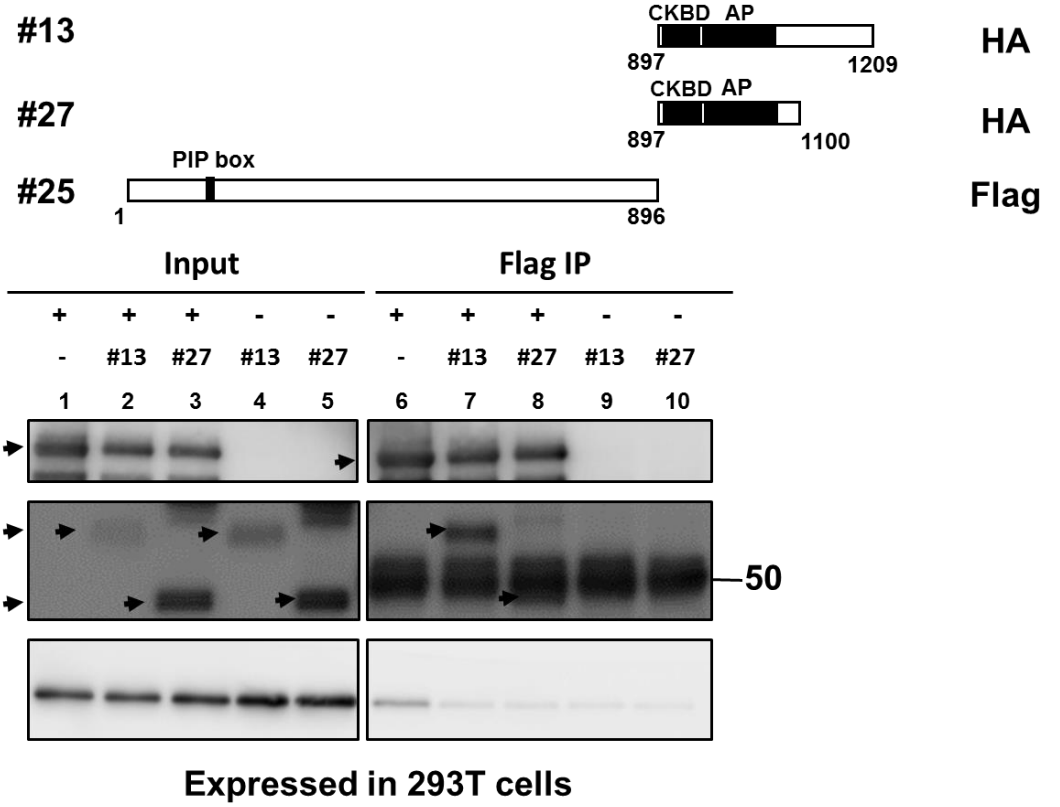


Figure 8C



Gel shift assay

Figure 8D



Expressed in 293T cells

Figure 8E

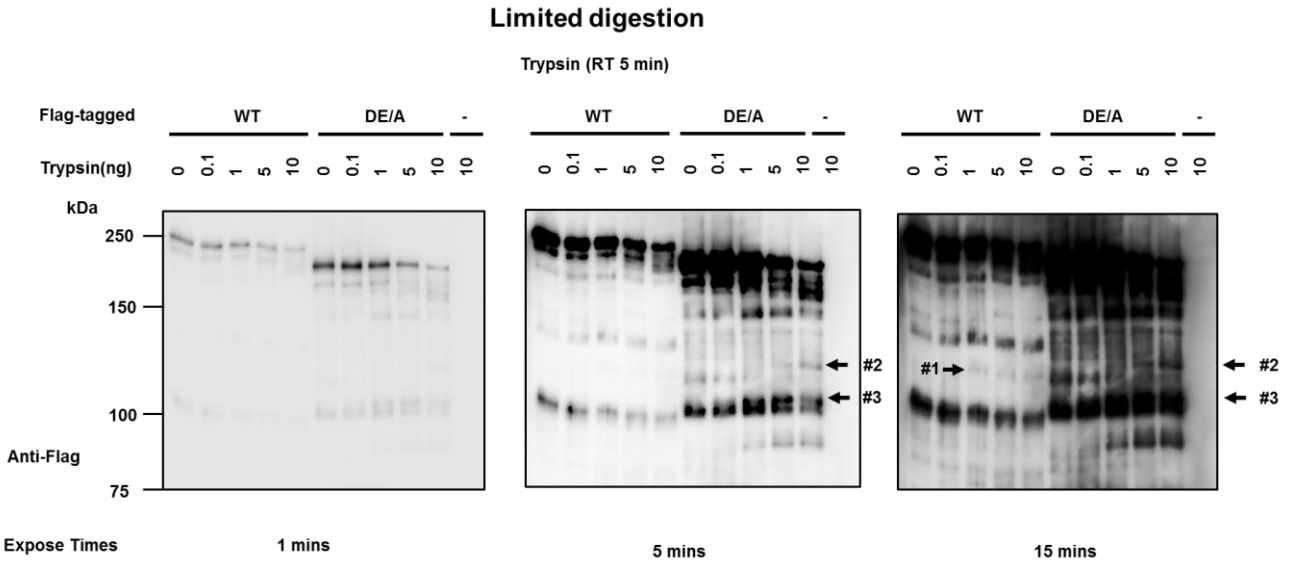


Figure 8F

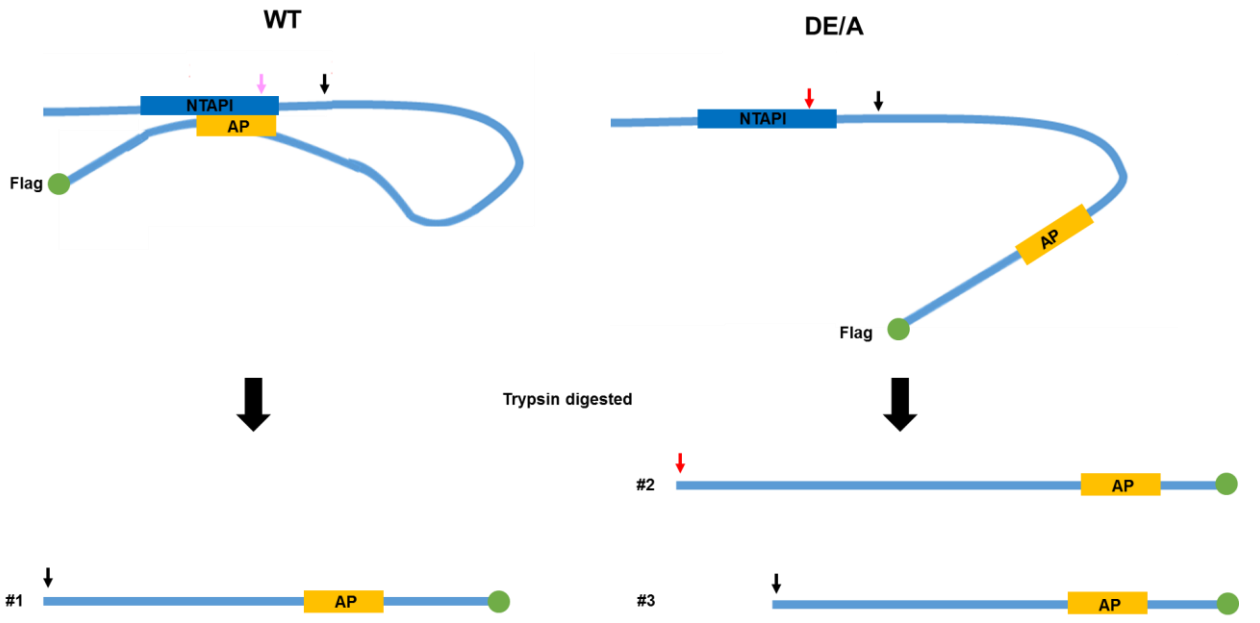
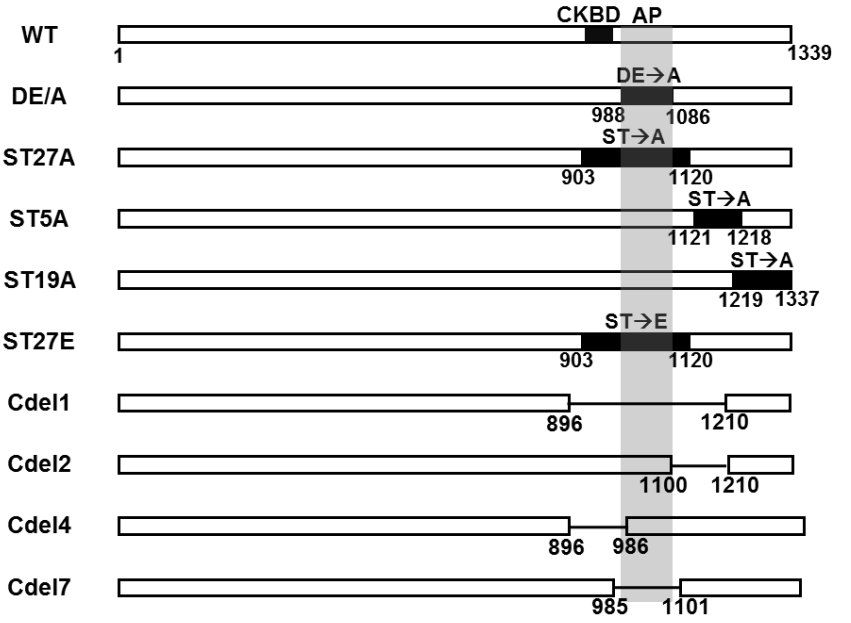
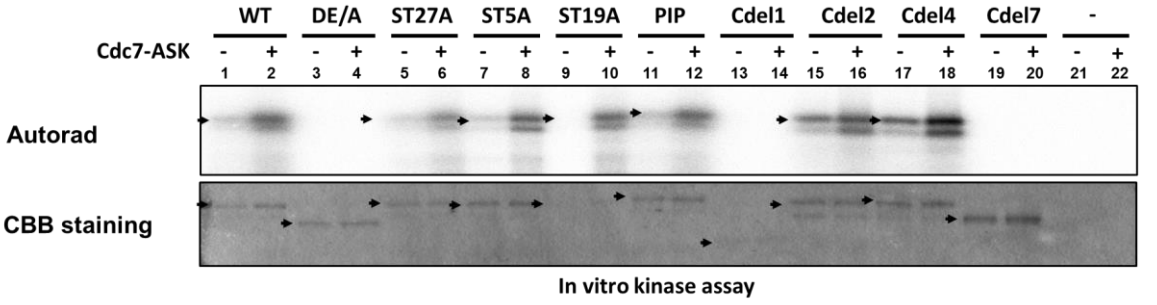


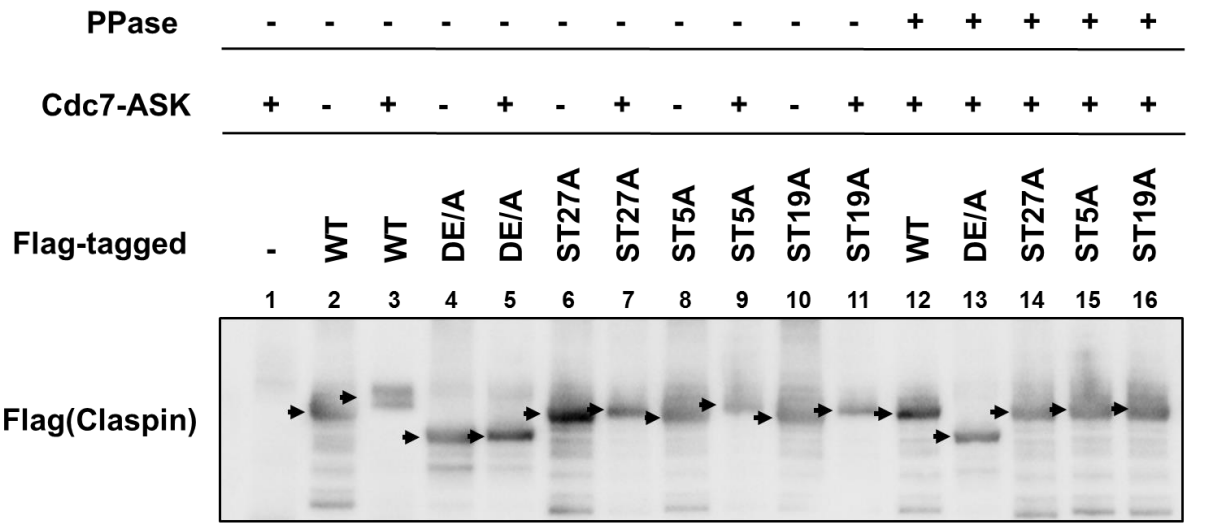
Figure 9A





In vitro kinase assay

Figure 9B



Expressed in 293T cells

Figure 9C

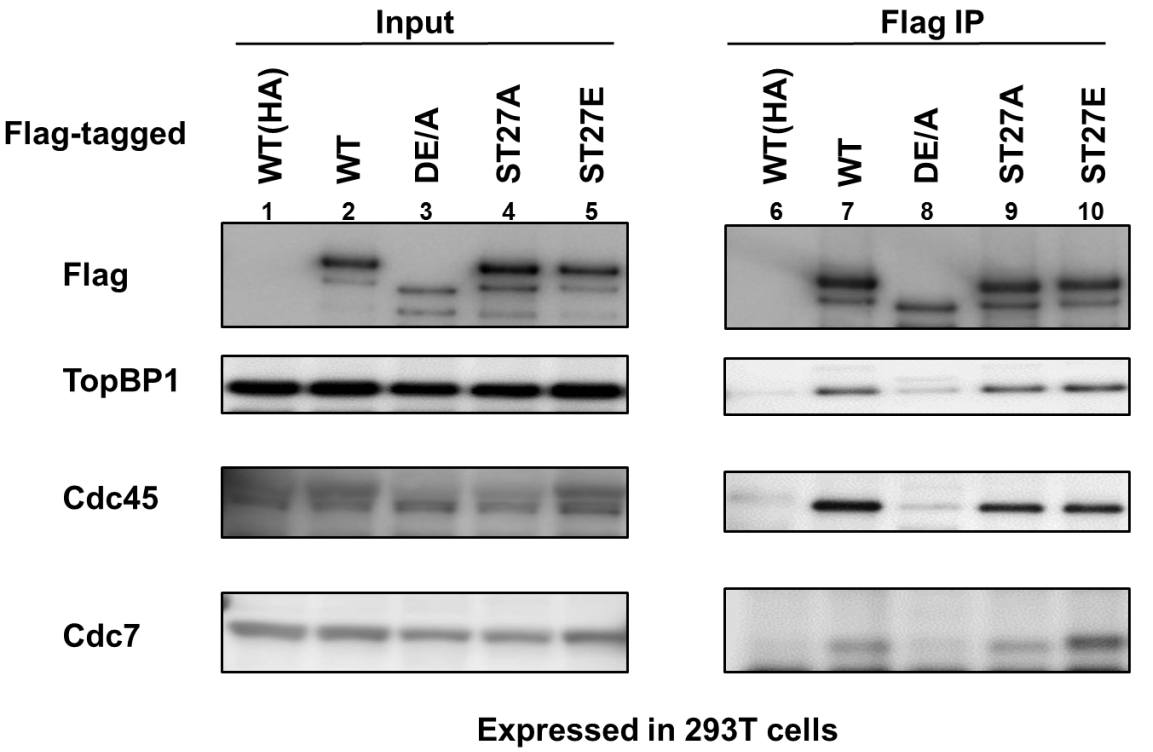
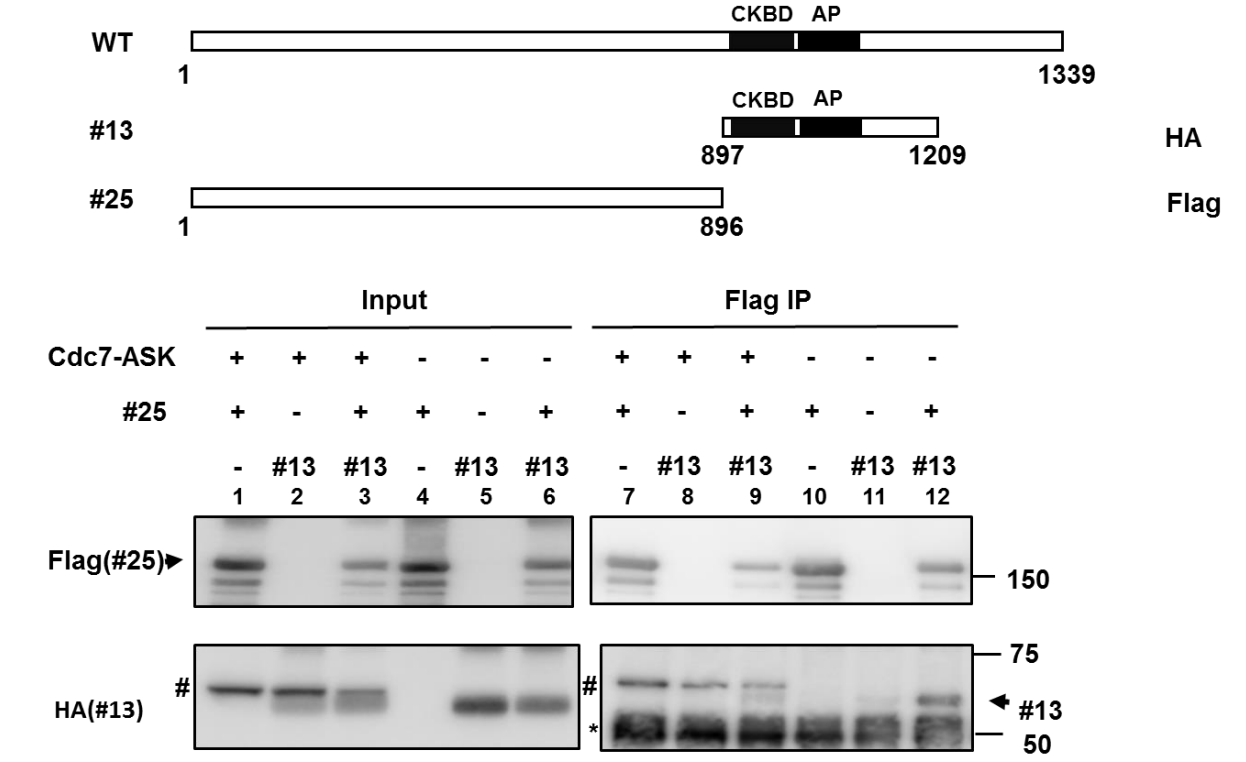


Figure 9D



* IgG

\# non-specifically reacting Cdc7 polypeptide

Figure 10A

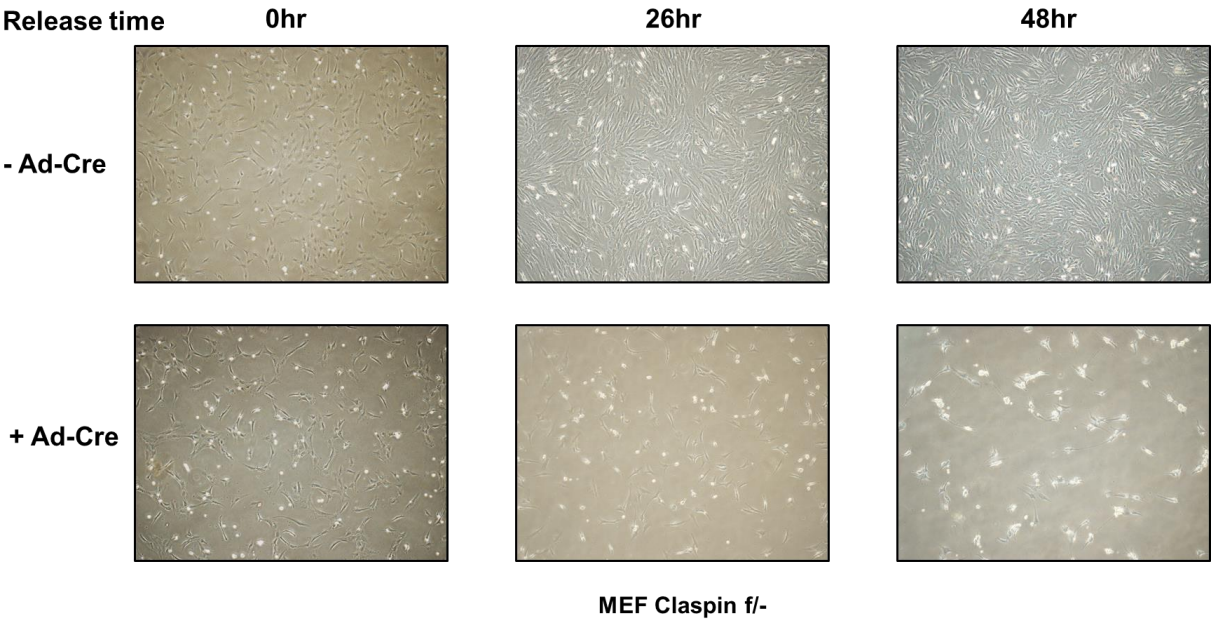


Figure 10B

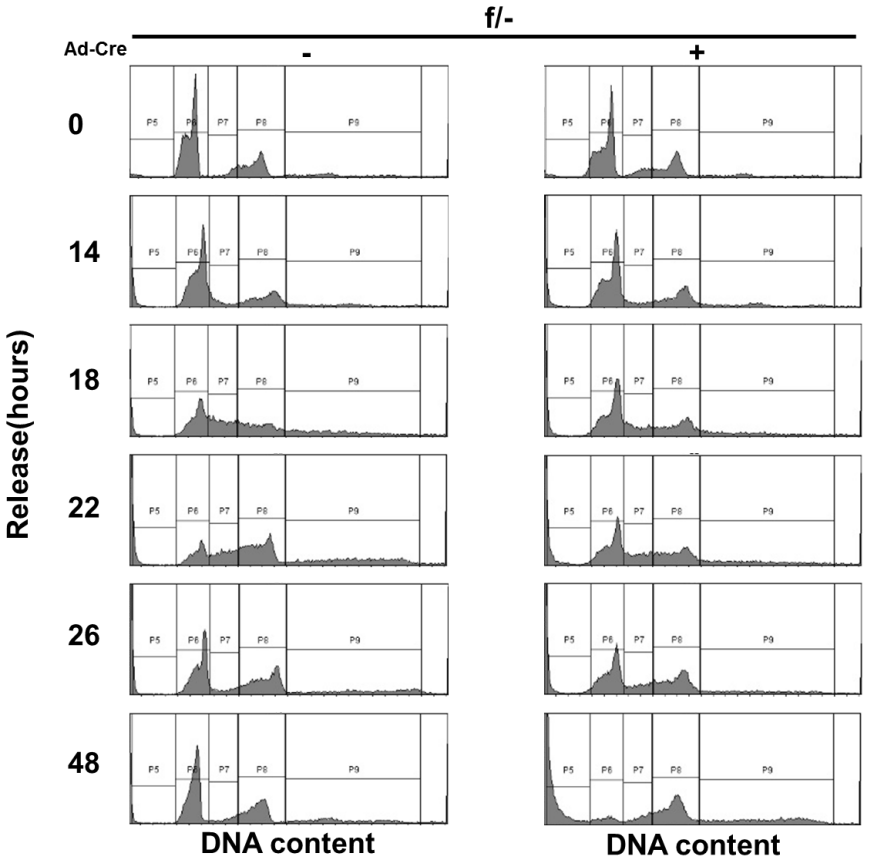


Figure 10C

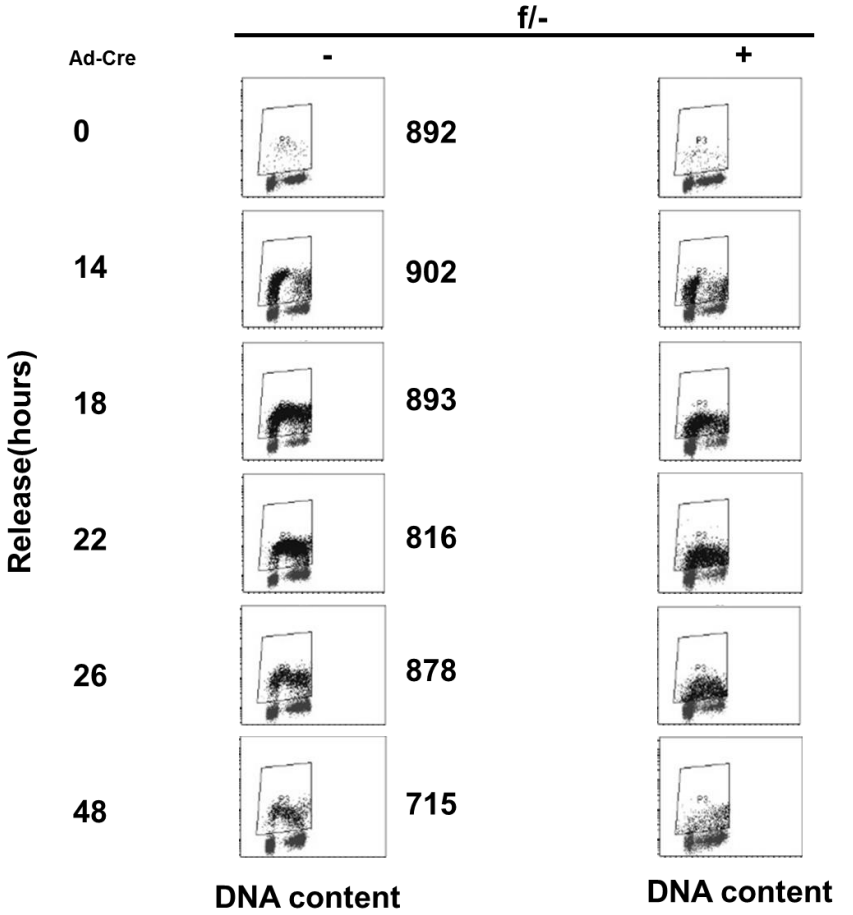


Figure 10D

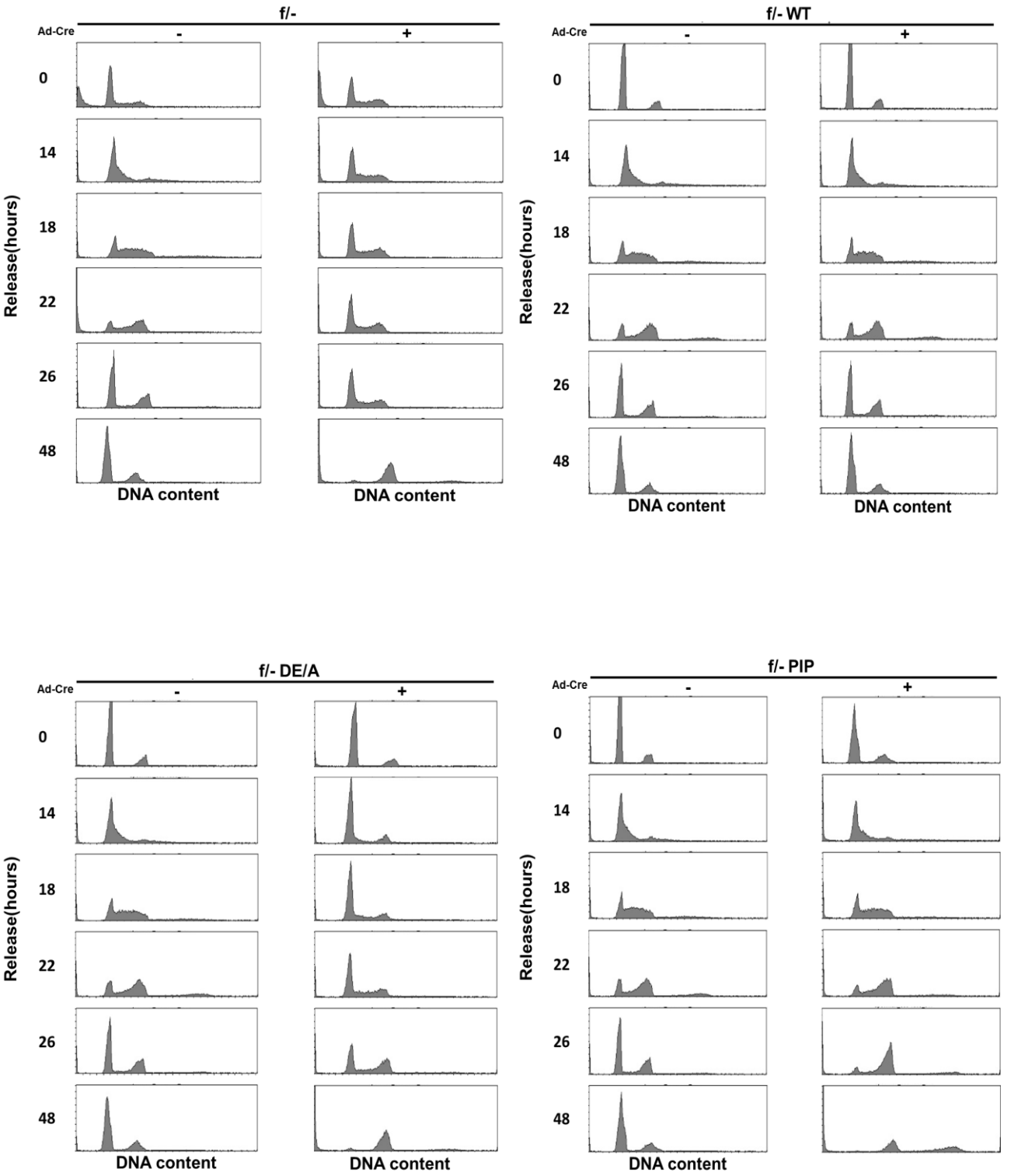

f/-
f/- WT
f/- DE/A
f/- PIP
Ad-Cre
-
+
0
14
18
22
26
48
Release(hours)
DNA content

Figure 10E

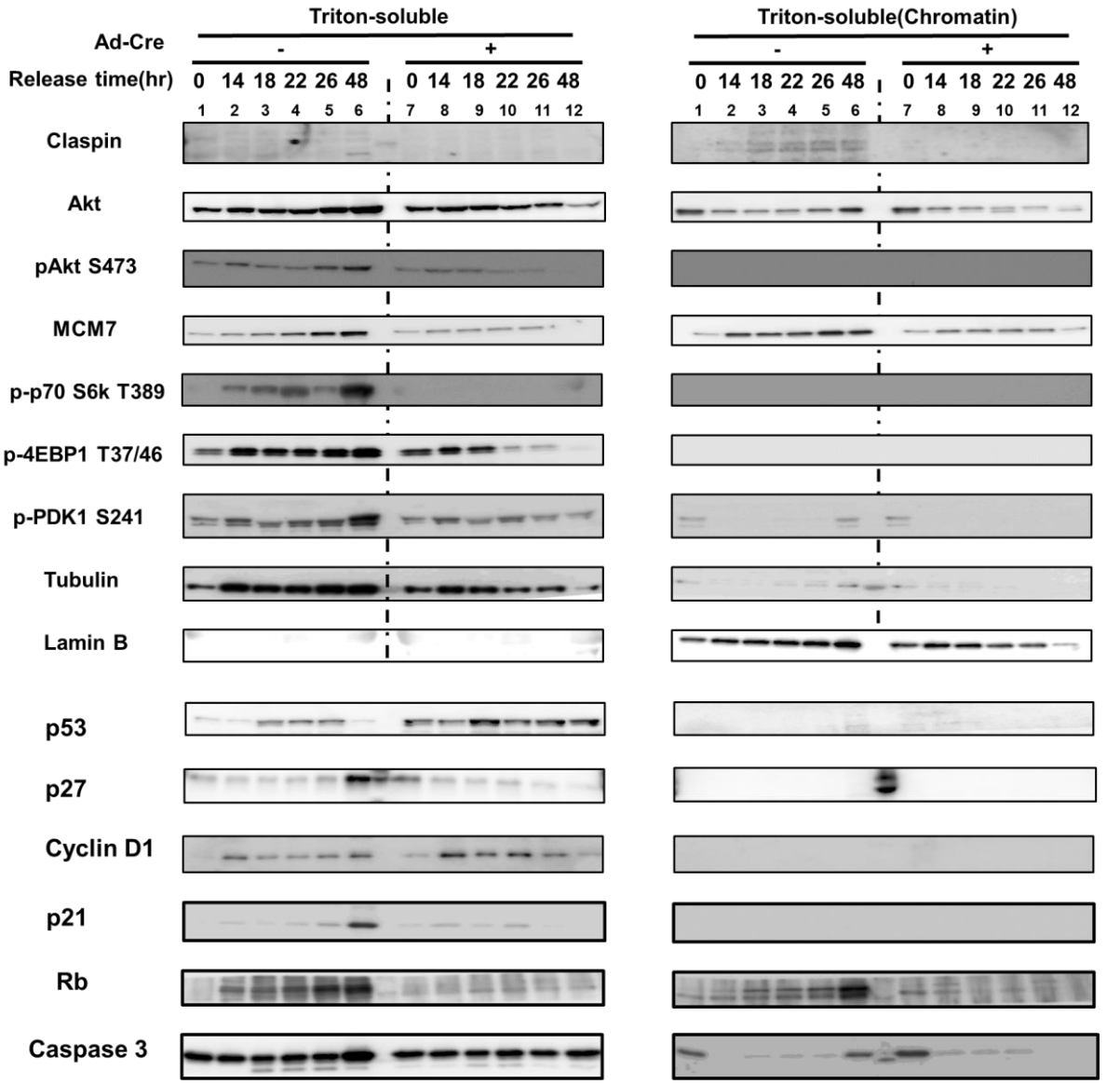

Triton-soluble
Triton-soluble(Chromatin)
Ad-Cre
-
+
Release time(hr)
0 14 18 22 26 48
0 14 18 22 26 48
1 2 3 4 5 6 7 8 9 10 11 12
Claspin
Akt
pAkt S473
MCM7
p-p70 S6k T389
p-4EBP1 T37/46
p-PDK1 S241
Tubulin
Lamin B
p53
p27
Cyclin D1
p21
Rb
Caspase 3
MEF Claspin f/-

Figure 10F