

# 学位論文

動物細胞を用いた

**DNA 複製/チェックポイント因子 Claspin の**

発現、精製と機能解析

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## Summary

Mrc1/Claspin are evolutionally conserved and has been known to play a major role in replication checkpoint regulation. Furthermore, checkpoint-independent roles at a replication fork have been suggested in yeasts. However, the information on biochemical functions of Claspin has been limited, due to difficulties associated with purification of this protein. I have improved expression and purification procedures for human Claspin as well as other high molecular weight replication proteins. I used a CSII-EF-MCS expression vector and Polyethyleneimine (PEI) as a transfection reagent for high-level expression in 293T cells. This method could be generally applicable to purification of large-sized proteins which have been difficult to handle. Purified Claspin stimulated the helicase activity of the MCM4-6-7 complex *in vitro*. This finding can at least partially explain a stimulatory role of Claspin at a replication fork. I also identified interactions of Claspin with replication and checkpoint factors, which may bear physiological significance.

### Abbreviations

ATM; ataxia telangiectasia mutated

ATR; ATM and Rad3-related

Cdc45; cell division cycle 45

CDK; cyclin-dependent kinase

GINS; Go Ichi Ni San

MCM; minichromosome maintenance

Mrc1; mediator of replication checkpoint

ORC; origin recognition complex

PEI; polyethyleneimine

Pol.  $\alpha$ ,  $\delta$ ,  $\epsilon$ ; polymerase alpha, delta, epsilon

pre-RC; pre-replicative complex

RPA; replication protein A

Tim; Timeless

Tipin; Timeless interacting protein

# **General Introduction**

## **1. DNA replication**

DNA replication initiates at replication origins. In bacteria, the initiator protein DnaA recognizes and binds to a single replication origin, called *oriC*, on the chromosome, and then it recruits DnaB helicase onto the partially unwound origin segment to start DNA synthesis. On eukaryotic chromosomes, in contrast, the origin recognition complex (ORC) binds to multiple sites on the genome, where the Cdc6 and Cdt1 proteins recruit MCM complex to generate pre-RC during G1 phase. Phosphorylation events by Cdc7 and Cdk now activates pre-RC and DNA replication is initiated (1). This step involves association of other replication factors including Cdc45 and GINS, which, along with MCM, may be required for generation of active helicase at the fork (2-4). DNA replication is initiated bidirectionally from the origins.

Replication fork machinery is composed of a number of factors which facilitate the unwinding of the duplex DNA and coordinate the DNA chain elongation and formation of chromatin structures. It also contains the factors which may play crucial roles when the progressing forks encounter obstacles along the chromosome. These obstacles include DNA damages, secondary structures on the template DNA, DNA binding proteins, shortage of nucleotide supply and so forth. Replication forks become stalled under these circumstances. The stalled fork could disassemble, if not attended. To prevent disintegration of a stalled replication fork, it needs to be stabilized first. The fork protection complex composed of Swi1-Swi3 in fission yeast plays an important role to maintain the fork integrity (54, 55). Mrc1 is also required for stabilization of stalled replication forks in yeasts (6-11). These factors are also required for replication checkpoint activation.

Cdc7 kinase and Cdk are required for transforming pre-RC into an active replication fork. In this process, Cdc45 and GINS may form a complex to generate an active replicative helicase termed CMG complex at the replication fork (2). Swi1, Swi3 and Mrc1 are conserved throughout evolution and appears to be an integral member of replication fork machinery (54). Mammalian orthologs of Tof1-Swi1, Csm3-Swi3 and Mrc1 are Tim, Tipin and Claspin, respectively, and similar roles in DNA replication have been suggested. Data in yeast indicate that Mrc1 is needed for DNA replication in a manner independent of the checkpoint function, although the precise role of Mrc1 in progression of replication forks has not been clarified mainly due to lack of biochemical characterization.

## **2. DNA replication checkpoint**

When DNA replication is perturbed by genotoxic reagents such as UV or HU (hydroxyurea), cells elicit checkpoint responses to temporally suspend the cell cycle progression until the cause of fork stall is removed and DNA replication can be “safely” resumed. The ATR-Chk1 pathway plays a major role in this signaling cascade.

### **3. Claspin**

Claspin was originally identified as a Chk1-interacting protein in *Xenopus laevis* (5). It is regarded as an ortholog of Mrc1 which was identified in yeasts and has been shown to function as both DNA replication and checkpoint reaction factor. Mrc1 has been shown to be present at both normal and stalled replication forks (6-11), and was shown to play a crucial role in stabilizing the stalled replication fork. In mammalian cells, lack of Claspin leads to slow-down of replication fork progression (12) and the knockdown or knockout of Claspin leads to significant reduction of DNA synthesis as measured by the incorporation of [<sup>3</sup>H]-thymidine in mammalian cells (**Fig. 1**). Thus, Claspin is required for efficient DNA replication, whereas Claspin is also required as a mediator to transmit the checkpoint signal from ATR to Chk1 kinase. During the course of this signal transduction, Claspin is known to be phosphorylated by ATR.

### **4. Aim of my thesis**

The aim of this study is to clarify the roles of the mammalian fork stabilizing factors (Claspin and Tim-Tipin) during normal replication as well as when fork is stalled. For this purpose, I attempted to purify these proteins and biochemically characterize them. Since these factors are associated with replication fork machinery, they are expected to interact with other fork factors. I have examined the interaction of Claspin with replication and checkpoint factors both on cell levels and *in vitro* with purified proteins. Once the interaction is confirmed, the interaction domains can be defined, and effect of mutations on cellular functions can be examined.

My particular focus was on biochemical effect of Claspin and Tim-Tipin on the functions of MCM complexes, including its helicase activity, since the roles of Mrc1/Claspin during the normal course of DNA replication have not been clear in spite of reports that they are required for efficient S phase progression. Furthermore, recent results in our laboratory indicated the interaction of Cdc7 and Mrc1/Claspin and phosphorylation of the latter protein by Cdc7 kinase. Thus, I also examined the interaction between Claspin and Cdc7 and assessed its functional significance. I also tried various conditions and established a novel, convenient and economical method to purify large-sized proteins from mammalian cells, which are normally hard to deal with.

# **Materials and Methods**

## **1. Plasmid construction**

### **mAG-hClaspin-3xFlag, mAG-Flag-Tim, mCherry-GST-Tipin**

mAG-CSII-EF-MCS-Geminin plasmid DNA was a gift from Dr. Atsushi Miyawaki (1). The entire human Claspin cDNA (GenBank: NM022111) were amplified from HeLa RNA by RT-PCR using primers listed in Table 1-1, and a DNA fragment digested by *XhoI* and *XbaI* was cloned in-frame into the *XhoI/XbaI* sites, replacing the geminin part of mAG-Geminin construct. 6xHis and 3xFlag tag sequences were inserted at *XhoI* and *NheI* sites, respectively. Truncation and deletion derivatives of Claspin was constructed on the basis of this plasmid (mAG-Claspin-3Flag) using primer sets listed in (**Table 1-1**).

The entire human Flag-Tim and GST-Tipin cDNA fragment was obtained from plasmid DNAs (pVL-2xFlag-Tim, and pVL1393 GST-Tipin, respectively; constructed by Kawashima and Masai, unpublished) by PCR using primers listed in Table 1-1. The 2xFlag-Tim and GST-Tipin fragments were digested by *Sall* and *XbaI*, and cloned into *XhoI/XbaI* sites of mAG vector, and mCherry vector, respectively.

### **Alanine substituted mutants**

The candidates of phosphorylation site of Claspin detected by nano-ESI-ms were substituted to alanine using QuikChangeR Multi Site-Directed Mutagenesis Kit (Stratagene, Cat. 200514). In this experiment, mAG-6xHis-hClaspin-3xFlag plasmid DNA were used as template, and primers listed in (**Table 1-1**). were used, and the operations were conducted as protocols attached to the kit.

### **Deletion mutants**

For the preparation of C-Del; Digestion of full length of mAG-6xHis-Claspin-3xFlag by *BalI* separated 897-1208a.a. from the plasmid DNA.

For the preparation of #2, 8, 9, 11, 12-del; Each deletion type of Claspin was made by the combinations of primers described in (**Table 1-1**).

## **2. Cell Culture**

293T cells were grown in D-MEM supplemented with 10% fetal bovine serum (HANA-NESCO) at 37°C, 5% CO<sub>2</sub>.

## **3. Transient expression of Claspin in 293T cells with PEI solution**

The solution “A” was made by mixing 0.8 µg of CSII-EF-MCS-mAG-6xHis-Claspin-3xFlag plasmid DNA and 50 µl of 150 mM NaCl, and the solution “B” was made by mixing 4 µg/ml of PEI solution (polyethyleneimine “MAX” (MW25,000) [Cat.24765; Polyscience, Inc.] dissolved in SQ, adjusted to pH7.0 and filtrated) and 50 µl of 150mM NaCl. Solution B was added to solution A and mixed gently. After

30 min incubation at a room temperature, the solution was added to dishes on which 293T cells are grown in fresh D-MEM (1 ml medium for 12-well plate). TransIT®-293 transfection reagent (Cat MIR2705; MIRUS) was used as recommended by the supplier for comparison of transfection efficiency. In expression of human Tim-Tipin complex, an equal amount (0.4 µg each) of Tim and Tipin expression plasmid DNA was mixed with 50 µl of 150 mM NaCl.

#### **4. Detection of mAG fluorescence**

Cells were grown on a 35 mm glass-bottom dish. At 42 hr after transfection, cells were observed under BZ-8000 microscope (Keyence) at 488nm to detect fluorescences of mAG, or under FSX100 microscope (OLYMPUS, **Supplementary Fig. S1C**). The cells were harvested, fixed by ethanol, and were treated by propidium iodide and RNaseA. The histograms of the numbers of cells versus DNA content or mAG fluorescence intensity were prepared by Fluorescent Activated Cell Sorter (Beckton Dickinson, Inc.)

#### **5. Purification of protein**

##### **human Claspin**

Cells (15cm dish x 10 plates) incubated for 42 hr after transfection were harvested and lysed in CSK buffer (10mM Pipes-KOH (6.8), 100mM potassium glutamate, 300mM sucrose, 1mM MgCl<sub>2</sub>, 1mM EGTA, 1mM DTT, 1mM Na<sub>3</sub>VO<sub>4</sub>, 50mM NaF, 0.1mM ATP, protease inhibitor (PI) tablet (Roche), and 0.5 mM PMSF) containing 0.06% TritonX-100 and 10U/ml Benzonase (Amersham plc.). After rotation for 60 min in cold room, the supernatant, harvested by centrifugation, was mixed with Ni-NTA agarose beads (QIAGEN) for 90 min in cold room. The beads were thoroughly washed with Ni Wash buffer (50mM NaPi (7.0), 10% Glycerol, 300mM NaCl, 15mM Imidazole, 0.5mM PMSF, PI tablet), and bound Claspin was eluted by Ni Elution buffer (50mM NaPi (7.0), 10% Glycerol, 300mM NaCl, 300mM Imidazole, 0.5mM PMSF, PI tablet). The Ni eluates were then mixed with anti-Flag M2 affinity beads (SIGMA) for 60 min in cold room. The beads were washed by Flag Wash buffer (50mM NaPi (7.5), 10% Glycerol, 300mM NaCl, 0.2mM PMSF, and PI tablet) and bound proteins were eluted with Flag Elution buffer (50mM NaPi (7.5), 10% Glycerol, 30mM NaCl, 200µg/ml 3xFlag peptide, 0.1mM PMSF, and PI tablet). Each eluate was analyzed by 5-20% gradient SDS-PAGE and proteins were detected by coomassie brilliant blue (CBB) or silver staining. The Flag eluate E3 fraction was used for monoQ purification using SMART. The buffer used in monoQ purification contained 20mM Tris-HCl (7.5), 1mM DTT, 0.01% TritonX-100, 2mM Mg-Acetate, 10% Glycerol, and 0.1mM PMSF. Proteins were eluted by 200mM to 600mM NaCl gradient.

##### **human Tim-Tipin complex**

Cells (10 cm dish x 1 plate) transiently expressing mAG-Flag-Tim and mCherry-GST-Tipin were harvested and lysed as described above. The lysate was mixed with Glutathione Sepharose 4B (GE) for 90 min in cold room. The beads were thoroughly washed with GST Wash buffer (50 mM Tris-HCl (8.0), 0.05 % TritonX-100, 1 mM DTT, 2 mM Mg-Acetate, 30 mM Na-Acetate, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.2 mM PMSF, and PI tablet) and bound proteins were eluted by GST Wash buffer containing 10 mM Glutathione.

The eluates were then purified by anti-Flag M2 affinity beads and a portion of the Flag eluates were applied to monoQ column in SMART system as described above and the proteins were eluted by 200 mM to 600 mM NaCl gradient. The fractions were dialyzed against monoQ buffer containing 30 mM NaCl.

#### **6. Immunoprecipitation for expression check**

Cells (3.5 cm plates) incubated for 42 hr after transfection were lysed by CSK buffer containing 0.06 % TritonX-100 and 10 U/ml Benzonase, and then the supernatants were mixed with anti-Flag M2 affinity beads for 60 min in cold room. The beads were washed by CSK buffer twice and boiled at 96°C for 5 min. Proteins in the supernatant were analyzed by SDS-PAGE and CBB staining.

#### **7. Cleavage by TEV protease and large-scale purification of 6xHis-Claspin-3xFlag**

The mAG-TEV-6His-Claspin-3Flag plasmid was constructed by inserting XhoI-TEV-6H oligonucleotides (shown in **Table 1-1**) at the XhoI site of mAG-Claspin-3Flag. The resulting mAG-TEV-6His-Claspin-3Flag was transiently expressed in 293T cells on 3,5 cm plates. The protein in the extract was trapped by anti-Flag M2 beads as described above, and then those proteins bound to the beads were incubated with TEV Protease (Invitrogen, cat; 10127-017, 10 U/sample) for 1.5 hr at room temperature. The released protein was analyzed by SDS-PAGE, followed by staining with CBB. After these pilot experiments, mAG-TEV-6His-Claspin-3Flag was expressed in 293T cells (15 cm x 10 plates) and purified by the combination of Ni-NTA Agarose beads, cleavage by TEV-protease, and anti-Flag® M2 agarose beads in a large scale.

#### **8. Native gel electrophoresis, glycerol gradient sedimentation, gel filtration and crosslinking**

Native gel electrophoresis was conducted on 5-20% gradient gel (ATTO) in 1x TBE, and the protein was analyzed by CBB staining. Glycerol gradient centrifugation (20-35 %) was conducted at 38,000 rpm for 18 hr at 4 °C in 20 mM TrisHCl (pH7.5), 0.5 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.1 mM PMSF, and 0.01 % TritonX-100 using TLS55 centrifuge tube on TL100 rotor with Optima™ MAX-XP Ultracentrifuge (BECKMAN COULTER). Gel filtration was conducted on Superdex™-200 PC 3.2/30 in SMART system in 50 mM NaPi (7.5), 10% glycerol, 30 mM NaCl, and 0.1 mM PMSF. In both glycerol gradient and gel filtration, high molecular weight native marker proteins (GE healthcare, Inc) were fractionated under the same condition. The fractions were analyzed by SDS-PAGE and silver staining (glycerol gradient) or CBB staining (gel filtration). The bands were quantified by LAS3000 (Fujifilm) and the precise peak position of each fractionation was estimated from Quartic function written in the Excel2007 application (Microsoft). Sedimentation coefficient and the Stokes radius were estimated on the basis of the fraction positions relative to those of markers. The Stokes radius and Sedimentation coefficient values of each marker protein used for calibration are shown in Table 5. The crosslinking of proteins was conducted at 37°C for 3 min in the buffer containing 10mM Tris-HCl (pH7.5), 1mM DTT, 0.5mM EDTA, 10mM K-glutamate, 8% Glycerol and 0.06 or 0.12% of glutaraldehyde (Wako, 073-00536). The reaction was terminated by the addition of 100 mM Tris-HCl (pH8.0) and directly electrophoresed on a native gel, or on

SDS-polyacrylamide gel after the addition of sample buffer containing SDS. Under this experimental condition, I confirmed that MCM4-6-7 protein complex (56) was efficiently crosslinked and migrated at around 600 kDa (hexamer) on SDS-PAGE.

### **9. siRNA transfection using PEI**

Each siRNA oligonucleotide (listed in **Table 2**) was mixed with 4 µg/ml of PEI in 150mM NaCl (or in Opti-MEM), and incubated for 30 min at room temperature. siRNA-PEI complex was added to the cells, which were incubated at 37°C with 5% CO<sub>2</sub>. At 24 hrs after addition of siRNA, medium was replaced to fresh D-MEM, and 2nd transfection of siRNA was conducted. Cells were further incubated for 24 hrs. In case where siRNA and expression plasmid was cotransfected, siRNA and plasmid DNA were mixed with 4 µg/ml of PEI in one tube and transfected into 293T cells as above. At the second transfection, only siRNA-PEI was transfected.

### **10. Preparation of mMCM4-6-7 helicase**

The recombinant baculoviruses expressing 6xHis-Mcm4/Mcm6 or Mcm7-Flag proteins were previously prepared (14). Sf9 and High five insect cells were cultured at 27°C in Sf-900 II SFM (Life Technologies, Inc.) and EX-CELL 405 medium (JRH Biosciences), respectively. For expression of the Mcm4/6/7 proteins, High five cells were coinfecting with recombinant baculoviruses expressing the His6-Mcm4/Mcm6 proteins and those expressing the Mcm7-FLAG, and were collected at 48 h post-infection. The recombinant Mcm4/6/7 complexes were purified as described in previous report (15).

### **11. Preparation of DNA substrates for assays**

#### **M13mp18<sup>37mer-dT<sub>50</sub></sup>**

[gamma-32P]-ATP was labeled to 37mer-dT<sub>50</sub> oligo (**Table 1-2**) by incubation with T4-PNK for 60min at 37°C. This labeling was stopped by adding EDTA and free ATP was removed by G50 column (Amersham). The labeled 37mer-dT<sub>50</sub> oligo was annealed to M13mp18 (TAKARA) and free 37mer-dT<sub>50</sub> oligo was removed by S-400 column (GE).

#### **Y-fork (80 + 110mer)**

[gamma-32P]-ATP was labeled to 50mer + dT<sub>60</sub> oligo (**Table 1-2**) by incubation with T4-PNK for 60min at 37°C. This labeling was stopped by adding EDTA and free ATP was removed by G50 column (Amersham). The labeled 50mer + dT<sub>60</sub> oligo was annealed to 50mer + dT<sub>30</sub> oligo (**Table 1-2**) and separated by 9% 29:1 TBE-PAGE gel containing 5% Glycerol.

#### **Other substrates**

dsDNA, Y-fork, A-fork[3'] and A-fork[5'] substrates were prepared by annealing a 5'-end-labelled 49mer and each unlabelled oligonucleotide and purification of the annealed product from the polyacrylamide gel (73)

### **12. Helicase assay**

Helicase assay was examined in the buffer containing 8% Glycerol, 60mM Hepes-NaOH (7.5), 60mM Na-Acetate, 20mM 2-ME (beta-mercaptoethanol), 0.6mM DTT, 0.3mM EDTA, 10ug/ml BSA, 0.006% TritonX-100, 10mM Mg-Acetate, 10mM ATP. Proteins were mixed in this buffer and incubated for 30min at 30°C with 10fmol/assay (M13mp18<sup>Δ</sup>37mer-dT<sub>50</sub>) or 50fmol/assay (Y-fork). Reacted samples were separated by 12% 19:1 TBE-PAGE gel and Imaging by BAS2500.

### **13. Gel shift assay**

Gelshift assay was examined in the buffer containing 8% Glycerol, 60mM Hepes-NaOH (7.5), 60mM Na-Acetate, 20mM 2ME, 0.6mM DTT, 0.3mM EDTA, 10ug/ml BSA, 0.006% TritonX-100.

Proteins were mixed in this buffer and incubated for 30min at 30°C with 10fmol/assay (37mer-dT<sub>50</sub>) or 50fmol/assay (110mer ssDNA, Y-fork). Reacted samples were applied to 5-20% gradient ATTO gel and electrophoresed in 1x TBE (200V 80min). This gel was analyzed by staining by C.B.B. and Imaging by BAS2500.

Gel shift assays were conducted for 60 min in 30°C with 50 fmol of ssDNA, dsDNA, Y-fork, A-fork[3'], or A-fork[5'] substrate in reactions (8 μl) containing 8 % glycerol, 10 mM Tris-HCl (pH7.5), 1 mM DTT, 0.5 mM EDTA, 40 μg/ml BSA, 50 mM K-glutamate, and the protein fraction of indicated amount which had been dialyzed using Dialysis Cup (Bio-Tech, MWCO8000) to remove the 3xFlag® peptide. The samples were loaded on polyacrylamide gel (5%, 29:1) in 1xTBE buffer and electrophoresed at 180V for 90 min. The gel was dried and analyzed by BAS2500 (Fujifilm). For antibody supershift assay, Claspin was preincubated with anti-Claspin antibody (made in our laboratory against a Claspin polypeptide [amino acids 876-1014]) for 30 min on ice before addition to gel shift assay reactions. The reactions were loaded on 3.5% polyacrylamide gel (29:1) containing 2.5% glycerol in 1xTBE buffer, and electrophoresed at 180V for 80 min.

### **14. Kinase assay of Cdc7-ASK**

The substrates Claspin polypeptides were prepared by immunoprecipitation from extracts of 293T cells transiently expressing each Claspin derivative. The immunoprecipitation was conducted by incubating each extract with Dynabeads-proteinG (Invitrogen, Cat. 100.03D) and anti-Flag M2 antibody for 40 min at room temperature, and followed by wash steps using Citrate-Phosphate buffer (5.0) described in Invitrogen's protocol. Substrate proteins attached to Dynabeads-proteinG-Flag antibody were directly used in kinase assays for Cdc7-ASK in 25 μl reactions containing 40 mM Hepes-KOH (7.6), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM glycerophosphate, 1 mM NaF, 2 mM dithiothreitol, 13.3mM MgOAc, 0.1 mM ATP, 50 ng purified human Cdc7-ASK, and 1 μCi of [ $\gamma$ -<sup>32</sup>P]-ATP. Reactions were incubated at 30°C for 60 min and loaded onto 8% SDS-PAGE (29:1). The gels were stained with silver and phosphorylations were detected by BAS2500. Truncated polypeptides (8 polypeptides covering all the coding region of Claspin) derived from Claspin were expressed as RGS-His fusions and were also used as substrates in the kinase assays as described above.

### **15. Immuno-precipitation with purified proteins**

Purified Claspin and MCM4-6-7 were mixed with Dynabeads proteinG (Invitrogen) and rotated for 2hr in cold room in the same buffer condition as helicase assay. The beads were washed twice by PBS(-) + 0.05% TritonX-100 (20x bed volumes of resin) and boiled with sample buffer for SDS-PAGE.

### **16. Analysis of phosphorylation sites of Claspin (nano-ESI-MS)**

15cm x 40plates of HCT-116 cells were synchronized by adding 2.5mM thymidine and incubation for 18hr. Then cells were released into fresh medium and incubated for 5hr. Next 30ng/ml of nocodazole was added and incubated for 7hr. The synchronized cells were released to fresh medium and incubated again. 6hr after this release, cells were harvested and lysed by adding CSK buffer containing 10mM Pipes-KOH(6.8), 100mM potassium glutamate, 300mM Sucrose, 1mM MgCl<sub>2</sub>, 1mM EGTA, 1mM DTT, 1mM Na<sub>3</sub>VO<sub>4</sub>, 50mM NaF, 0.1mM ATP, protease inhibitor (PI) tablet (Roche), 0.5 mM PMSF, and 1% TritonX-100. Lysate was mixed with Dynabeads proteinG (Invitrogen) and anti-Claspin #6 a.b. and rotated for 2hr in cold room. Bound sample was washed with PBS(-) + 0.05% TritonX-100 + 300mM NaCl (5 times by 20x bed volumes). Washed beads were boiled with sample buffer for SDS-PAGE and the about 4pmol of endogenous human Claspin was separated by cut-out. The denaturized Claspin was further alkylated reductively and analyzed by nano-ESI-MS (APRO life Science Institute, Inc).

### **17. Protein-protein interaction on PVDF-membrane**

Purified MCM2-3-4-5-6-7 or MCM2-4-6-7 was applied onto SDS-PAGE gel (7% 29:1). The separated MCM proteins were transferred to Immobilon-P PVDF Transfer Membrane (IPVH00010, Pore size: 0.45µm, Millipore), followed by blocking with 5% skim-milk (30 min at room temperature), wash, and blotting with 500 ng of purified Claspin (incubation in cold room O/N). The Claspin protein bound to membrane was detected by anti-Claspin antibody, as normally conducted in western blotting analyses.

# **Chapter 1.**

## **Expression and Purification of Claspin**

## **Introduction**

The sizes of eukaryotic proteins are generally large, and purification of large-sized proteins in an intact form is technically challenging. Expression in *E. coli* or in insect cells often results in low-level expression or failure to recover proteins in soluble fractions. To overcome these difficulties, I have used expression in mammalian systems for purification of large-sized proteins. The features of my systems include high-level expression using a vector containing a strong EF1 $\alpha$  promoter with Kozak sequence prior to ATG (derived from CSII-EF-MCS [URL1]) and use of high-molecular-mass polyethyleneimine reagent prepared in house for easy and economical transfection into human cells.

### **1. High-level transient expression of proteins in mammalian cells**

Numerous vectors for expression in mammalian cells have been developed. I have used CSII-EF-MCS vector, originally developed as a lenti-virus vector for gene therapy [URL1]. The cloning of the gene of interest at the MCS permits its expression as a fused polypeptide with mAG (monomeric Azami-Green), under the strong EF1 $\alpha$  promoter. Expression is facilitated also by the presence of the Kozak sequence prior to the ATG codon of the mAG. The presence of the fluorescent tag permits easy detection of the expression. I have incorporated a TEV cleavage site immediately after the fluorescent protein, enabling the removal of the fluorescent polypeptide from the purified protein (**Fig. 4**).

### **2. Transfection into mammalian cells with an efficient and low-cost transfection reagent**

One drawback in the use of the mammalian system for large scale expression is the efficiency and cost of gene transfer. I have used high-molecular-mass polyethyleneimine "MAX" reagent (PEI, MW25,000, Polyscience, Inc. Cat. 24765, Linear) solution for easy and efficient transient transfection into 293T cells. PEI is a cationic polymer with abundant positive surface charges, and its potential as a gene carrier has been recognized (16). PEI has been developed as a non-viral gene delivery system (17). It is soluble in water, and is a linear (or branched) polymer with a protonable amino group in every third position (18, 19). Due to their high cationic charge density at physiological pH, PEI can form a non-covalent complex with DNA (**Fig. 2**).

Viruses, as a biological carrier of gene, are used widely in the ongoing clinical trials ([www.wiley.co.uk/genetherapy/clinical](http://www.wiley.co.uk/genetherapy/clinical)), but there are a lot of safety concerns associated with viral vectors, such as induction of immune reactions, possibility of insertional mutagenesis, and the appearance of viruses in the treated patients (20-23), which downrates their clinical utility. Therefore, non-viral vectors such as cationic liposomes or polycations have been explored (24-26), although these synthetic carriers, including PEI solution (27, 28), have not been able to surpass the viral carriers in terms of the efficiency and specificity of gene delivery. However, the recent improvement of PEI solution has led to dramatically boosted efficiency and specificity (29). Thus, the PEI solution, among the polycationic polymers, is

regarded as a hopeful system for DNA delivery (30, 31). According to recent reports, PEI is also useful for siRNA experiments (32-34). The PEI-DNA or PEI-RNA complexes are easily incorporated into cells with endocytosis.

Another practical issue is the cost for the transfection. Although marketed transfection reagents are convenient and could offer a good result, they are generally very expensive and are not suitable for large scale transfection experiments which would be required for purification of a large amount of protein for biochemical characterization. The cost for the in house protocol by the use of the PEI solution is 1/1000 of the commercial products. Furthermore, I found that transfection by the protocol described here lead to less degradation of the expressed recombinant proteins with an identical expression level (**Fig. 3**).

## **Experiments and results**

### **1. Detection of expression**

With the use of the current expression vector, the expression of the gene of interest can be readily confirmed by mAG fluorescence. mAG emits strong green fluorescence unaffected by pH (35). I have also developed a vector containing a TEV cleavage site downstream of mAG, permitting the removal of the mAG portion from the purified protein.

Human Claspin cDNA (4020bp) was amplified by PCR and the fragment was cloned into the CSII-EF-MCS vector. The resulting expression plasmid DNA was transfected into 293T cells with polyethyleneimine “MAX” reagent. DNA (in 150 mM NaCl) and PEI (in 150 mM NaCl) were mixed and pre-incubated for 30 min at a room temperature, and then the PEI-DNA complex was added to 293T cells (**Fig. 1**). The combination of PEI solution and CSII-EF-MCS-based vector system significantly improved the expression levels of recombinant Claspin (**Fig. 2B**). Three conditions (SQ water, Opti-MEM, or 150mM NaCl) for preincubation were examined. Opti-MEM or 150 mM NaCl gave similar efficiency, but water gave a very poor result. High transfection efficiency was achieved over a wide range of PEI concentration (5µg/ml). At a higher concentration (8~15 µg/ml), toxicity against cell viability was observed. The transfection efficiency by PEI or TransIT293 was quantitatively analyzed by FACS (**Fig. 3C**). The transfection efficiency by PEI was slightly lower than that by TransIT293, but the numbers of viable cells after transfection by PEI exceeded those after transfection by TransIT293, reflecting lower toxicity of PEI. Furthermore, degradation of the expressed Claspin was reduced with the PEI method compared to the commercially available TransIT293 reagent (**Fig. 3D**). This may be related to the fact that the slightly lower expression level in each cell by PEI transfection. The Tim-Tipin complex was also successfully expressed by the same method (**Fig. 3D**).

### **2. Purification of the expressed protein**

I inserted a TEV protease recognition sequence between mAG and Claspin, because the mAG tag (226a.a.) is relatively big and may potentially affect the function of Claspin. The mAG-TEV-6His-Claspin-3Flag (or mAG-2x[TEV-6His]-Claspin-3Flag) protein was expressed and trapped with anti-Flag M2 affinity beads as above, and then the mAG portion was removed by incubation of the beads with TEV protease (**Fig. 4**). The resulting 6His-Claspin-3Flag protein could be obtained by further purification by TEV protease cleavage of the Flag-beads bound protein (**Fig. 5**). The total yield of Claspin was about 200 µg from 15cm x 10 plates of 293T cells.

### **3. siRNA experiments with PEI**

It would be very convenient if one can express a protein from the exogenous plasmid and simultaneously

knockdown the endogenous gene, since that will permit the functional analyses of the mutant forms of the protein of interest. I first examined whether efficient siRNA transfection could be achieved with PEI (**Fig. 6A**). I used siRNAs for Cdc7 and Claspin, that have shown the knockdown of the endogenous genes with oligofectamine reagent. I showed that both Cdc7 and Claspin could be knocked-down in 293T cells by using PEI in 150mM NaCl (**Fig. 6B**). Two consecutive transfection with a 24 hr interval resulted in more efficient inhibition of Cdc7 expression (**Fig. 6A**).

I then examined whether siRNA and an expression vector could be transfected simultaneously. I transfected siRNA for Cdc7 and mAG-hClaspin-3xFlag vector. Cdc7 knockdown was also observed also when mAG-Claspin-3xFlag was overexpressed (**Fig. 6C**). I then transfected and tested the effect of Claspin siRNA targeted to non-coding segment along with mAG-hClaspin-3Flag vector (**Fig. 6D**). While endogenous Claspin was downregulated, the expression of mAG-hClaspin-3Flag Del #2 was detected (**Fig. 6E**). The knockdown or knockout of Claspin leads to significant reduction of DNA synthesis as measured by the incorporation of [<sup>3</sup>H]-thymidine in mammalian cells (**Fig. 1**). Whether the expression of the wild-type of mAG-hClaspin-3Flag can restore the level of DNA synthesis is now being investigated.

## **Discussion**

### **1. Expression and purification of high-molecular weight proteins using mammalian cells as a host**

Various expression systems have been developed to facilitate purification of functional proteins. The expression and purification of large-sized proteins have sometimes been technically difficult due to low level expression, degradation, or failure to recover in soluble fractions. I report here that high-level expression of large-sized proteins in mammalian cells by combining a highly efficient vector system and an efficient and low-cost transfection method permits purification of functional and full-length proteins with relative ease.

### **2. Level of expression**

I applied my method to purification of human Claspin and Tim-Tipin complex known to play important roles in DNA replication checkpoint control. The size of Claspin is more than 200kDa on SDS-PAGE, and it has been difficult to purify the intact protein in *E. coli*. Claspin could be expressed in Sf9 insect cells, but the expression level was not sufficiently high to obtain amount enough for biochemical characterization. It was reported that 2 µg of Claspin protein could be obtained from  $1 \times 10^7$  cells of Hi5 cells (37). In my current protocol, more than 5 µg of highly purified Claspin was reproducibly acquired from  $1 \times 10^7$  293T cells (**Fig. 2**). Tim and Tipin were also coexpressed in 293T cells and could be purified as a complex.

The high-level expression may be due to the vector used. pcDNA3.1(+)-Claspin plasmid did not give sufficient expression of Claspin in the same cells, using the identical transfection reagents. The EF-1 $\alpha$  promoter and the Kozak sequence present on the vector might facilitate the expression. I could remove the mAG tag by TEV protease, and obtained the purified protein without mAG (**Fig. 5**). In heterologous expression systems, Claspin might be recognized as a foreign substance and may be targeted for degradation or secluded into insoluble fractions. The presence of highly acidic segments consisting of many asparatic and glutamic acid (~50%) might render the protein prone to degradation or precipitation into insoluble materials.

### **3. Cost for transfection**

Polyethyleneimine "MAX" reagent used in this report is very inexpensive, yet gave rise to transfection efficiency comparable to commercially available reagents (**Fig. 2**). The 50 µl PEI reagent required for transfection on 10 cm dish costs 0.55 yen (0.65 cents), 1/1000 of the commercially purchased reagent (such as TransIT293). Transfection into 293T cells on twenty 15cm-plates (25 ml culture/ plates), would cost 27.5 yen (30 cents) with PEI reagent, in contrast to over 34,000 yen (\$400) with TransIT293.

### **4. Prevention of degradation during expression and purification**

Unexpectedly, I found that transfection by PEI results in less degradation of the expressed proteins although I do not know the precise reason for this. The PEI exhibited the transfection efficiency almost comparable to TransIT293 (Fig. 3A), and was less toxic, as judged from the numbers of the viable cells after transfection (data not shown). However, in terms of the level of expression, TransIT293 gave rise to more cell populations expressing a high level of the fusion protein than PEI did (Fig. 3C). This may lead to higher toxicity as well as to more degradation of the expressed protein with TransIT293. Large proteins tend to be degraded and prevention of degradation is crucial for purification of full-length polypeptides. Reduced degradation of the expressed proteins would be added advantage in using the PEI reagent (**Fig. 1C**).

### **5. Solubility of the expressed proteins and purification**

Claspin expressed at a high level in 293T cells was largely soluble and could be readily purified by the two affinity columns (**Fig. 5**). Similarly, GST-tagged Tipin formed a complex with co-expressed Tim1 and could be purified as a Tim-Tipin complex (**Fig. 9**). These data suggest recombinant proteins expressed in this system can be purified as functional proteins.

### **6. Other advantages of the current expression and purification system**

Other advantages of using mammalian transient expression system for purification of proteins include; 1) Presence of post-translational modification (such as phosphorylation, acetylation or glycosylation) on the expressed proteins. 2) Ease by which the interacting proteins can be analyzed using the tag attached to the expressed proteins. Due to overexpression of the target protein, weakly interacting proteins can be detected. 3) Multiple proteins can be coexpressed simply by cotransfecting 293T cells with multiple expression plasmids. This will facilitate the purification of a protein complex. 4) Recombinant Lentiviruses can be generated by simply transfecting 293T cells with the expression plasmid together with pCAG-HIVgp [URL2] and pCMV-VSV-G-RSV-Rev [URL3].

### **7. Other utility of PEI for protein analysis**

Recent reports have indicated the utility of PEI for siRNA transfection (18-20) and therefore I applied this reagent to Cdc7 and Claspin knockdown (**Fig. 6**). Successful knockdown was confirmed for both Cdc7 and Claspin. siRNA transfection worked even when plasmid DNA was transfected at the same time (**Fig. 6C, 6E**). Cdc7 was efficiently silenced while the exogenous Claspin was expressed. The double transfection worked also in the combination of the Claspin siRNA and Claspin expression vector. The simultaneous double transfection method would enable rapid functional characterization of various mutant forms of mammalian proteins on a cellular level.

### **8. The effect of mAG-tag, transfection reagent on the expression level of protein**

Three types of fluorescent tag (mAG, mKO2, and mCherry) do not seem to affect the expression level, although at present it is possible that the presence of a fluorescent tag may inhibit the degradation since I have not compared the expression level between the tagged and non-tagged proteins. The kinds of

transfection reagent used, although may affect the stability, do not affect the expression level perse. The level of protein expressed may mostly depend on the promoter and Kozak sequence used on the vector.

### **9. The effect of mAG-tag on the function of Claspin**

The presence of the mAG-tag does not seem to affect the biochemical functions of Claspin. mAG-tagged Cdc7 and tag-free Claspin exhibited the same biochemical activities in interaction with Cdc7 (**Fig. 11**).

### **10. The potential contamination of DNA in the purified preparation**

I used Benzonase (or DNase) in lysis step. 10 U/ml of Benzonase is present in the buffer used for lysis of the cells and that used for Ni-beads binding. I speculate that almost all the nucleic acids are degraded during these steps. The viscosity of the lysate is in fact low. Moreover, as for Claspin protein, it binds rather inefficiently to DNA except for Y-fork. Therefore, it is likely that there is vert little contamination of nucleic acids in the purified Claspin fractions.

## **Chapter 2.**

### **Biochemical characterization of Claspin**

# **Introduction**

## **1. Discovery of Claspin**

Claspin, originally discovered as a Chk1-interacting protein in *Xenopus laevis* (1), may play a crucial role in stabilizing the stalled replication fork and mediating the checkpoint signal responses.

The expression level of Claspin changes during the cell cycle, being low during early G1, high in G1/S-phase, and degraded at G2/M in a manner controlled by the caspase and/or ubiquitin proteasome pathway (38-41).

## **2. Role as a checkpoint factor**

Claspin binds to both Chk1 and ATR, and is necessary for ATR-dependent phosphorylation of Chk1 kinase in both *Xenopus* and human systems (42-44). Claspin has been shown to be an essential protein for the ATR-dependent activation of the DNA replication checkpoint responses, and has been regarded as a mediator of checkpoint signaling. Claspin is required for ATR to phosphorylate Chk1, and the phosphorylation of Chk1 depends on its association with Claspin (1). In fact, activation of checkpoint induces Claspin phosphorylation, which is essential for Claspin-Chk1 association and subsequent activation of Chk1 (3).

Similar to *Xenopus* Claspin, human Claspin is also required for phosphorylation of Chk1, it interacts with Chk1, and the association of two proteins depends on the phosphorylation of Claspin (2). Claspin also interacts with other checkpoint proteins, ATR and Rad9 (2).

## **3. Role as a replication factor**

The reduction of endogenous Claspin protein level by siRNA in human cells lead to slow rate of replication fork movement (12, 45), while the overexpression of a non-degradable form of Claspin resulted in delay in the inactivation of Chk1, and recovery from the DNA replication checkpoint (39, 46, 47). It was proposed that a proper amount of Claspin is required for regulated checkpoint responses, and therefore the regulation of its expression levels would be important for proper regulation of the ATR-mediated checkpoint responses as well as for faithful DNA replication.

Previous reports indicate that the yeast ortholog of Claspin, Mrc1, moves with replication fork and is also necessary for a normal rate of DNA replication (6-11). Claspin and Mrc1 were reported to bind to DNA and have a high affinity for branched DNA *in vitro* (37, 48). The association of Claspin in *Xenopus* egg extracts with chromatin depends on Cdc45 and occurs at approximately the same time as polymerase  $\epsilon$  (49).

## **4. Interaction at a DNA replication fork**

Interactions among other DNA replication factors have been reported (Cdc45, replication protein A (RPA),

and DNA polymerase  $\epsilon$ .) (50-52). Moreover, in addition to the interactions among checkpoint factors (ATR, Rad9 and Rad17), interactions with Caspase-7, SCF- $\beta$ TrCP have been also reported (41, 53). It was also reported recently that Mrc1 directly interacted with MCM6, a subunit of the MCM helicase, in *Saccharomyces cerevisiae* (57).

### **5. Role of Claspin/Mrc1 in cell growth and replication fork**

It was reported that depletion of Claspin in human cells lead to inefficient growth, whereas its overproduction stimulated growth. The replication fork rate was reduced and inter-origin distances became shorter in Claspin-depleted cells (45). It was also shown in yeast that Mrc1 was needed for proper rate of DNA replication fork progression in a manner independent of its checkpoint function. These results indicate that Claspin/Mrc1 plays a positive role during the normal course of DNA replication, and it may be related to its checkpoint-independent function at the replication fork.

### **6. Tim-Tipin complex**

Tim-Tipin protein complex is involved in the activation of Chk1 by ATR in the DNA replication checkpoint system (63, 64). It is also suggested that the complex is required for inhibition of the replication fork movement (63-66). Tim-Tipin complex was reported to be co-immunoprecipitated with MCM from cell lysates (64, 65). Direct interaction of Tim-Tipin with MCM was also observed (67). It has been reported that the yeast ortholog of Tim-Tipin, Tof1-Csm3 (*Saccharomyces cerevisiae*) or Swi1-Swi3 (*Schizosaccharomyces Pombe*), are required for the association of Mrc1 with chromatin at the replication fork (68).

## **Experiments and results**

### **1. Purified Claspin exists mainly as a monomer**

Claspin is 1339 amino acid long and the calculated molecular weight is 151.2 kDa. However, both endogenous and recombinant Claspin migrates around 230 kDa in SDS-PAGE. This may be because of the presence of the clusters of aspartic acids and glutamic acids which cause anomalous migration on SDS-PAGE. In order to clarify the subunit structures of Claspin, I analyzed the purified Claspin protein on glycerol gradient and gel filtration. On glycerol gradient, however, it sedimented around 140 kDa (**Fig. 7A**). On the other hand, Claspin was eluted between 440 kDa and 600 kDa on gel filtration (**Fig. 7B**). The Claspin bands were quantified to generate the calibration curves, and the precise peak fraction values were estimated from quartic functions. The sedimentation coefficient predicted from glycerol gradient is 6.9S and the Stokes radius predicted from the gel filtration pattern is 75Å. Therefore, the molecular weight is estimated to be 213kDa (75). Since the calculated molecular weight of 6His-Claspin-3Flag is 156 kDa, this value is closer to the monomer size.

On a native gel, Claspin migrates between 232 and 440 kDa markers (**Fig. 7C**). In order to evaluate the size of Claspin more precisely, Claspin was treated with glutaraldehyde (76) and analyzed on a native gel and SDS-PAGE (**Fig. 7C**). It migrated a little faster on a native gel after glutaraldehyde crosslinking. On the other hand, its migration was slightly retarded on SDS-PAGE, but not to the extent that it can be regarded as a dimer size. On the basis of all the data, I have concluded that the purified Claspin exists as a monomer.

### **2. DNA binding properties of purified Claspin protein**

Sancar's group previously reported that purified human Claspin protein binds to branched DNA structures (37). Using various DNA substrates, I confirmed DNA binding properties of the Claspin protein expressed and purified in this system (**Fig. 8A**). Claspin bound to Y-fork substrate most efficiently among the DNA structures examined. The reduction of the shifted band intensity and its supershift induced by the addition of anti-Claspin antibody showed that the shifted band indeed contained Claspin protein (**Fig. 8B**). Very little binding was observed on ssDNA and that to dsDNA was much weaker than that to Y-fork. Next, effect of the presence of an annealing strand on an unreplicated arm was examined. A-fork[3'] or A-fork[5'] containing a nascent lagging or leading strand, respectively, was bound by Claspin less efficiently compared to Y-fork. The results are mostly consistent with the previously reported binding specificity of Claspin (37), and suggest that the most preferred fork structure bound by Claspin is that containing two single-stranded unreplicated arm DNA.

I also confirmed the binding properties of Claspin, Tim/Tipin with other substrates in gel shift assays (**Fig. 9B**). Two distinct protein-DNA complexes (IV and III) were detected in gel shift assays on a Y-fork (**Fig. 9C**). When a small amount of Tim-Tipin (**Fig. 9A**) was added in the presence of Claspin, the fast migrating complex (IV and III) disappeared and the intensities of the slow-migrating complex (II) increased. The

addition of anti-Claspin antibody (**Fig. 9D**) caused supershift of the complexes (IV, III, and II migrated to I), while the addition of anti-GST antibody (against GST-Tipin; **Fig. 9E**) caused no shift, suggesting that these complex (IV, III, and II) contained only Claspin. At the concentrations used, Tim-Tipin alone did not show any DNA binding. These results indicate that Tim-Tipin somehow remodels the Claspin-DNA complexes (see below).

### **3. Analyses of protein-protein interactions using the expressed protein.**

An advantage of using human cells as a host for expression is that one can characterize the expressed proteins in the cell level. Using the tagged protein as a bait, one can examine the interaction with other proteins or search for novel interactors by mass spectrometry analyses. Overexpression may permit the detection of the weak interactions which could be difficult to detect at the endogenous level.

I examined the interaction between Claspin and endogenous Cdc7 in 293T cells expressing the tagged Claspin protein at a high level. Although physical interaction between Claspin and Cdc7 was previously reported at the endogenous level (36), the interaction was very weak and preparation of the enriched chromatin fraction was needed to detect the interaction. I expressed Claspin fused to three different fluorescent tags (mAG, mKO2 and mCherry) in the presence or absence of 3Flag tag for each case. Pull-down with anti-Flag® M2 affinity beads showed that Cdc7 was coprecipitated only when the bait contained a Flag-tag in each three fluorescent tag construct (**Fig. 11**). This experiment clearly demonstrates that Claspin interacts with Cdc7. It also shows that even weak interactions can be efficiently detected in my overexpression system.

I examined the presence of other checkpoint and DNA replication factors in the pulled-down samples. I was able to detect ATR, ATM, Chk1, Mcm subunits, MCM10, Tim, Cdc45 and DNA polymerase  $\alpha$ ,  $\beta$ , and  $\epsilon$  in the immunoprecipitates from the Flag-tagged Claspin extract, but not from non-tagged Claspin extract (**Fig. 10A**). These results indicate that Claspin would interact with panels of replication factors as well as with checkpoint factors (**Fig. 10B**).

### **4. Molecular dissection of Claspin.**

Human Claspin (1339a.a.) sequence was applied to BLAST search engine in NCBI site ([URL 4], **Fig. 12**), and Coiled-Coils search engine ([URL 5], **Fig. 13**). This BLAST search suggested the presence of two highly conserved domains at N-terminus (amino acid 250-300) and C-terminus (1030-1200), while the coiled-coil search showed high score at 160-300, 580-700, and 910-1200. The N-terminus segment, 160-300, which is conserved and highly scored for coiled-coils, would be a DNA binding domain because the sequence of GTTRKERKAARLSKEALKQLHS (270-291) was also detected by Helix-turn-Helix prediction engine [URL 6]. Indeed, this sequence was already suggested to be a part of DNA binding domain in Claspin (37). This N-terminus region was conserved in Mrc1 (*S. pombe*) and was shown to be required for interaction with DNA (49). In pursuit of the potential functions of the middle (351-896, especially in 600-700) and C-terminal domains (897-1209), various deletion mutants were made.

I generated a series of truncation or deletion mutants of Claspin and determined which segment

of Claspin is required for interaction with these factors. The results indicate that Tim-Tipin interacts with the N-terminal segment of Claspin, whereas Cdc7 interacts with the C-terminal segment of Claspin more preferentially (**Fig. 14**). The central and C-terminal segments of Claspin can bind to MCM.

### **5. Phosphorylation of Claspin by Cdc7 kinase**

It was previously reported that Claspin undergoes phosphorylation upon fork arrest with HU. This hyperphosphorylation depends on Cdc7 function. The purified Claspin was efficiently phosphorylated by Cdc7 kinase *in vitro* (**Fig. 15A**, lane 18). I then examined the truncation derivatives of Claspin expressed in mammalian cells described in **Fig. 14**. The expressed proteins were pulled down with anti-Flag antibody and the beads containing the mutant Claspin were used as substrates in *in vitro* Cdc7 kinase reaction (**Fig. 15A**). The pulled down Claspin and its derivatives were associated with unknown kinase which phosphorylates the Claspin. This unknown kinase appears to interact with the N-terminal segment (amino acid 1-350) of Claspin. The C-terminal 443 amino acid (897-1339) is not associated with an endogenous kinase but is efficiently phosphorylated by Cdc7 kinase (lane 10).

The truncation or deletion mutant polypeptides expressed were purified by Ni, Flag, and gel filtration column, and were used as substrates for kinase assays (**Fig. 15B**). The results of this experiment showed that the major phosphorylation sites on Claspin by Cdc7 are located at C-terminal segment of Claspin.

I also expressed and purified 8 polypeptides derived from the entire Claspin coding frame and used them as substrates for *in vitro* Cdc7 kinase assays (**Fig. 15C**). The fragments 1 (1-194), 2 (195-335), and 7 (1015-1143) were efficiently phosphorylated by Cdc7 *in vitro*. These results indicate that Cdc7 may interact preferentially with the C-terminal segment (1209-1339) and phosphorylate the segment 1015-1143. Cdc7 also phosphorylates the N-terminal 335 amino acid segment. Consistent with this, Cdc7 can interact with the N-terminal 350 amino acid polypeptide albeit with reduced efficiency (**Fig. 14**).

I synchronized HCT116 cells by thymidine and nocodazole at G2/M phase, and harvested the early S phase cells at 6 hr after release. The cells were lysed and endogenous Claspin was acquired by Immuno-precipitation using anti-Claspin antibody immobilized on Dynabeads proteinG (**Fig. 15D, E, F**). Claspin protein bound to the beads were boiled in sample buffer for SDS-PAGE and electrophoresed. Four pmol of Claspin was cut out from the gel and analyzed by nano-ESI-MS (APRO life Science Institute, Inc). Potential S phase-specific phosphorylation sites were identified (**Fig. 15G**).

## **Discussion**

### **1. Size of purified human Claspin**

Claspin was purified by combination of Ni-NTA agarose beads and anti-Flag® M2 affinity beads. The mAG moiety was removed by TEV protease digestion. The 6His-Claspin-3Flag protein (1377 amino acids, 156 kDa) was examined in native gel, glycerol gradient, or gel filtration (**Fig. 7**). The data from glycerol gradient and gel filtration were used to estimate the size of the Claspin protein as 213 kDa. On a native gel electrophoresis, the protein migrated around 232kDa. Crosslinking with glutaraldehyde did not significantly affect its migration on a native gel or on SDS-PAGE. Based on these data, I speculate that Claspin exists as a monomer. Claspin is highly enriched in acidic amino acids (298 aspartic acids and glutamic acids out of 1339 amino acids) and calculated pI is 4.74. The highly negatively charged surface may affect the stokes radius and lead to a larger estimated molecular weight.

### **2. DNA binding activity of human Claspin**

As reported before, the Claspin protein purified in this system also bound strongly to Y-fork DNA. Claspin did not bind to ssDNA and dsDNA efficiently. A-fork[3'] or A-fork[5'], carrying an annealing strand on the leading or lagging arm, respectively, was bound by Claspin, but less efficiently than Y-fork (**Fig. 8**). These results mostly confirm previous reports (37) and suggest that Claspin prefers the fork-like structures with two single-stranded DNA arms.

### **3. Claspin interacts with various replication factors**

Using the efficient expression of a tagged protein, I was able to examine interaction of Claspin with various replication/ checkpoint factors (**Fig. 10A**). I detected binding of ATR, Chk1, MCM4, MCM10, Cdc7, Tim, Cdc45 and DNA polymerase  $\alpha$ ,  $\beta$ , and  $\epsilon$ . with ectopically expressed Claspin protein. Interaction between ATR and Chk1 is expected from its important role in replication stress-induced checkpoint signaling. Indeed, Claspin was originally identified in *Xenopus* egg extracts as a Chk1-interacting factor (5).

Interaction of Claspin with Mcm and Cdc45 is consistent with its association with replication fork machinery (58). The interaction between Mrc1 (counterpart of Claspin) and MCM/ Cdc45 was previously reported in yeast and *Xenopus* egg extracts (49, 51, 57), but not in human cells. The combination of high level expression of Claspin as well as the use of the efficient tag (3Flag) may facilitate the detection of inefficient interaction. Interaction of Claspin and Tim-Tipin was previously reported in human cells and *Xenopus* egg extracts (64, 72). I also reported interaction between fission yeast Mrc1 and Swi1-Swi3 complex (73). Novel and most striking interaction was detected with DNA polymerases. Claspin interacted with three DNA polymerases known to be required for replication. Interaction with  $\delta$  and  $\epsilon$  was efficient, whereas that with  $\alpha$  was weak. Interaction between Mrc1 and DNA polymerase  $\epsilon$  was previously reported in budding yeast (11). Strong interaction with DNA polymerases is unexpected, but this may indicate a

possibility that Claspin modulates the activities of DNA polymerases during the normal course of DNA replication as well as when the fork is stalled (**Fig. 10B and C**). Claspin did not interact with ORC, Cdc6, Geminin, Cdt1, RPA, PCNA, AND-1, Rif1, Chk2, and CDK2 in my current assays. Based on these data, I propose that Claspin exists at replication fork in contact with MCM helicase and DNA polymerases (**Fig. 10C, upper**). Interaction with both leading and lagging strand DNA polymerases suggest a possibility that Claspin may act as a sensor for the fork stall signals. Upon encounter with the stalled fork, the replisome may be reorganized so it can facilitate the recruitment of Cdc7, Chk1 and ATR for checkpoint activation (**Fig. 10C, lower**).

Interaction between Cdc7 and Claspin was previously reported in human cells (36), although the efficiency of the interaction was low. In the current experimental setting, significant interaction was detected. Claspin is hyperphosphorylated upon replication stress, and this hyperphosphorylation depends on Cdc7 (36). Claspin undergoes phosphorylation also during normal early S phase, which also depends on Cdc7 (Uno *et al.* unpublished data). Precise role of Cdc7-Claspin interaction in replication checkpoint and normal DNA replication needs further studies.

The interactions described above were observed in the protein fractions pulled down by anti-Flag affinity beads from lysates (**Fig. 10**) but they mostly disappeared after further purification (**Fig. 5B**). For examples, some unknown kinases are present in the pulled down materials of full-length Claspin and phosphorylated Claspin (lane 3 of Fig. 15A). However, the purified Claspin did not show any phosphorylation on its own under the same condition (lane 19 of Fig. 15A). Claspin was purified through three steps involving Ni-column after washing with 15mM Imidazole solution, anti-Flag affinity beads following washing with 300mM NaCl solution, and gel filtration. These purification steps could have removed almost all the associated proteins.

#### **4. Domains of Claspin involved in binding and phosphorylation by Cdc7**

I generated the deletion and truncation derivatives of Claspin and pulled them down with anti-Flag antibody on Dynabeads proteinG. The pull down was efficient and the polypeptides were readily detectable after silver staining of the gel (**Fig. 15A**). Examination of the presence of Tim, Cdc7 and MCM revealed that Tim-Tipin bound to the N-terminal 350 amino acid segment, whereas Cdc7 interacts mainly with the C-terminal 444 amino acid segment. Cdc7 interacts also with the central and N-terminal segments, albeit with reduced efficiency. MCM interacts with the central and C-terminal segments (**Fig. 14**).

The truncated polypeptides containing the N-terminal 350 amino acids appear to coimmunoprecipitate unknown kinase which could phosphorylated the pulled down Claspin fragments. The pull down with C-terminal 444 amino acids (del #8) did not have associated kinase which is evident in this kinase assay but was efficiently phosphorylated by added Cdc7 kinase, consistent with the efficient association of Cdc7 with this segment in the pull down assay. The truncation or deletion mutant polypeptides were purified by Ni, Flag, and gel filtration and were used as substrates for kinase assay. The C-terminal 444 amino acid polypeptide (#8) was phosphorylated by Cdc7 to the highest level and C-Del

polypeptide was phosphorylated to a much lower level compared to the full-length polypeptide. The kinase assays using the individual recombinant polypeptides derived from Claspin as substrates also gave the similar results, showing that segment from 1015 to 1143 is most efficiently phosphorylated by Cdc7 kinase *in vitro*. It should be noted that the N-terminal segments (del #2 [1-350] and polypeptide #1 [1-194] and #2 [195-335]) can be also phosphorylated by Cdc7, consistent with the interaction of Cdc7 with these regions as well.

Phosphorylation sites were identified in the C-terminal and N-terminal region of endogenous Claspin from early S phase (**Fig. 15D-G**), and these sites were conserved in mammalian cells. Phosphopeptide-specific antibodies recognizing these phosphorylation events are now being developed to examine whether phosphorylation indeed depends on Cdc7 in the cells. Mutant Claspin molecules in which these sites are mutated are being constructed and their *in vivo* functions will also be examined.

## **Chapter 3.**

### **Stimulation of MCM helicase by Claspin**

## **Introduction**

MCM plays an essential role in the initiation and progression of eukaryotic DNA replication (59-62), and it is thought to form the MCM2-3-4-5-6-7 helicase complex *in vivo*, which unwinds DNA at the replication forks. MCM4-6-7 complex (hexamer composed of two trimers) was shown to have a DNA helicase activity *in vitro* (14, 15, 56), but the helicase activity has not been demonstrated in the isolated MCM2-3-4-5-6-7 complex. Recent reports indicate that the complex of MCM, Cdc45 and GINS has DNA helicase activity and this CMG complex may function as a replicative helicase at the fork.

In addition to the reports that Mrc1 interacts with replication factors and exists in the replication fork complex (7, 58), direct interaction between Mrc1 and MCM6 was reported in *Saccharomyces cerevisiae* (57). On the basis of these observations, I decided to examine the biochemical effect of purified Claspin on the MCM complexes.

## **Experiments and Results**

### **1. Confirmation of the helicase activity of MCM4-6-7**

MCM4-6-7 was expressed in Hi5 insect cells and purified by Nickel agarose beads and anti-Flag M2 affinity beads (**Fig. 16A**). The helicase activity of MCM4-6-7 was confirmed by a standard helicase assay (**Fig. 16B**).

### **2. Claspin promotes the helicase activity of MCM4-6-7**

To investigate whether the Claspin modulates the helicase activity of MCM4-6-7, Claspin fractions were added to helicase assays with MCM4-6-7 (**Fig. 17A**). The protein peak comigrated with the stimulation of the helicase activity. Titration of Claspin showed the extent of unwinding correlated with the amount of Claspin added to the reaction mixtures (**Fig. 17B**). These results suggest that Claspin can promote the helicase activity of MCM4-6-7 *in vitro*.

### **3. Claspin interacts with MCM4-6-7**

Claspin may stimulate the MCM helicase by directly interacting with it. To examine the interaction between Claspin and MCM4-6-7, both fractions were mixed and immunoprecipitation was conducted with anti-Claspin antibody. The pellet contained MCM subunits (**Fig. 18A**), showing that Claspin can physically and directly interact with MCM4-6-7. Interaction between Claspin and MCM was also indicated by *in situ* binding assay with MCM proteins immobilized on PVDF-membrane (**Fig. 18B**). The results indicate that Claspin binds to MCM4 subunit. MCM4-6-7 binds to ssDNA in gel shift assays. The complexes derived from a single hexamer and potentially double hexamers were detected. This binding was stimulated by the addition of Claspin fraction (**Fig. 19, lane 2 and 7**), although addition of excess Claspin lead to loss of stimulation (**Fig. 19, lanes 8 and 9**).

## Discussion

MCM helicase is believed to be the major component of the replicative helicase complex at the replication fork. It has been thought that DNA replication fork machinery could be disintegrated when stalled replication fork is not properly stabilized. MCM is a central factor for the replication fork and regulation of the activity of the “MCM helicase complex” would be crucial for maintenance of replication fork integrity. Mrc1/Claspin has been suggested to be a component of the replication fork and has been shown to play an important role in stabilization of stalled replication fork and also in transmitting checkpoint signals.

On the other hand, it has been shown in yeast that Mrc1 has a checkpoint-independent role for efficient progression of the replication fork. In mammals as well, positive roles of Claspin for S phase have been suggested. However, the precise nature of checkpoint-independent roles of Mrc1/Claspin has not been known.

In this thesis, I overexpressed and purified human Claspin protein and have shown that it can stimulate the helicase activity of MCM4-6-7 complex *in vitro* (**Fig. 17**). The purified Claspin and MCM4-6-7 physically interacted with each other (**Fig. 18**) and this interaction between MCM and Claspin was also detected in mammalian cells by pull down experiment (**Fig. 10**). Claspin purified in this work bound to DNA as reported before (**Fig. 8-9, 19**). I showed that depletion of Claspin in human cells or conditional knockout of Claspin in DT40 cells resulted in decreased DNA synthesis (**Fig. 1**). This is consistent with previous reports that Claspin is required for efficient cell growth. My finding that Claspin stimulates the helicase action of the MCM complex may provide molecular basis for these findings.

However, at present, trivial explanation for stimulation of the helicase activity on M13mp18<sup>37mer</sup>-dT50 substrate would also be possible. This substrate contains a very long segment of ssDNA on M13mp18 and Claspin might non-specifically bind to this region, and MCM's access to the 3'-tail segment of the 37mer-dT50 oligonucleotide may be indirectly facilitated. In order to prove physiological significance of my biochemical finding, I need to identify a mutant Claspin which does not stimulate the helicase activity of MCM4-6-7, and show that the mutant is defective in cellular function during S phase.

## **Conclusions and General Discussion**

In this study, I developed a highly efficient transient expression system in mammalian cells using a low cost PEI transfection reagent and a highly efficient expression system. The system is particularly suitable for expression and purification of large-sized proteins that are generally difficult to handle in bacterial or insect cell expression system. Moreover, combination with siRNA transfection would permit rapid functional characterization of mammalian proteins involved in cell cycle regulation, signal transduction or other cellular physiological pathways. Using this method, full length Claspin and Tim-Tipin complex were expressed in human cells, and were successfully purified with a high purity and purified proteins were analyzed for the DNA binding activity as well as for phosphorylation by a kinase and interaction with other proteins. Using this expression system, systematic analyses of binding proteins were conducted, particularly the binding with polymerases newly found in this thesis may suggest the possibility that Claspin modulates the activities of DNA polymerases during the normal course of DNA replication as well as when the replication fork is stalled.

The results indicated that Claspin interacted with Tim-Tipin and Cdc7 through the N-terminal and C-terminal segments, respectively. Tim-Tipin may stabilize the Claspin-DNA complex and may promote the association of more Claspin molecules on the DNA substrate. The functional assays of mutant Claspin molecules using Claspin-depleted cells indicated that the C-terminal segment (896-1209) is essential for its functions.

I also showed the requirement of Claspin for efficient DNA replication. This requirement might be explained at least partially by its ability to stimulate MCM helicase at the replication fork. The detailed interaction between Claspin and MCM proteins were revealed. The ability of Claspin to stimulate the helicase activity of MCM4-6-7 is intriguing, considering the positive role of Claspin/Mrc1 in replication fork progression in both yeasts and mammalian cells. Claspin may facilitate the loading of the MCM onto the template DNA.

Among the interactions of Claspin with replication and checkpoint factors observed in human cells, the interaction with Cdc7 kinase is significant, since this interaction is conserved in fission yeast as well as in *Xenopus* egg extracts. In both *Xenopus* egg extracts and yeast, checkpoint independent roles of Cdc7 were suggested. It is of interest to examine the precise role of this interaction during initiation of DNA replication. Claspin is efficiently phosphorylated by Cdc7 kinase *in vitro* and the phosphorylation of Claspin observed at the onset of the S phase might also be mediated by Cdc7 kinase. I conducted comprehensive analyses of phosphorylation of Claspin polypeptide by Cdc7/ASK *in vitro*. The results lead to identification of potential Cdc7-mediated phosphorylation sites on Claspin. The predicted phosphorylation sites are consistent with the segment of Claspin that interact with Cdc7 (the major site, C-terminal; the secondary site, N-terminal). I have already identified the S phase specific phosphorylation

sites on Claspin (**Fig. 15**) and generated the Claspin mutants lacking these phosphorylation sites, whose functions are now being examined.

## Future Perspectives

The Claspin was found to promote the helicase activity of MCM4-6-7 on a conventional heteroduplex template. However, at the fork, all the MCM subunits are required and MCM2-3-4-5-6-7 heterohexamer is likely to be the functional form. Furthermore, it has been suggested that the CMG complex (MCM2-3-4-5-6-7 in complex with Cdc45 and GINS) may be an active replicative helicase. It would be important to examine the effect of Claspin on the MCM heterohexamer or CMG complex. It would be also important to clarify the mechanisms of helicase stimulation by Claspin.

The domains interacting with replication or checkpoint factors were identified through molecular dissection of Claspin. I am currently examining the *in vivo* functions of these mutants in cells depleted of endogenous Claspin to define the region required for stimulation of DNA replication. These data will provide insight into the regulatory mechanisms of DNA replication initiation and replication fork progression.

In addition to the functional significance of interaction of Claspin with many replication/ checkpoint factors, the physiological significance of Cdc7-mediated phosphorylation is being investigated. I observed that Claspin was phosphorylated not only after replication stress but also during normal G1/S transition (**Fig. 14**). Cdc7, phosphorylated N-terminal and C-terminal segments of Claspin *in vitro*, and the phosphorylation sites determined by nano-ESI-MS were also clustered in the N-terminal and C-terminal regions of Claspin. I am now developing the phosphorylation-specific antibodies to examine if these phosphorylation is indeed dependent on Cdc7 in the cells.

Claspin interacts with the Tim-Tipin complex *in vivo* and this interaction could be important not only for the loading of Claspin onto chromatin but also for efficient fork progression, since Tim-Tipin is also required for efficient S phase progression in yeast. Analyses using purified MCM, Claspin and Tim-Tipin complex would yield important information on the precise regulation of helicase at the fork.

Finally, the expression-purification system I developed in this thesis is useful for purification of factors whose biochemical characterization has been difficult to achieve. I have already constructed expression plasmids for several key replication factors and in the process of purifying the complexes involved in initiation and progression of DNA replication.

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[URL2] pCAG-HIVgp

[http://www.brc.riken.jp/lab/cfm/Subteam\\_for\\_Manipulation\\_of\\_Cell\\_Fate\\_J/Plasmid\\_List\\_J\\_files/pCAG-HIVgp%20map.pdf](http://www.brc.riken.jp/lab/cfm/Subteam_for_Manipulation_of_Cell_Fate_J/Plasmid_List_J_files/pCAG-HIVgp%20map.pdf)

[URL3] pCMV-VSV-G-RSV-Rev

[http://www.brc.riken.jp/lab/cfm/Subteam\\_for\\_Manipulation\\_of\\_Cell\\_Fate\\_J/Plasmid\\_List\\_J\\_files/pCMV-VSV-G-RSV-Rev%20map.pdf](http://www.brc.riken.jp/lab/cfm/Subteam_for_Manipulation_of_Cell_Fate_J/Plasmid_List_J_files/pCMV-VSV-G-RSV-Rev%20map.pdf)

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DelCla#9/10F	acgc-GTCGAC-caccaccaccaccac-gcaaataactactgaaatgaac
DelCla#9/12R-2	cg-TCTAGA-tta-gctagc-caatggcaatcgaggcttcaaag
DelCla#10R	cg-TCTAGA-tta-gctagt-tttagctttaacacctgaagc
DelCla#10R-2	cg-TCTAGA-tta-gctagc-tttagctttaacacctgaagc
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DelCla#11R	cg-TCTAGA-tta-gctagt-attatctttatccattcttttcatct
DelCla#11R-2	cg-TCTAGA-tta-gctagc-attatctttatccattcttttcatct
DelCla#12F	acgc-GTCGAC-caccaccaccaccac-gatggcagtagtgaaattggc
S169A-F	aaagcaaaagtaaaagcaaaaagaagacttgag
S169A-R	caagtctcttttgcctttacttttgcctttcc
S1129A-F	gatgggatctgcacccgatggctctggcgcaatg
S1129A-R	tcgccaggaccatcggcgtgcagatccccatcagc
S1147A-F	aacatagatgatgctgccagatggactgttcc
S1147A-R	acaagccatctgggcagcatcatctatgttttcc
S1156A-F	ttgtccacagagacgctgatgatgatcagac
S1156A-R	gtctgatcatcatcagcgtctctgtggaacaag
S1156A T1161A-F	ttgtccacagagacgctgatgatgatcagcgtgaagaacagcttgatg
S1156A T1161A-R	caagctgttctcagcctgatcatcatcagcgtctctgtggaacaag
S1168A-F	gaacagcttgatgaggcagaagccaggtggagg
S1168A-R	tccacctgcttctgcctcatcaagctgttcttc

**Table 1-2.**

Oligo sequences for gelshift assay, helicase assay

37mer-dT <sub>50</sub>	GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGT-T <sub>50</sub>
50mer-dT <sub>60</sub>	CACTCGGGCTCGTTTTACAACGTCGTGACTGGGCACTTGATCGGCCAACC-dT <sub>60</sub>
dT <sub>30</sub> .50mer	dT <sub>30</sub> -GGTTGGCCGATCAAGTGCCAGTCACGACGTTGTAAAACGAGCCCGAGTG

**Table 2.**

siRNA oligo sequences used in this study

siCdc7-D sense	GCAGUCAAGACUGUGGAU-TT
siCdc7-D antisense	AUCCACAGUCUUUGACUGC-TT
siClaspin #2 sense	GGAAUCAGAGACAGCUCCG-TT
siClaspin #2 antisense	CGGAGCUGUCUCUGAUUCC-TT
siClaspin #3 sense	GGAAAGAAAGGCAGCCAGA-TT
siClaspin #3 antisense	UCUGGCUGCCUUUCUUUCC-TT
siClaspin #7 sense	GCACAUACAUGAUAAAGAA-TT
siClaspin #7 antisense	UUCUUUAUCAUGUAUGUGC-TT
siClaspin-nc#6 sense	UCCAGCCAAUGAUCCUUC-TT
siClaspin-nc#6 antisense	GGAAGGAUCAUUGGCUGGA-TT
siClaspin-nc#7 sense	GCCAAUGAUCCUCCUUCU-TT
siClaspin-nc#7 antisense	AGAAGGAAGGAUCAUUGGC-TT
siClaspin-nc#8 sense	UUGGCCACUGAUUUCAAUU-TT
siClaspin-nc#8 antisense	AAUUGAAAUCAGUGGCCAA-TT

**Table 3.**

Composition of amino acids in Claspin protein

		pl	a.a.	%
<b>Glu</b>	<b>E</b>	<b>3.22</b>	<b>194</b>	<b>14.48842</b>
Ser	S	5.68	139	10.38088
Lys	K	9.75	128	9.559373
Leu	L	5.98	118	8.812547
<b>Asp</b>	<b>D</b>	<b>2.77</b>	<b>104</b>	<b>7.76699</b>
Ala	A	6.00	71	5.302465
Gly	G	5.97	69	5.153099
Gln	Q	5.65	62	4.630321
Thr	T	6.16	62	4.630321
Arg	R	10.76	60	4.480956
Pro	P	6.30	58	4.331591
Asn	N	5.41	57	4.256908
Val	V	5.96	51	3.808813
Phe	F	5.48	42	3.136669
Ile	I	6.05	37	2.763256
His	H	7.59	28	2.091113
Met	M	5.74	26	1.941748
Tyr	Y	5.66	18	1.344287
Cys	C	5.05	11	0.821509
Trp	W	5.89	4	0.29873
		5.68 (Ave.)	1339 (Total)	100

**Table 4.**

Different names of replication factor among interspecies

<b><i>S. cerevisiae</i></b>	<b><i>S. pombe</i></b>	<b><i>H. Sapiens</i></b>
Orc	Orc	Orc
Cdc6	Cdc6	Cdc6
Cdt1	Cdt1	Cdt1
Mcm	Mcm	Mcm
Cdc7	Hsk1	Cdc7
Dbf4	Him1 / Dfp1	Dbf4
Sld3	Sld3	Treslin ?
Sld2	Sld2	RecQ4
Cdc45	Cdc45	Cdc45
Mcm10	Mcm10	Mcm10
GIN5	GIN5	GIN5
Tof1	Swi1	Tim
Csm3	Swi3	Tipin
Mrc1	Mrc1	Claspin
Ctf4	Mcl1	AND-1
DNA polymerase $\alpha$	DNA polymerase $\alpha$	DNA polymerase $\alpha$
DNA polymerase $\beta$	DNA polymerase $\beta$	DNA polymerase $\beta$
DNA polymerase $\gamma$	DNA polymerase $\gamma$	DNA polymerase $\gamma$
Tel1	Tel1	ATM
Rad3	Mec1	ATR
Rad26	Ddc2	ATRIP
Rad9	Ddc1	Rad9
Rad1	Rad17	Rad1
Hus1	Mec3	Hus
Rad17	Rad24	Rad17
Crb2	Rad9	BRCA1
Chk1	Chk1	Chk1
Cds1	Rad53	Chk2
Smc1	Psm1	Smc1
Smc3	Psm3	Smc3

**Table 5.**

The values for the Stokes radius and Sedimentation coefficient of marker proteins used in glycerol gradient sedimentation and gel filtration

	kDa	Stokes radius	Sedimentation coefficient
Thyroglobulin	669	85	19.5
Ferritin	443	67	17.6
Catalase	232	52	11.3
Dehydrogenase	140	46	7.7
BSA	66	35	4.3

**Table 6.**

Antibodies used in this study

Antibody	Supplier	Cat. no.	Reference
Claspin	In house		
MCM2-7	BD biosciences	559541	
Tim	In house		(63)
Tipin	In house		(63)
Cdc45	In house		(1)
RPA70	SantaCruz	sc-28304	
RPA34	NeoMarkers	MS-691-P0	
Cdc7	MBL	K0070-3	
CDK2	SantaCruz	sc-6248	
ATR	SantaCruz	sc-1887	
ATM	SantaCruz	sc-7230	
Chk1	SantaCruz	sc-7898	
Chk2	SantaCruz	sc-17747	
Cdt1	SantaCruz	sc-28262	
ORC2	MBL	3B7	
Geminin	SantaCruz	sc8450	
MCM4	In house		(1)
MCM10	Bethyl	A300-131A	
Pol.α	SantaCruz	sc-5920	
Pol.δ	From Dr. Toshiki Tsurimoto		
Pol.ε	From Dr. Toshiki Tsurimoto		
PCNA	SantaCruz	sc-56	
AND-1	In house		(77)
Rif1	Bethyl	A300-568A	

## **Figure legends**

### **Fig. 1. Efficient DNA replication requires Claspin**

(A) Left, Claspin protein level in HeLa (upper) and NHDF (lower) cells. Right, level of [<sup>3</sup>H]-thymidine incorporation per 1x10<sup>4</sup> cells. Column 1, control siRNA; column 2, treated with 2 mM HU for 12 hrs; column 3, treated with Claspin siRNA #1; column 4, treated with Claspin siRNA #7.

(B) Left, generation of conditional Claspin knockout DT40 cells. Right, growth curve of wild-type and Claspin knockout cells (in the presence and absence of Dox).

(C) Left, Claspin protein level in DT40 cells. Lanes 1 and 3, wild-type; lanes 2 and 4, Claspin<sup>-/-</sup>hClaspin-HA. Lanes 1 and 2, no Dox added; lanes 3 and 4, Dox added at 1 μg/ml. Right, level of [<sup>3</sup>H]-thymidine incorporation per 2x10<sup>4</sup> cells. Columns 1 and 2, wild-type; columns 3 and 4, Claspin<sup>-/-</sup>hClaspin-HA. Columns 1 and 3, no Dox added; columns 2 and 4, Dox added at 1 μg/ml.

### **Fig. 2. An easy to use and low cost transfection method using PEI solution.**

(A) Phosphate group of DNA is captured by the amino group in the PEI reagent.

(B) Plasmid DNA is mixed with PEI in 150mM NaCl and incubated for 30 min at room temperature. The DNA - PEI complex is added to 293T cells cultured in D-MEM.

(C) The mAG-6His-Claspin-3Flag cassette is present between *Eco*RI and *Xba*I site of CSII-EF-MCS vector [URL1] carrying an EF-1α promoter upstream of MCS.

### **Fig. 3. High-level and degradation-free expression of large-sized proteins by combination of a CSII-EF-MCS-based vector and PEI-mediated transfection into 293T cells**

(A) PEI at various concentrations was mixed with DNA (0.8 μg) in SQ, 150 mM NaCl or Opti-MEM. After incubation for 30 min, each DNA-PEI complex was added to 293T cells cultured in 12 well plates (1ml medium / well). At 42 hr after transfection, the cells were observed by Biozero fluorescence microscope (BZ-8000, Keyence). The excitation at 488nm was used to detect the fluorescence of mAG protein.

(B) 293T cells were transfected as in (A) by PEI (5 μg/ml in 150 mM NaCl; 1) or by TransIT293 (2.25 μl; 2). The numbers of mAG-positive cells were visually counted and the ratio versus total numbers of the cells are presented as transfection efficiency.

(C) 293T cells were transfected with mAG-Claspin-3Flag plasmid using various concentrations of PEI or TransIT293, and transfection efficiency was quantified by FACS. mAG fluorescence intensity was divided into 4 levels which are marked as P1~4. Populations (%) of the cells in each fraction are presented in the bar graphs.

(D) Extracts from the 293T cells transfected with the Claspin (left) or Tipin or combination of Tim and

Tipin (right) expression vector(s) using PEI solution or Trans-IT293 were mixed with anti-Flag® M2 affinity beads (SIGMA) or Glutathione Sepharose™ 4B beads (GE healthcare), respectively, and rotated for 60 min in cold room. The beads were washed and bound proteins were eluted by boiling in SDS-PAGE sample buffer. With this procedure,  $7-8 \times 10^5$  cells produced 6-10 µg of full-length Claspin or full-length Tim-Tipin. In both cases, less degradation of purified proteins was noted with the PEI method compared to transfection with TransIT293. Bold arrows show full-length Claspin, Tim or Tipin protein. Thin arrows indicate the degradation products of Claspin.

**Fig. 4. Insertion of a TEV recognition site and cleavage of the fluorescent tag by TEV protease.**

mAG-TEV-6His-Claspin-3Flag was constructed, as described in “Materials and methods” and was expressed in 293T cells. The expressed protein was adsorbed to anti-Flag M2 affinity beads, which was incubated by TEV protease. The protein was almost completely digested by TEV protease, giving rise to a smaller 6His-Claspin-3Flag protein.

**Fig.5. Purification of mAG-6xHis-Claspin-3xFlag.**

(A) Flow-chart diagram of purification strategy of Claspin. This diagram shows a stepwise procedure that was used for purification of the proteins in this study.

(B) mAG-TEV-6His-Claspin-3xFlag was expressed in 293T cells (15 cm x 10 plates) and purified by combination of Ni-NTA Agarose beads and anti-Flag® M2 affinity beads, followed by cleavage of the beads-bound protein by TEV protease and elution by Flag peptide. The Flag eluates were analyzed on SDS-PAGE, which was detected by C.B.B. staining.

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**Fig. 6. siRNA transfection using PEI.**

(A) Increasing concentrations of Cdc7-D siRNA (0.1, 0.2 and 0.3 µM) were transfected into 293T cells once (1x transfection) or twice (2x transfection), as described in “Materials and methods”. (B) Increasing concentrations of Claspin siRNA (0.1, 0.2 and 0.3 µM) or 0.3 µM Cdc7 siRNA was transfected into 293T cells once. (C) Cdc7-D siRNA (0.3 µM) and mAG-hClaspin-3Flag expressing plasmid (0.8 or 2.4 µg) were cotransfected into 293T cells as described in “Materials and methods”. (D) Claspin siRNA (0.2µM) targeting non-coding region of Claspin were tested (2x transfection). (E) A deletion mutant of Claspin (Del-#2) was transiently expressed by transfection of plasmid DNA (0.2 µg) when the endogenous Claspin was knocked-down by siClaspin-nc#7 (0.2 µM x 2). In (A)~(E), whole cell extracts were analyzed by Western Blotting using the antibodies indicated.

**Fig. 7. Claspin exists as a monomer**

(A) Glycerol gradient sedimentation analysis. Purified Claspin (50 µl of the E3 fraction in Fig. 5B) was fractionated in 20-35% glycerol gradient centrifugation.

(B) Analyses by gel filtration column. Purified Claspin (50  $\mu$ l each of the E1 fraction in Fig. 5B) was fractionated by gel filtration column (Superdex-200) in SMART system. In both (A) and (B), the buffer used was 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.1 mM PMSF, and 0.01 % TritonX-100. High molecular weight native marker proteins (GE Healthcare, Inc.) were also fractionated under the same condition. The fractions were analyzed by SDS-PAGE and silver staining (A) or CBB staining (B). The Sedimentation coefficient and Stokes radius of Claspin were estimated to be 6.9 (A) and 75 (B), respectively, on the basis of its fraction positions relative to those of markers (graphs). The band intensities of Claspin in fractions of glycerol gradient sedimentation or gel filtration (Fig. 5A and B) were quantified and blotted in the Excel2007 sheet. The calibration lines in quartic functions were drawn (graphs) and the X-values which gave maximal Y-values were calculated.

(C) [Left] Analyses in a native gel. Purified Claspin (1, 2, and 4  $\mu$ l each of E1 fraction in Fig. 5B) was separated on 5-20% gradient gel (ATTO) in 1x TBE. [Right] Purified Claspin (2 $\mu$ l of E2 fraction in Fig. 5B) was crosslinked with 0.06 or 0.12% of glutaraldehyde and analyzed by native gel or SDS-polyacrylamide gel. The proteins were visualized by CBB staining.

#### **Fig. 8. DNA binding properties of Claspin expressed and purified in 293T cells**

(A) Purified Claspin (1 and 3 $\mu$ l each [65 and 195 nM, respectively] of the dialyzed Flag E2 fraction in Fig. 5B) was analyzed in gel shift assays. The substrates (6.25nM) used are schematically indicated at the bottom of each panel. Filled circles indicate the  $^{32}$ P-labeled 5'-ends. Reactions were analyzed in 5 % (29:1) polyacrylamide gel in 1x TBE buffer. The gel was dried and the labeled substrates were detected by BAS2500. Claspin preferentially binds to the Y-fork structure.

(B) The binding of Claspin to Y-fork structure (Fig. 8A) was confirmed by supershift using anti-Claspin antibody. Purified Claspin (1 and 2 $\mu$ l each [65 and 130nM, respectively] of the dialyzed Flag E2 fraction in Fig. 5B) was pre-mixed with 90, 180, 360, and 720 ng of anti-Claspin antibody (affinity-purified), and incubated for 30 min on ice, and then Y-fork substrate was added. Reactions were electrophoresed in 3.5% 29:1 polyacrylamide gel containing 2.5% glycerol in 1x TBE buffer. The gel was dried and the labeled substrates were detected by BAS2500.

#### **Fig. 9. DNA binding properties of purified Claspin and Tim-Tipin complex**

(A) mAG-2xFlag-Tim and mCherry-GST-Tipin was transiently expressed in 293T cells and purified by GST, Flag, and monoQ. MonoQ fractions (fr.11~13) were dialyzed to decrease the concentration of NaCl to 30mM.

(B) Claspin and Tim-Tipin fractions were applied to gel shift assay with 50 fmol/assay of ssDNA or Y-fork. Reactions were applied to 5-20% gradient PAGE gel and electrophoresed in TBE buffer. The gel was stained by C.B.B. for the detection of size marker and the radioactivity was detected by BAS2500.

(C, D, and E) Claspin and Tim-Tipin fractions were used in gel shift assays with Y-fork. Four hundred ng of anti-Claspin antibody or anti-GST antibody were added in the lanes indicated.

**Fig. 10. Interaction of Claspin with various replication and checkpoint factors in 293T cells**

(A) The proteins pulled down by anti-Flag M2 affinity beads from the cell extracts expressing mAG-Claspin or mAG-Claspin-3Flag were analyzed by western blotting using the antibodies indicated. In, input; Un, unbound; B, bound to beads. Bound fractions are derived from the 4x amount of the sample compared to the input. Bold letters indicate the proteins that can interact with Cdc7.

(B) Summary of proteins interacting with Claspin. Bigger letters indicate the proteins more efficiently interacting with Claspin.

(C) Hypothetical and schematic drawing for protein interactions involving Claspin at a replication fork. Upper, propagating replication fork under normal condition. Only relevant replication fork factors are shown and others are omitted. Lower, stalled replication fork. Only the key factors required for replication checkpoint are shown. Factors shown to interact with Claspin in this study are in gothic, whereas others are in italics. Dotted arrows indicate the potential phosphorylation events by Cdc7 kinase.

**Fig. 11. Interaction between transiently expressed Claspin and endogenous Cdc7 kinase.**

Plasmids expressing mAG-, mKO2-, and mCherry-Claspin fusion proteins were transfected into 293T cells by PEI. Extracts were prepared and immunoprecipitation using anti-Flag® M2 affinity beads was conducted. Cdc7 protein is detected in extracts expressing the Claspin fused with 3xFlag tag, but not in those expressing Claspin without 3xFlag tag.

**Fig. 12. BLAST search on human Claspin and fission yeast Mrc1**

(A) human Claspin amino acid sequence was applied to BLAST search engine (NCBI).

(B) *S. pombe* Mrc1 amino acid sequence was applied to BLAST search engine (NCBI).

**Fig. 13. Coiled-Coil score of Claspin and fission yeast Mrc1 polypeptide sequences**

human Claspin, *Xenopus* Claspin, and *S. pombe* Mrc1 amino acid sequence was applied to Coiled-Coil Scoring engine.

**Fig. 14. Interaction of Claspin with replication factors: truncation mutants**

(A) Pull down experiments were conducted with 293T cells expressing each deletion mutant of Claspin. Interacting proteins were detected by western blotting using the antibody shown to the right of the panel.

(B) Summary of results of pull down experiments.

**Fig. 15. Phosphorylation of Claspin**

(A) Each Claspin derivative was used as a substrate for kinase assay with Cdc7-ASK. Purified human Cdc7-ASK and 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]-ATP were mixed with immunoprecipitated Claspin on Dynabeads, and they were incubated for 60 min at 30°C. Reactions were analyzed by 8% SDS-PAGE (29:1). The gels were stained with silver and phosphorylation was detected by BAS2500. (B) Each Claspin derivative was largely expressed and purified by Ni, Flag, and gelfiltration column (Superdex-200) in SMART system. The each

fraction was used as a substrate of Kinase assay with Cdc7-ASK. (C) Eight polypeptides of truncated Claspin were prepared and were also used as substrates for kinase assay with Cdc7-ASK and Cdk2-CyclinE. (D) The synchronization of HCT116 cells by thymidine block-nocodazole release protocol. (E) FACS analyses of DNA content of the HCT116 cells released from G2 arrested cells. (F) At various time points after release, cells were harvested and whole cell extracts were analyzed by western blotting. Claspin and tubulin are detected. Claspin is mobility-shifted at 4-8 hrs after release. For a large scale preparation, 15 cm x 40 plates ( $2 \times 10^9$  cells) of HCT116 were synchronized and released. At 6hr after nocodazole release (when cells are in S phase), cells were harvested and lysed, and endogenous Claspin was harvested from extract by Immunoprecipitation with anti-Flag antibody (3,16). Antibody-bound proteins were boiled in SDS-PAGE sample buffer and separated by 7.5% SDS-PAGE (19:1). (G) 4 pmol of Claspin was isolated from this gel and analyzed by nano-ESI-MS. Identified potential phosphorylation sites are listed.

#### **Fig. 16. Purification of MCM4-6-7 helicase and measurement of its helicase activity**

(A) Purified MCM4-6-7 fraction was analyzed by SDS-PAGE and C.B.B. staining. (B) The fraction shown in (A) was assayed in a standard helicase assay as described in 3.12 using M13mp18<sup>37mer</sup>-dT<sub>50</sub> substrate. Unwound 37mer-dT<sub>50</sub> ssDNA was separated on 12% PAGE (19:1 TBE).

#### **Fig. 17. Stimulation of helicase activity of MCM4-6-7 by Claspin**

(A) Claspin fractions from gel filtration (Superdex-X200) were assayed in a standard helicase reaction of MCM4-6-7.

(B) Titration of Claspin fractions from monoQ column in SMART in helicase assays.

(A, B) Upper, autorad of helicase assay; left, silver staining of SDS-PAGE of fractions from gel filtration; lower right, quantification of the helicase assays shown in upper panels. B, outer buffer of dialysis used as a negative control.

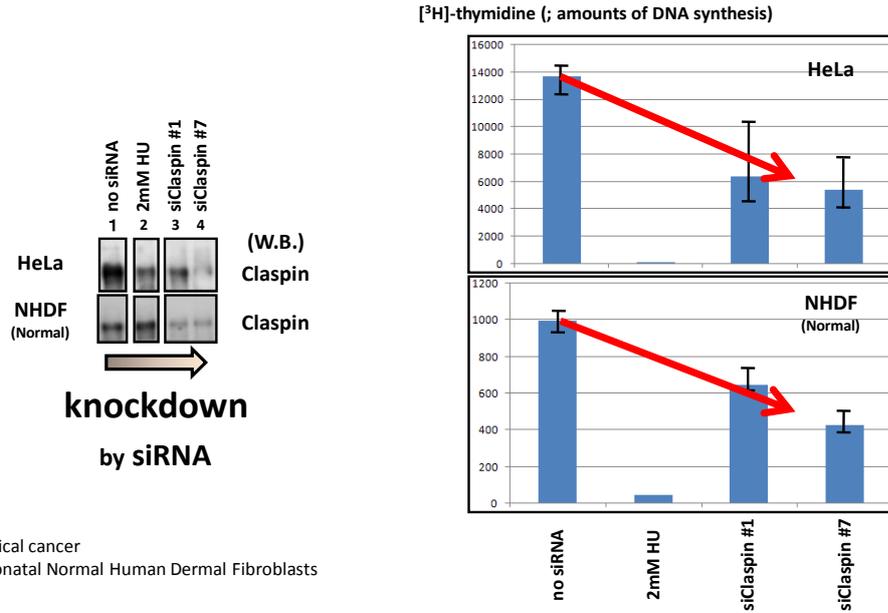
#### **Fig. 18. Interaction between MCM4-6-7 and Claspin**

(A) Purified Claspin and MCM4-6-7 were mixed with Dynabeads-linked protein G and anti-Claspin antibody. Unbound [Un] and bound [B] fractions were analyzed by Western blotting using anti-MCM4 antibody. (B) Flow-chart diagram of protein-protein interaction analysis strategy on PVDF-membrane. (C) MCM2-3-4-5-6-7 or MCM2-4-6-7 were separated by 7% 29:1 SDS-PAGE and transferred to PVDF-membrane. The Claspin bound to each membrane was detected by anti-Claspin antibody. The positions of each MCM protein was confirmed by SDS-PAGE, followed by C.B.B. staining. Red arrows indicate each MCM protein. (D) A model of interaction between Claspin and MCM helicase.

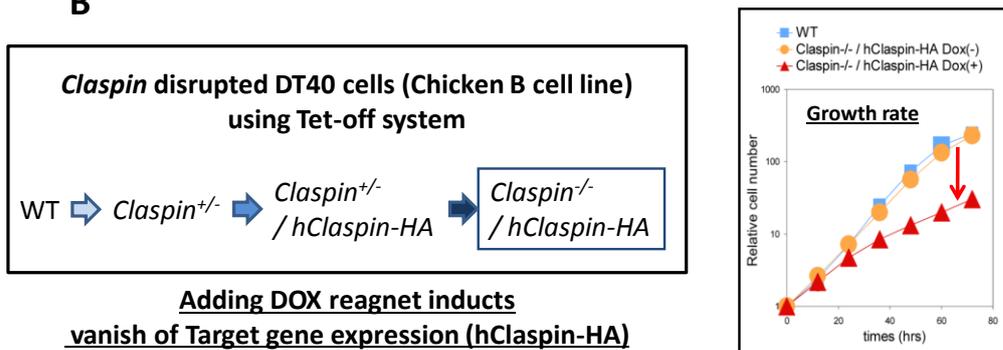
#### **Fig. 19. DNA binding assays of Claspin and MCM**

Flag E1 of Claspin, MCM4-6-7 were applied to gel-shift assay with 37mer-dT<sub>50</sub> ssDNA. Reactions were applied to 5-20% PAGE and stained by C.B.B. for the detection of size markers. The gel was dried and RI bands were detected by BAS2500.

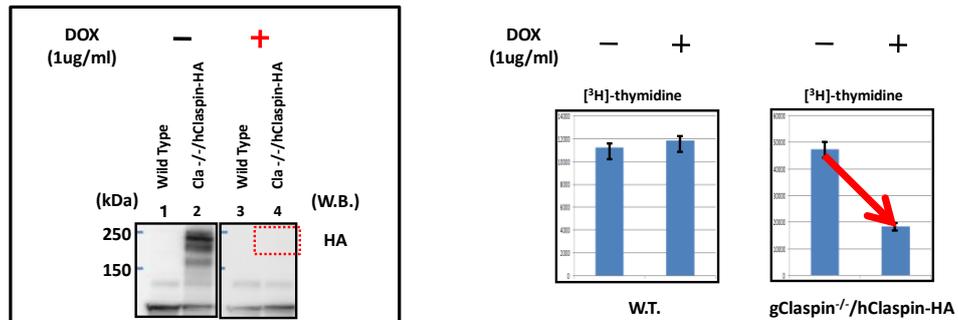
**A**



**B**

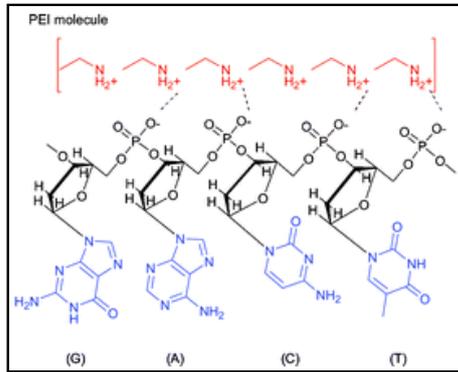


**C**



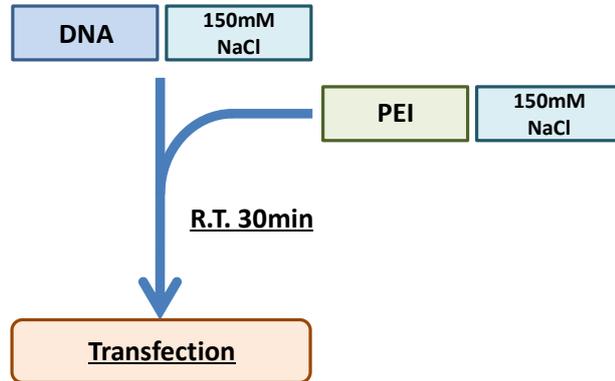
**Fig. 1. Efficient DNA replication requires Claspin**

**A**

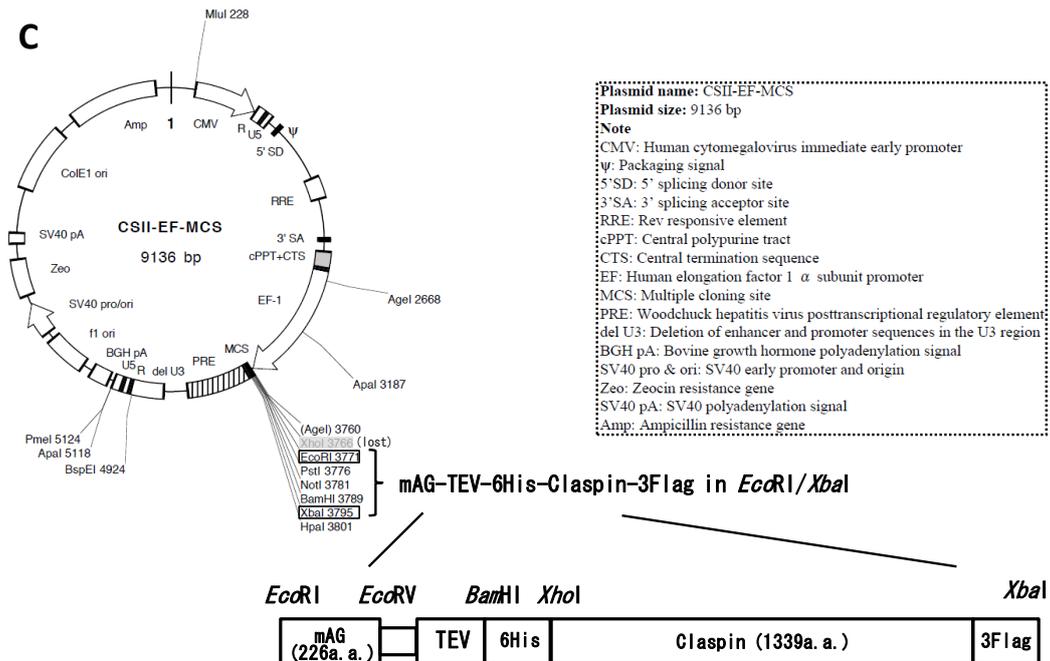


**[J.Mater.Chem. 17, 2561-2565 [2007]]**

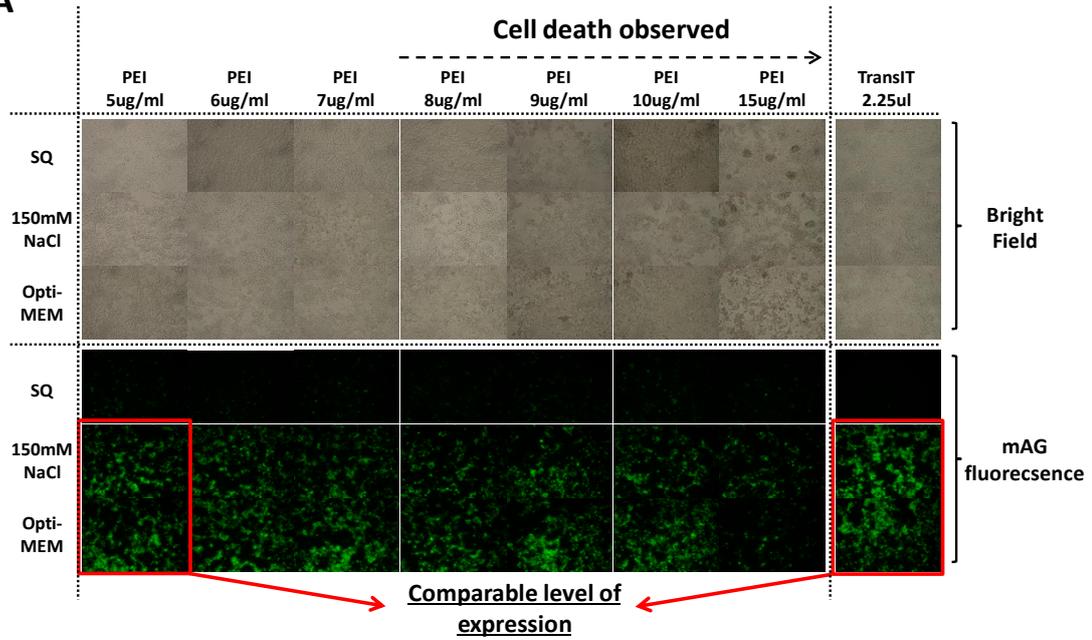
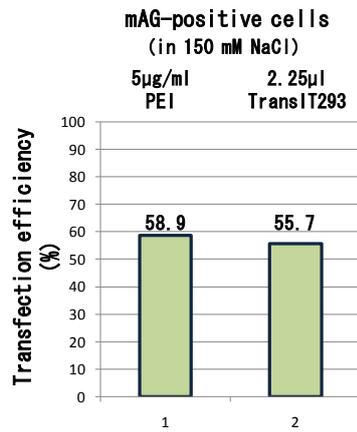
**B**

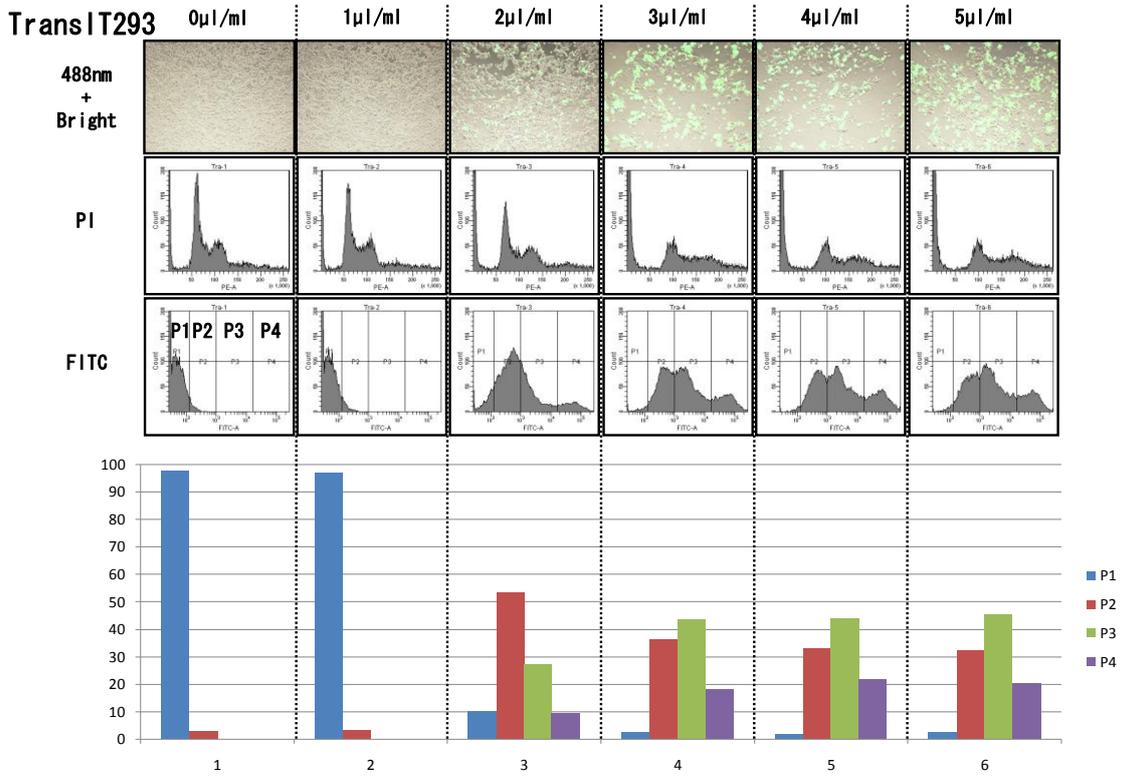
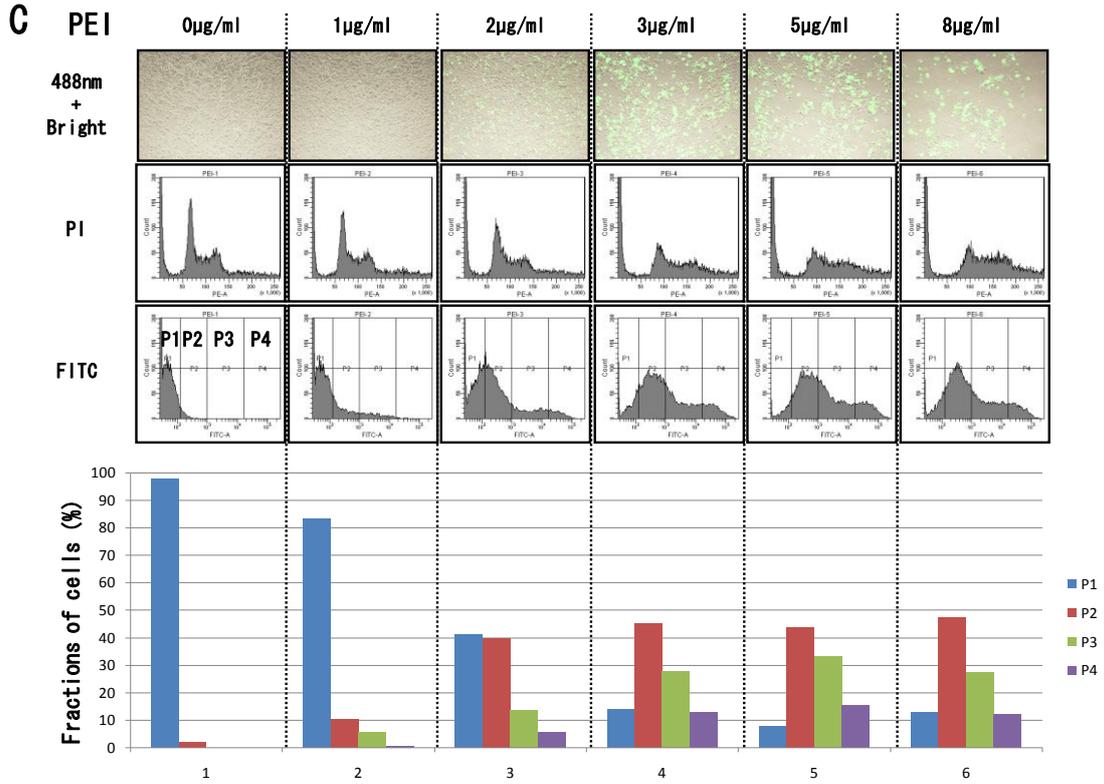


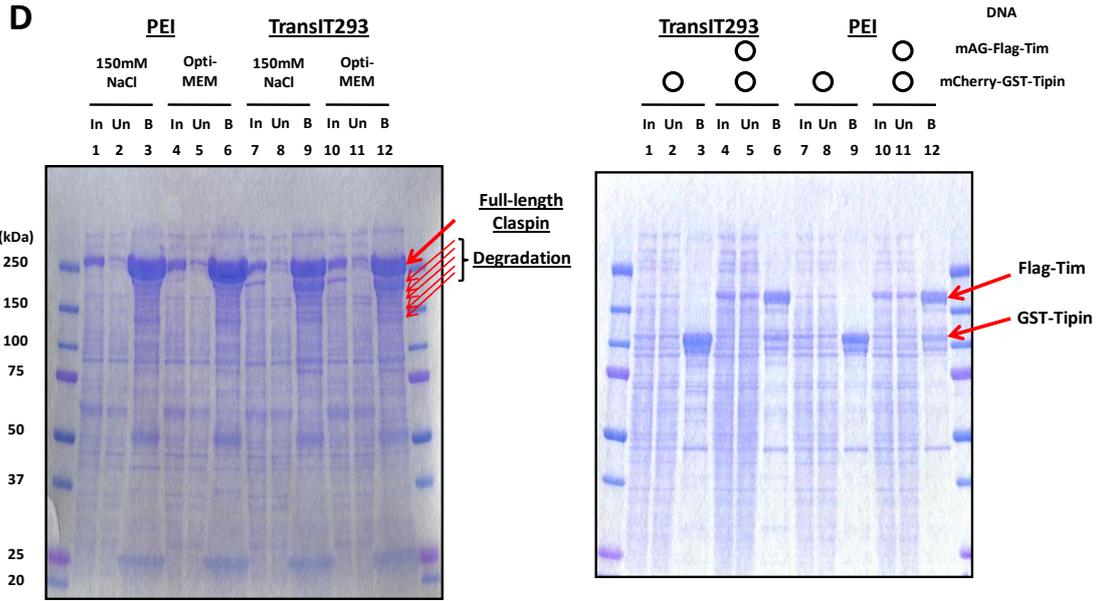
**C**



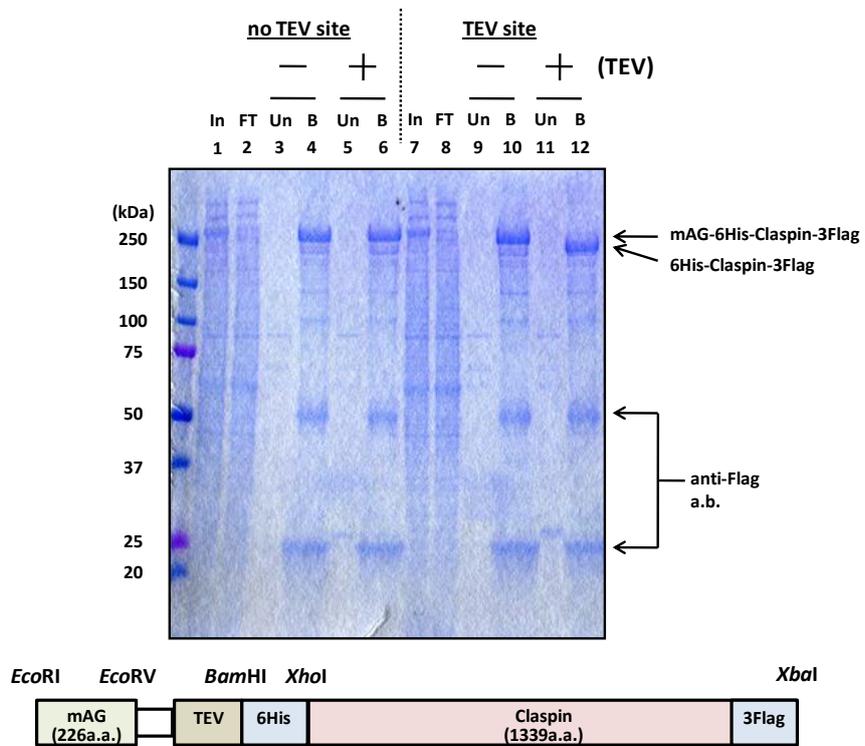
**Fig. 2. An easy to use and low cost transfection method using PEI solution.**

**A****B**

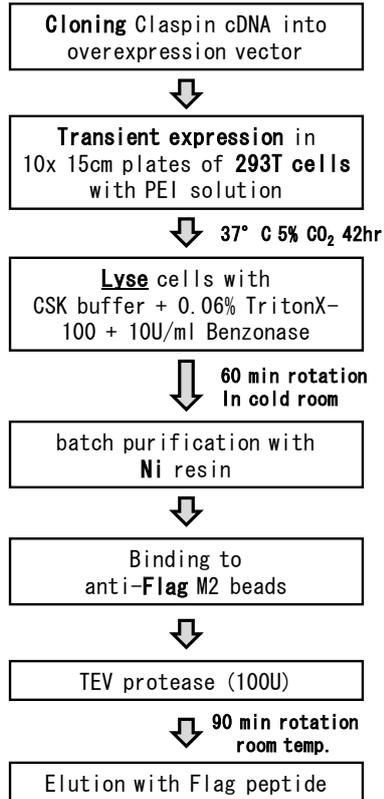
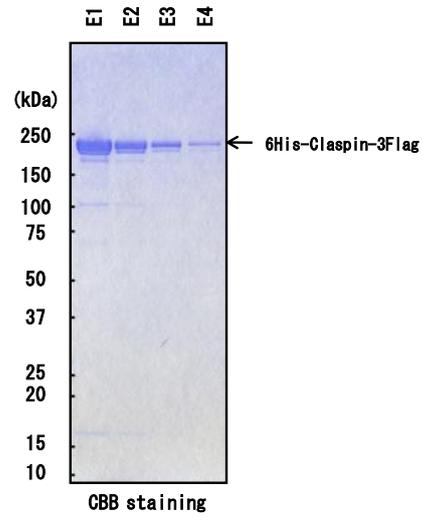




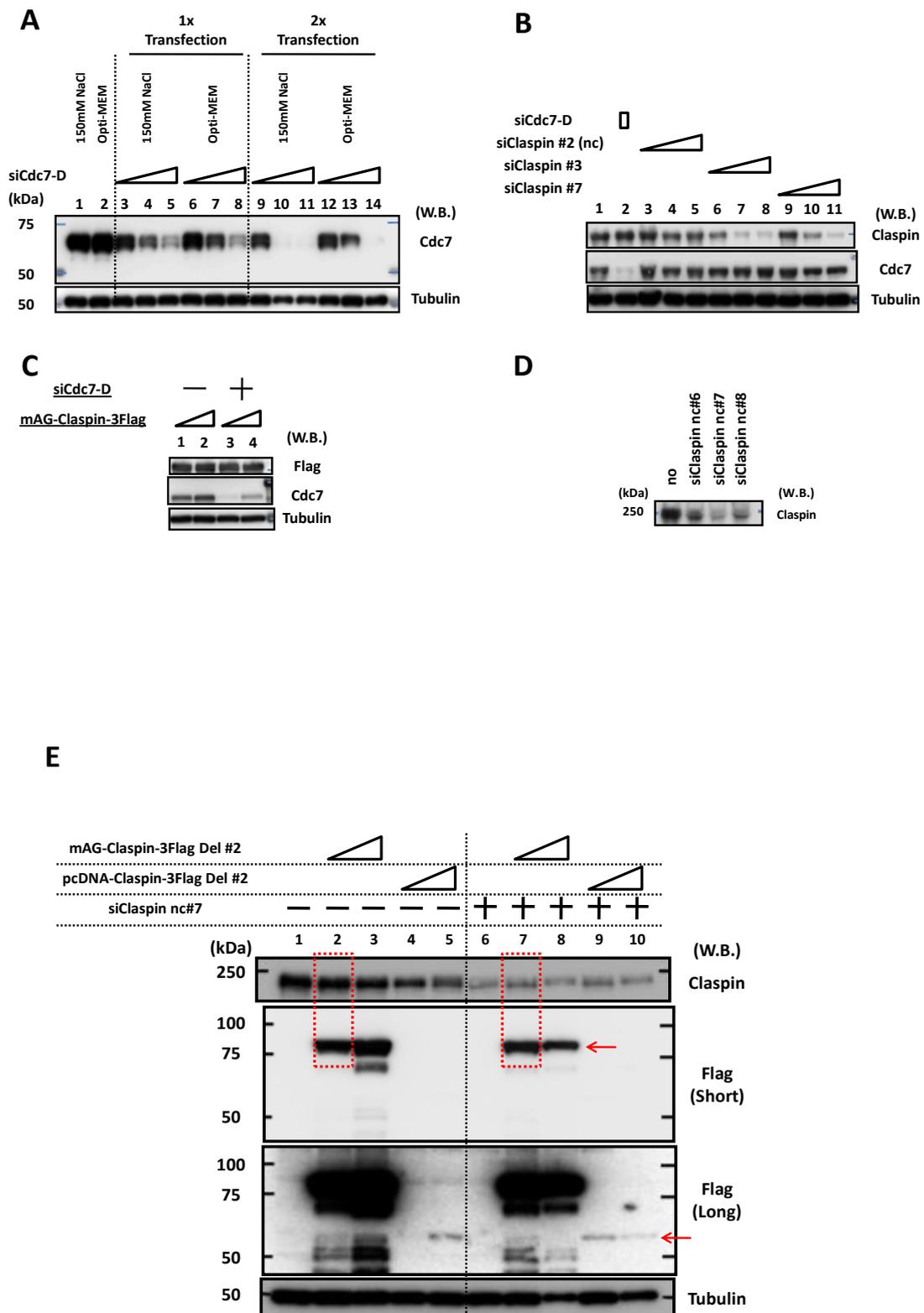
**Fig. 3. High-level and degradation-free expression of large-sized proteins by combination of a CSII-EF-MCS-based vector and PEI-mediated transfection into 293T cells**



**Fig. 4. Insertion of a TEV recognition site and cleavage of the fluorescent tag by TEV protease.**

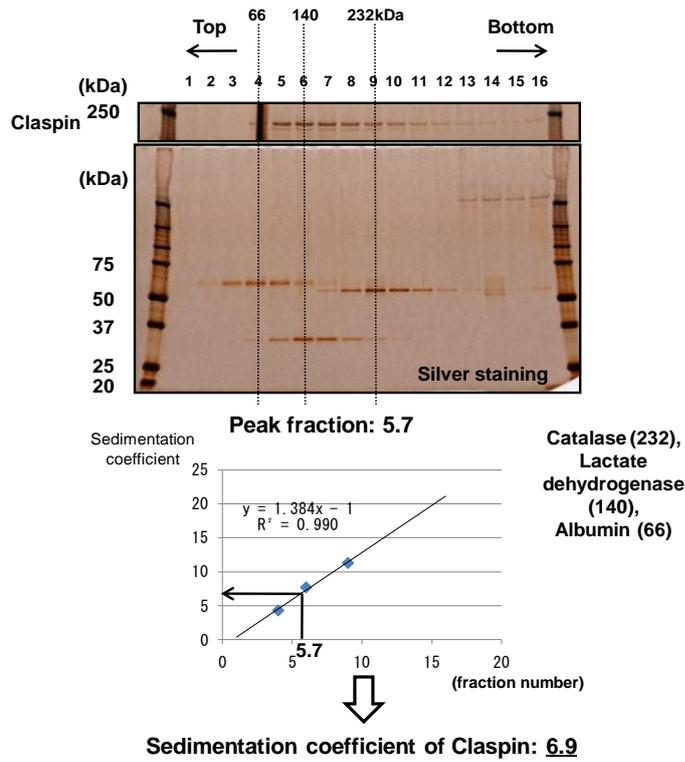
**A****B**

**Fig. 5. Purification of 6His-Claspin-3Flag.**

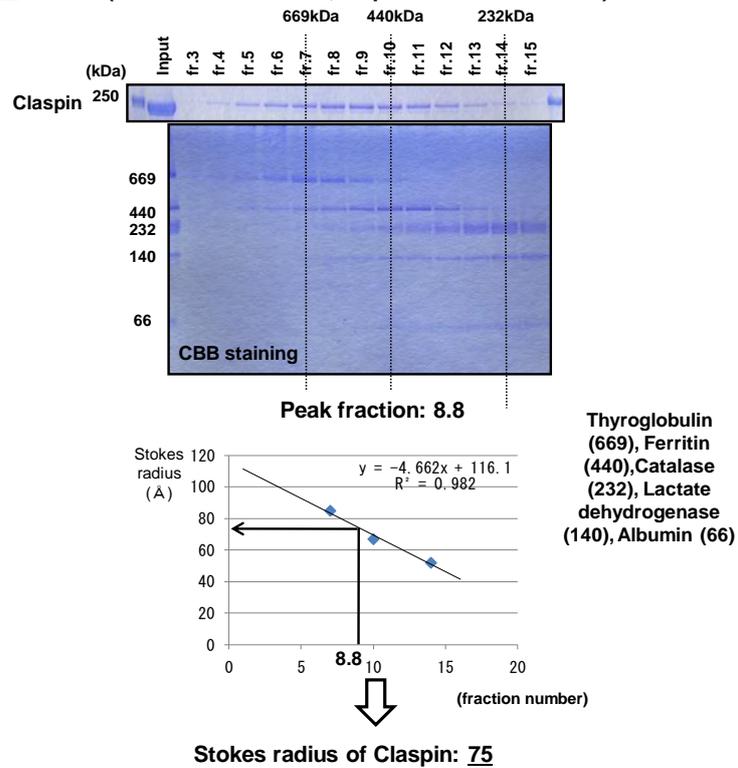


**Fig. 6. siRNA transfection using PEI.**

**A** (Glycerol gradient sedimentation; 20~35%, 38,000rpm x 18 hr)



**B** (Gel filtration column; Superdex-200 in SMART)



## Glycerol gradient sedimentation

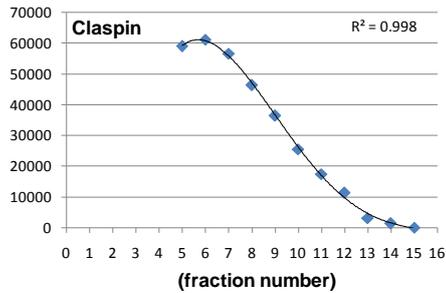
$$y = -19.80x^4 + 638.1x^3 - 6581x^2 + 17149x + 48072$$

( $R^2 = 0.998$ )

$$0 = -79.2x^3 + 2865x^2 - 32282x + 10550$$

$$\begin{aligned} X1 &= 1.688 \\ X2 &= 11.24 + 1.366i \\ X3 &= 11.24 - 1.366i \end{aligned}$$

$$1.688 + 4 = \underline{5.688}$$



## Gel filtration

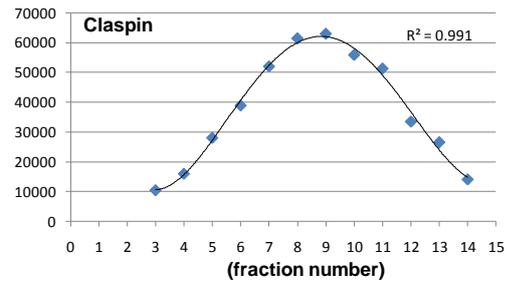
$$y = 44.99x^4 - 1224x^3 + 9490x^2 - 15580x + 17950$$

( $R^2 = 0.991$ )

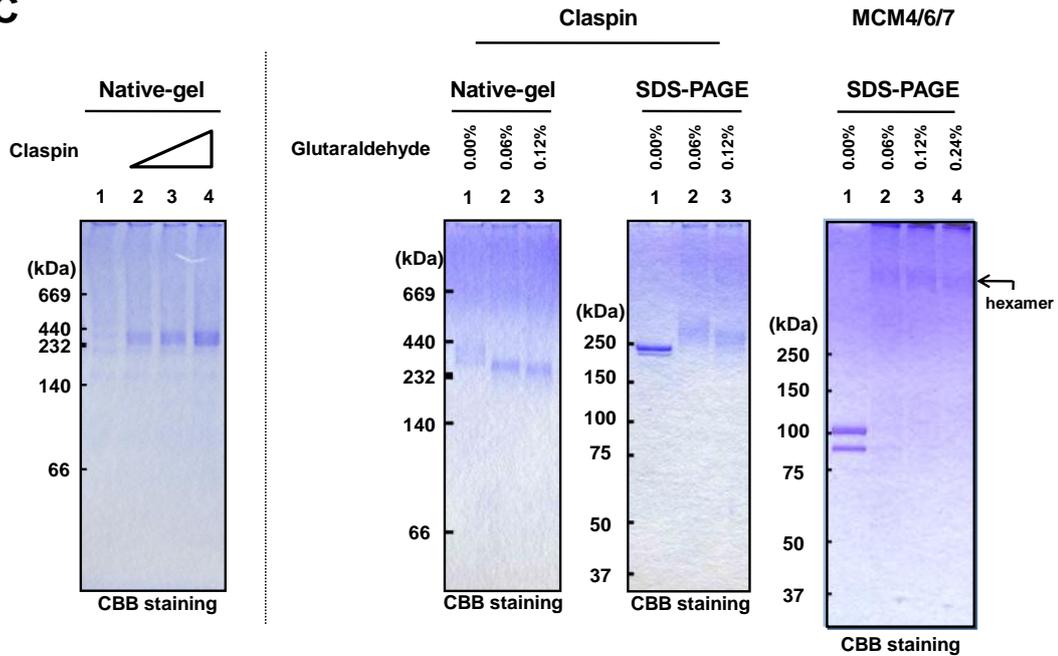
$$0 = 179.96x^3 - 3672x^2 + 18980x - 15580$$

$$\begin{aligned} X1 &= 12.55 \\ X2 &= 1.007 \\ X3 &= 6.846 \end{aligned}$$

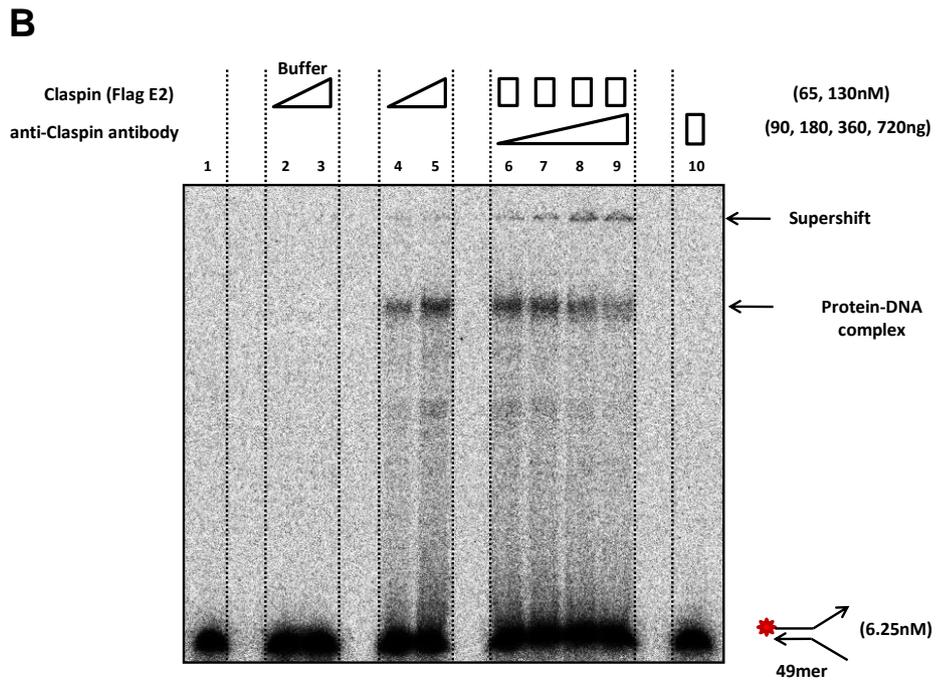
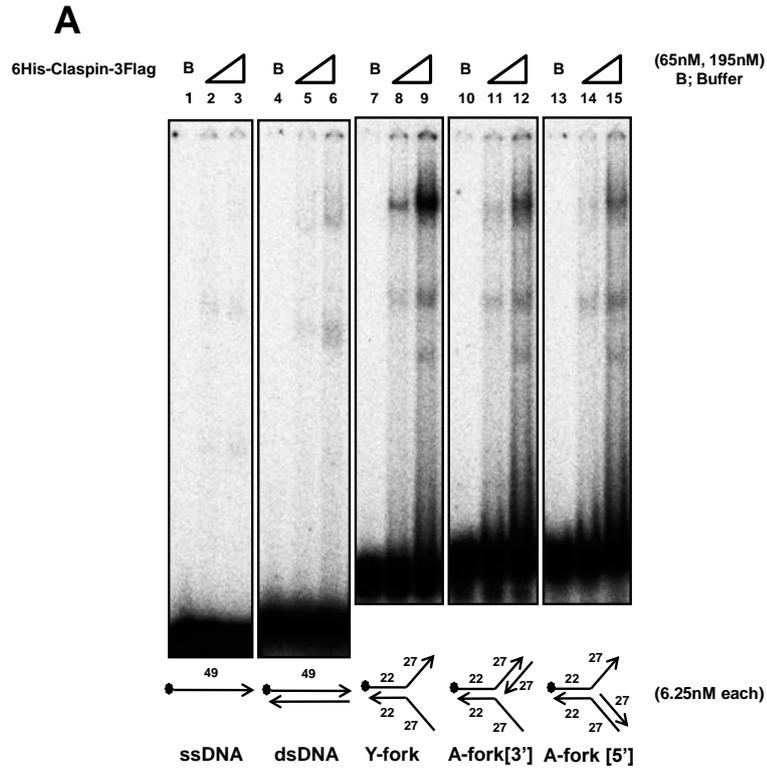
$$6.846 + 2 = \underline{8.846}$$



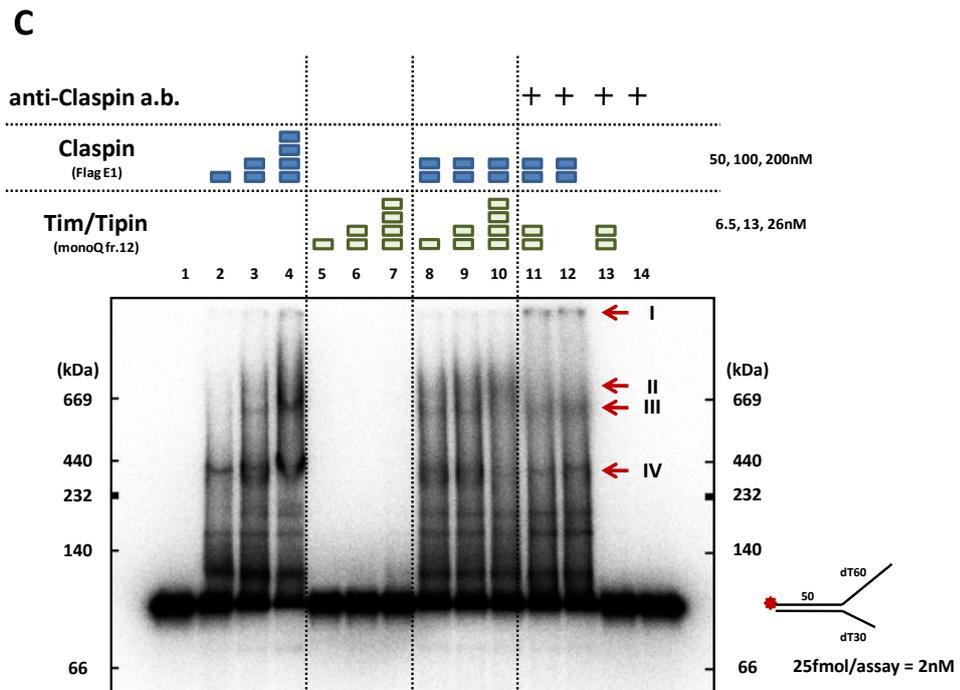
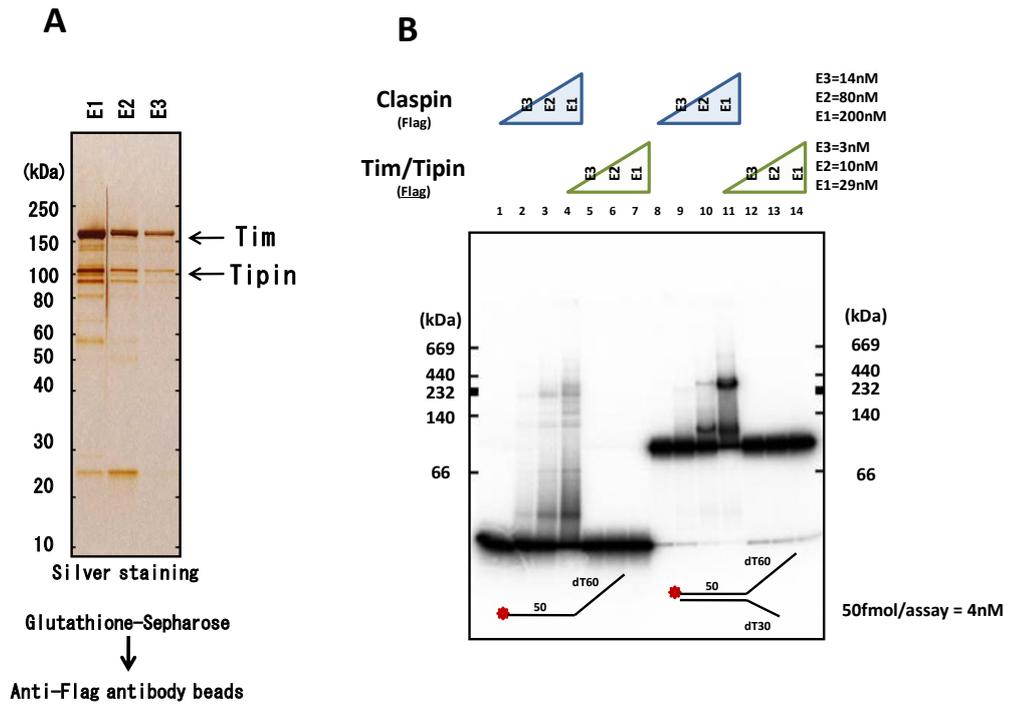
**C**



**Fig. 7. Claspin exists as a monomer.**



**Fig. 8. DNA binding activity of Claspin expressed and purified in 293T cells.**

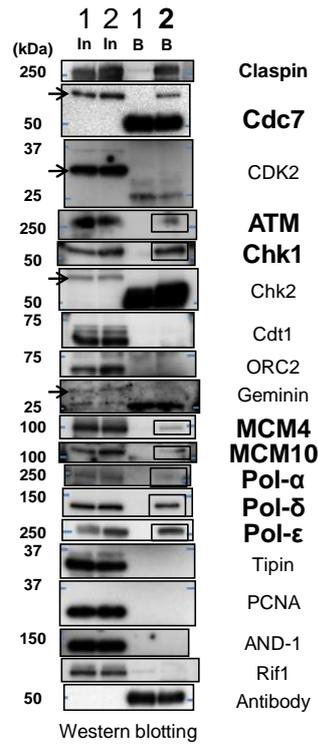
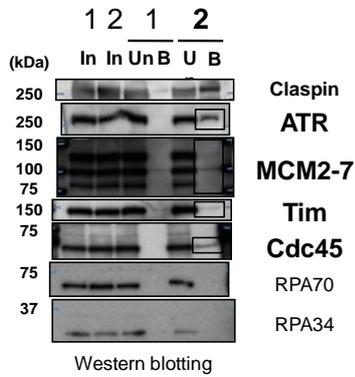




**A**

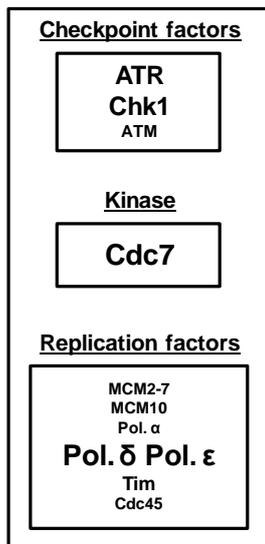
Transient Expression  
in 293T cells  
1: mAG-Claspin  
2: mAG-Claspin-3Flag

↓  
Pull Down with  
anti-Flag antibody beads

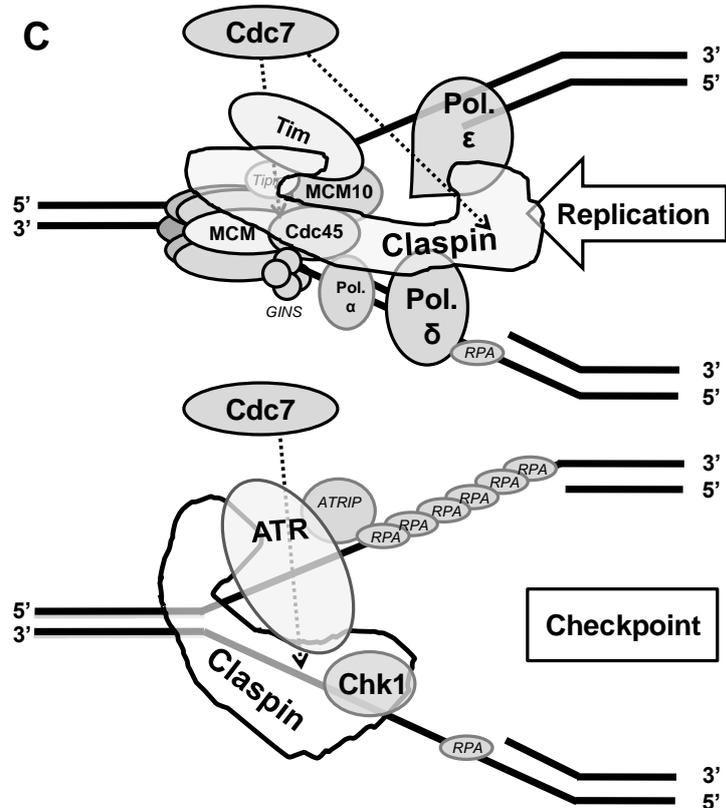


**B**

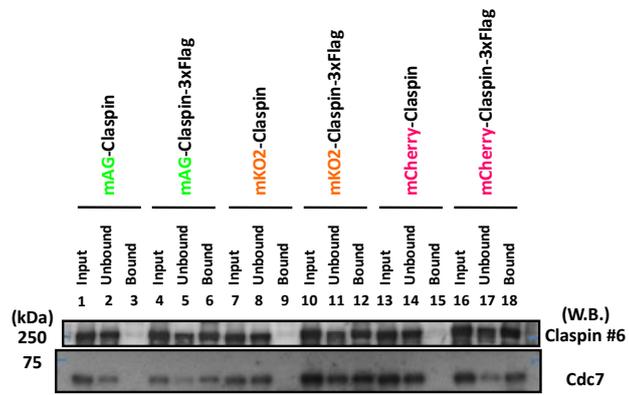
Proteins  
interacting with Claspin



**C**

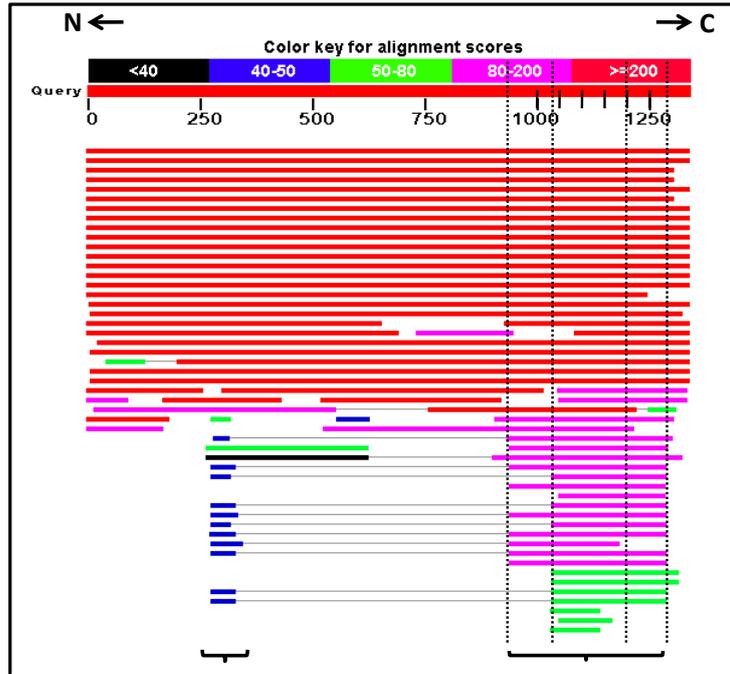


**Fig. 10. Interaction of Claspin with various replication and checkpoint factors in 293T cells**

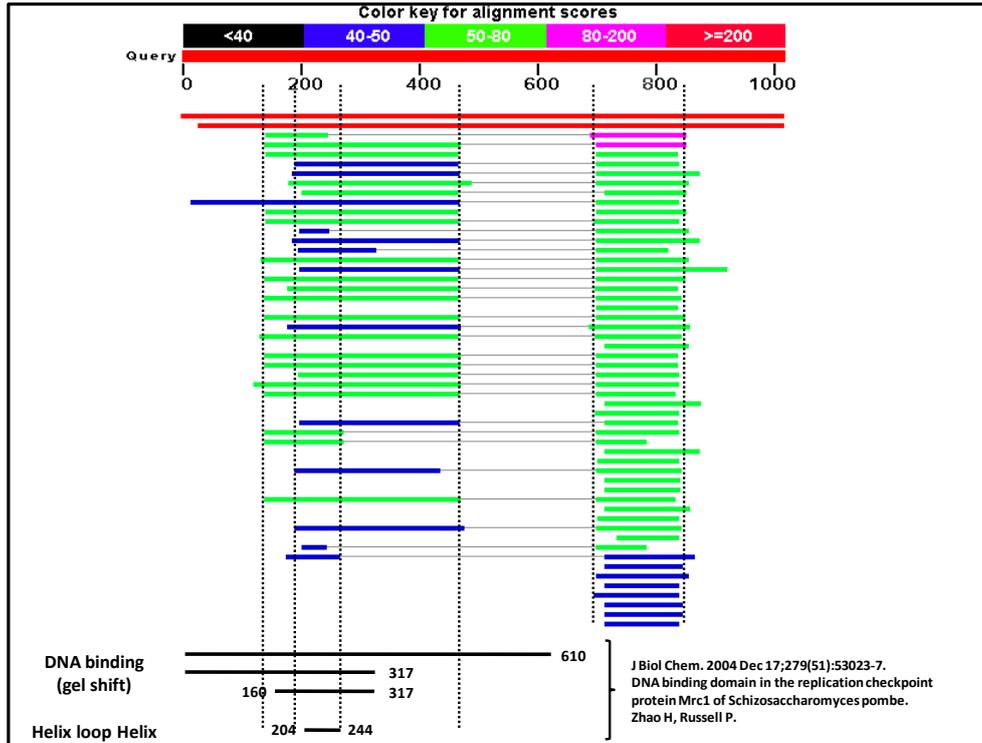


**Fig. 11. Interaction between transiently expressed Claspin and endogenous Cdc7 kinase**

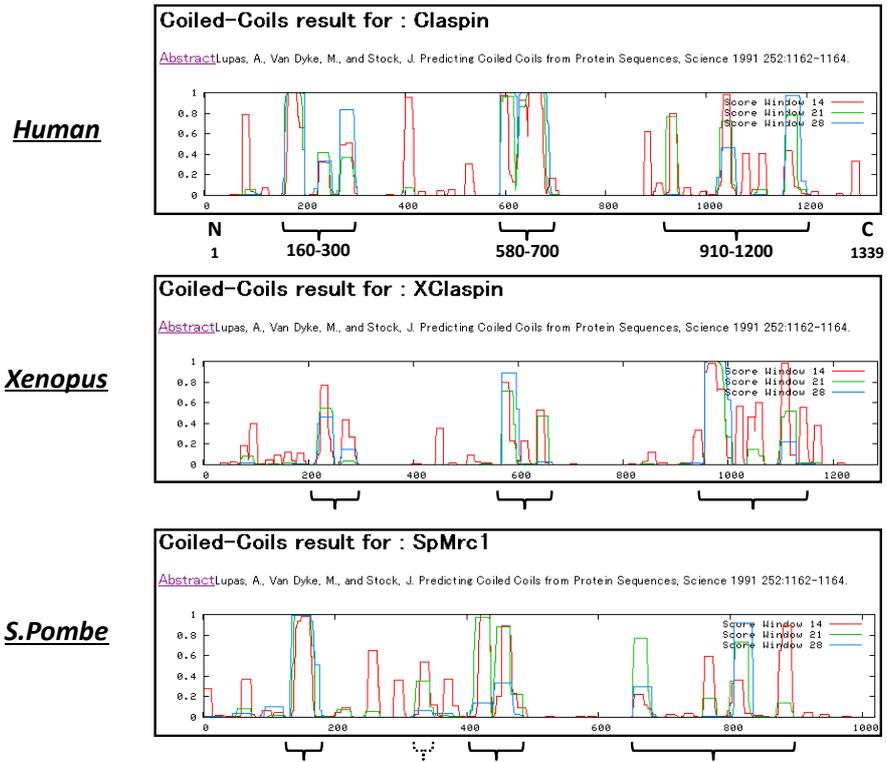
**A** Blast Search about human Claspin  
<http://blast.ncbi.nlm.nih.gov/> ->protein blast)



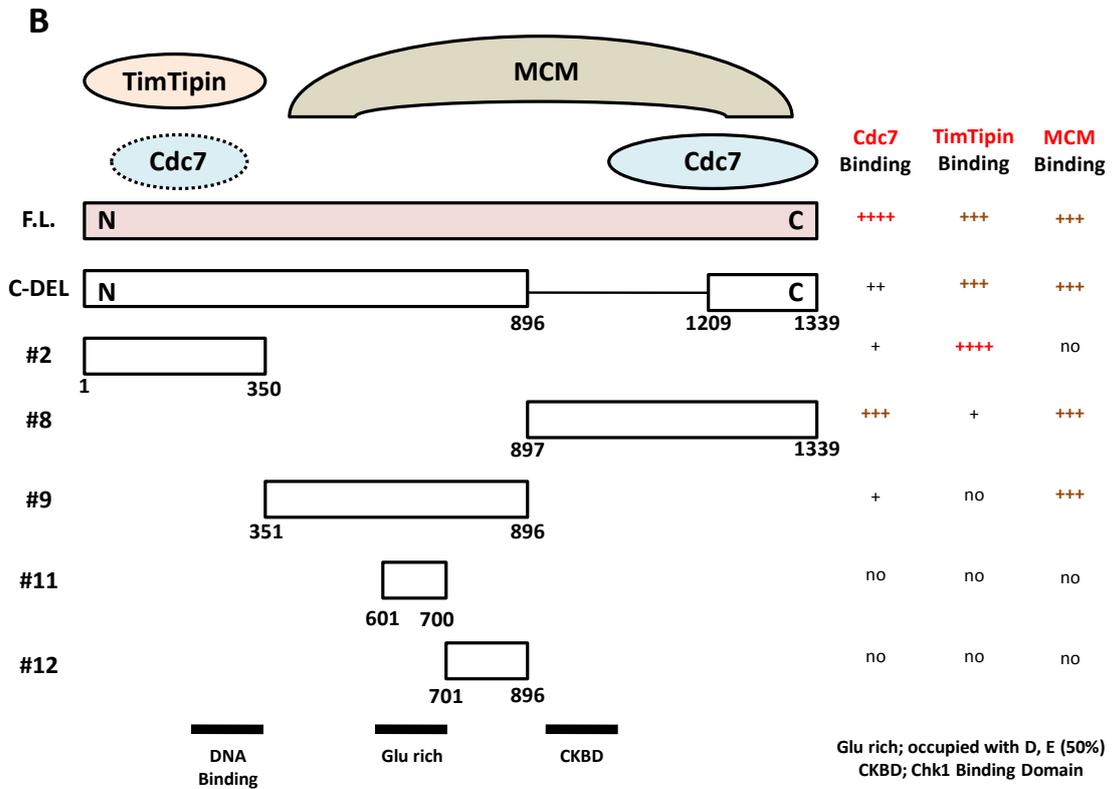
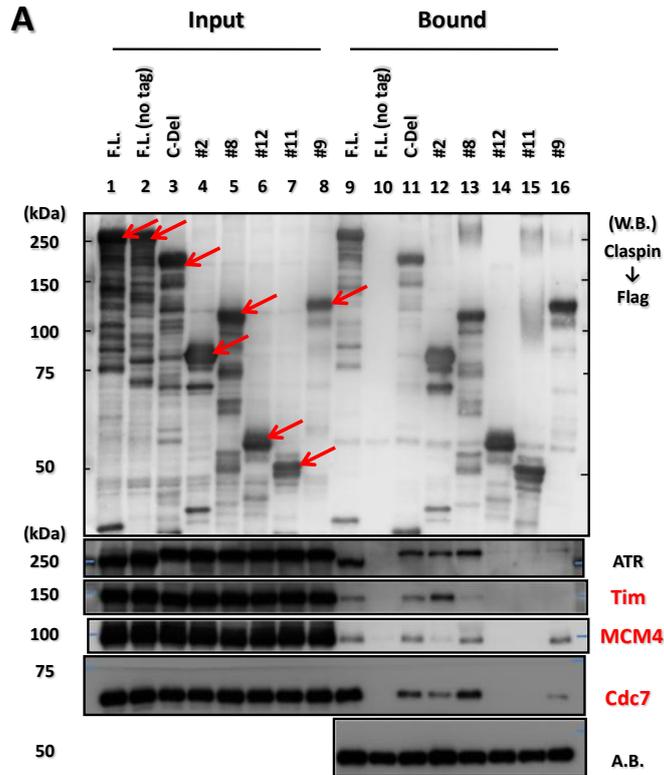
**B** Blast Search about spMrc1



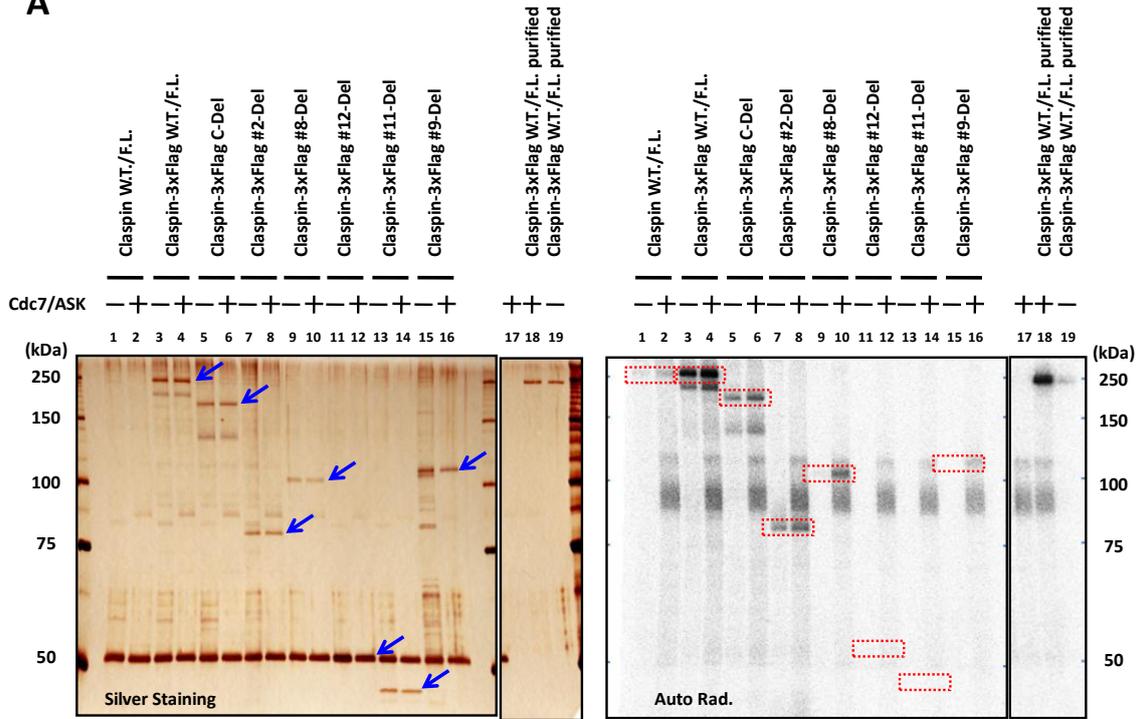
**Fig. 12. BLAST search on human Claspin and fission yeast Mrc1**



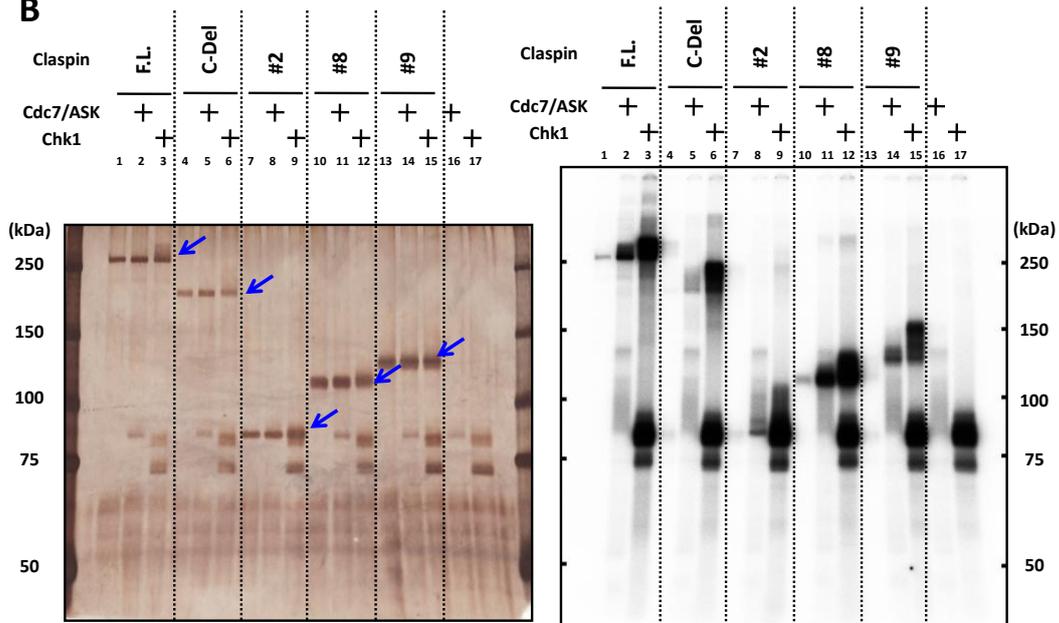
**Fig. 13. Coiled-Coil score of Claspin and fission yeast Mrc1 polypeptide sequences**



**Fig. 14. Interaction of Claspin with replication factors: truncation mutants**

**A**

[Kinase assay of Claspin pulled-down on anti-Flag M2 affinity beads]

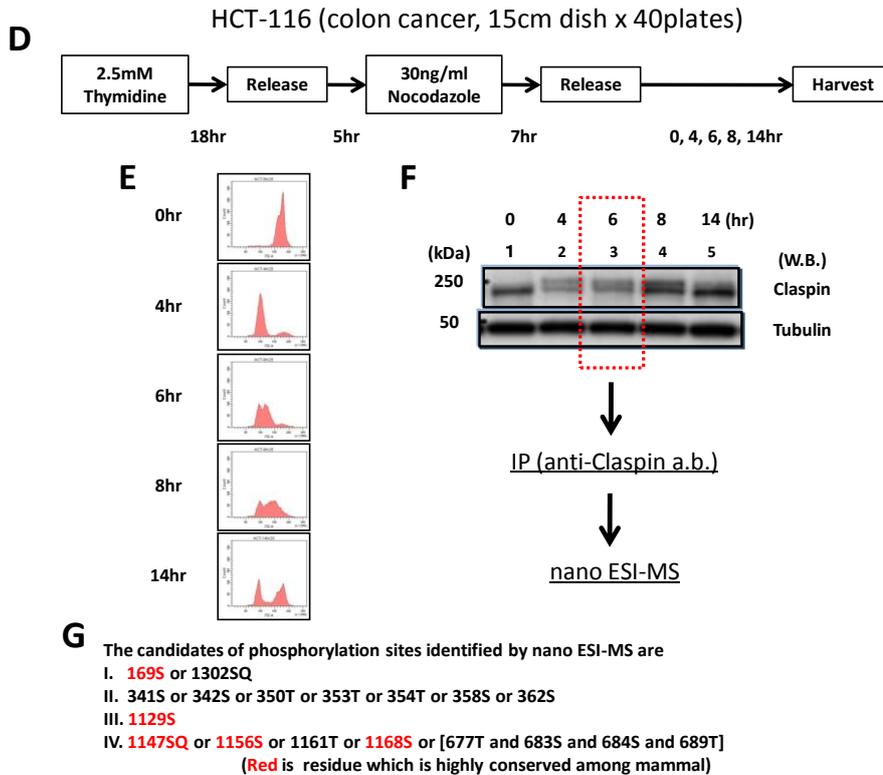
**B**

[Kinase assay of Claspin expressed and purified from 293T cells]

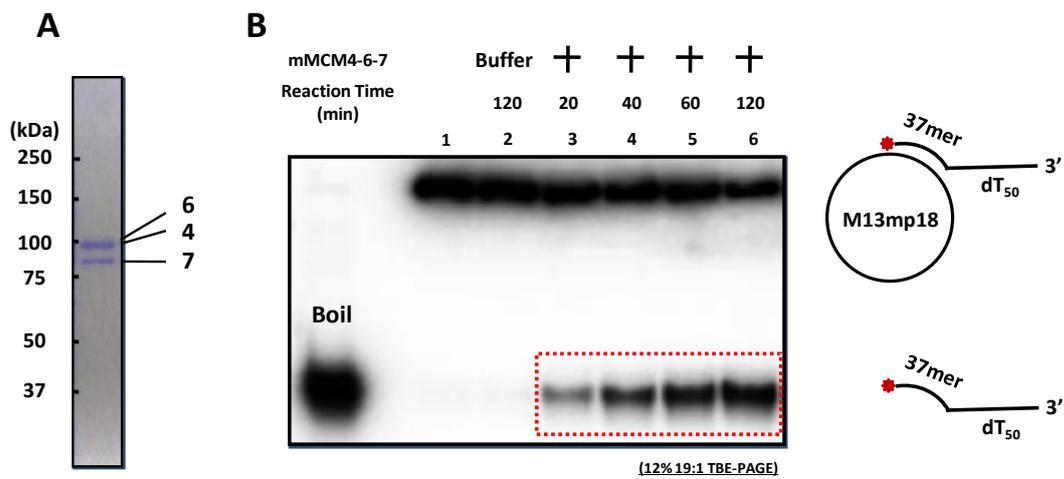
C

Claspin	#1 1-194	#2 195-335	#3 336-526	#4 527-734	#5 735-875	#6 876-1014	#7 1015-1143	#8 1144-1319
phosphorylation by Cdc7	++	++	-	+/-	-	-	+/-	+
phosphorylation by Cdc7 + polyamine	++	++	+/-	+/-	+	+	+++	+
phosphorylation by Cdk2-CyclinE	++	++	-	-	++	+	+	+++
SQ TQ site	1	1	1	0	3	2	1	3
Chk1 Binding Site						910-919 939-948 976-985		
Ser, Thr content	35/194 18.04%	18/141 12.77%	31/191 16.23%	22/208 10.58%	34/141 24.11%	19/139 13.67%	10/129 7.75%	25/176 14.20%
Asp, Glu content	21.13%	19.15%	20.42%	31.25%	15.60%	23.74%	32.56%	15.91%
Lys, Arg, His content	19.07%	21.99%	13.61%	16.35%	8.51%	12.23%	18.60%	18.18%

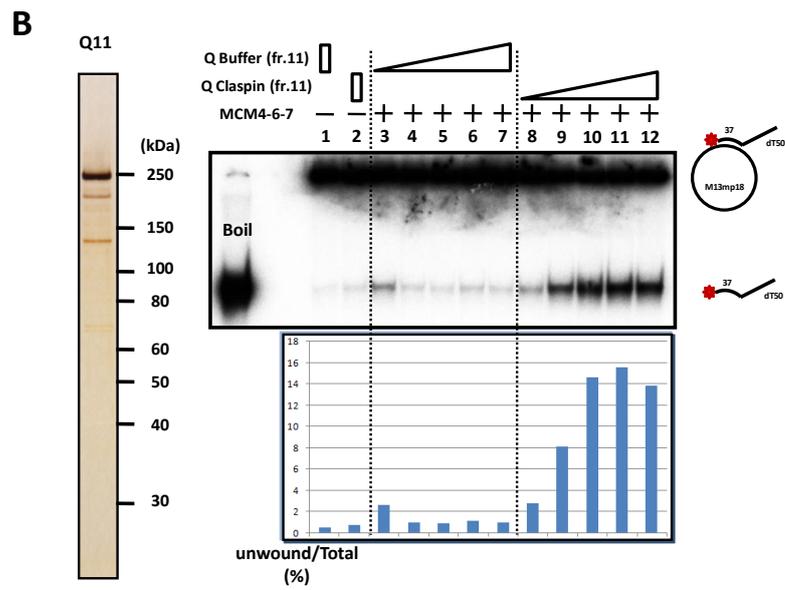
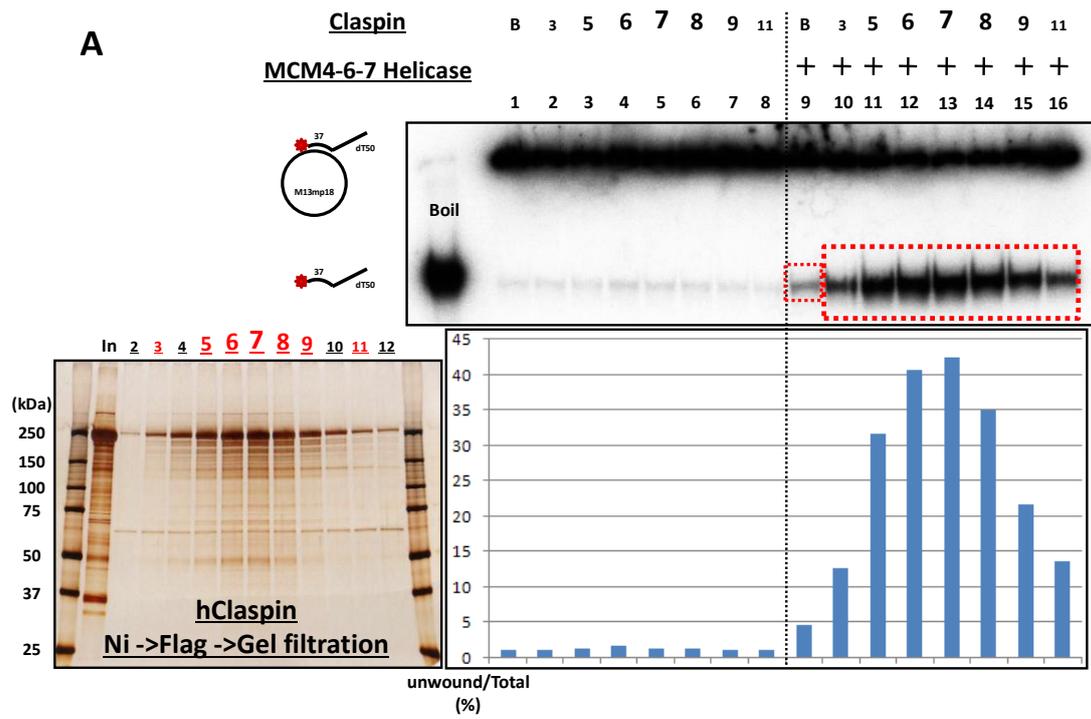
[Kinase assay with proteins expressed and purified from *E. coli.*]



**Fig. 15. Phosphorylation of Claspin**

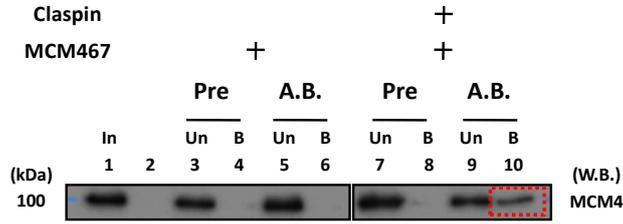


**Fig. 16. Purification of MCM4-6-7 helicase and measurement of its helicase activity**

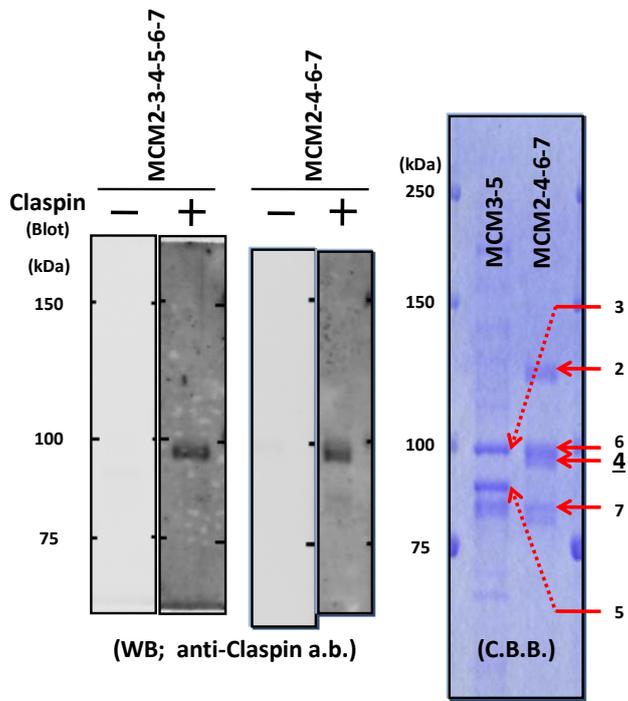
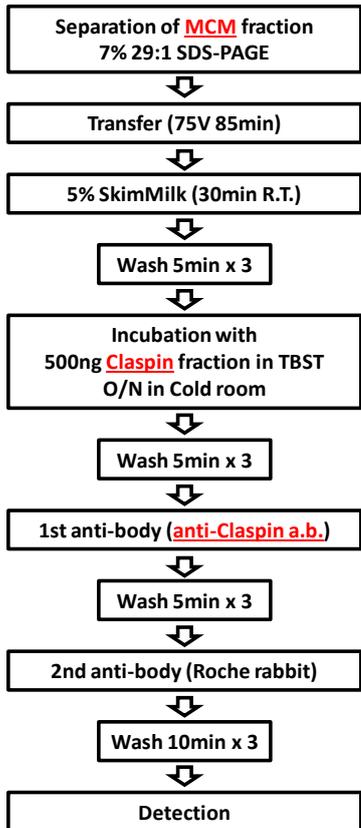


**Fig. 17. Stimulation of helicase activity of MCM4-6-7 by Claspin**

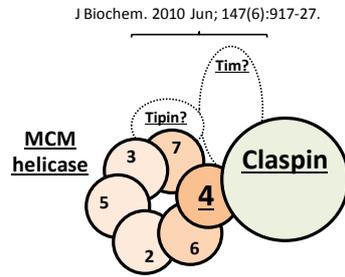
**A**



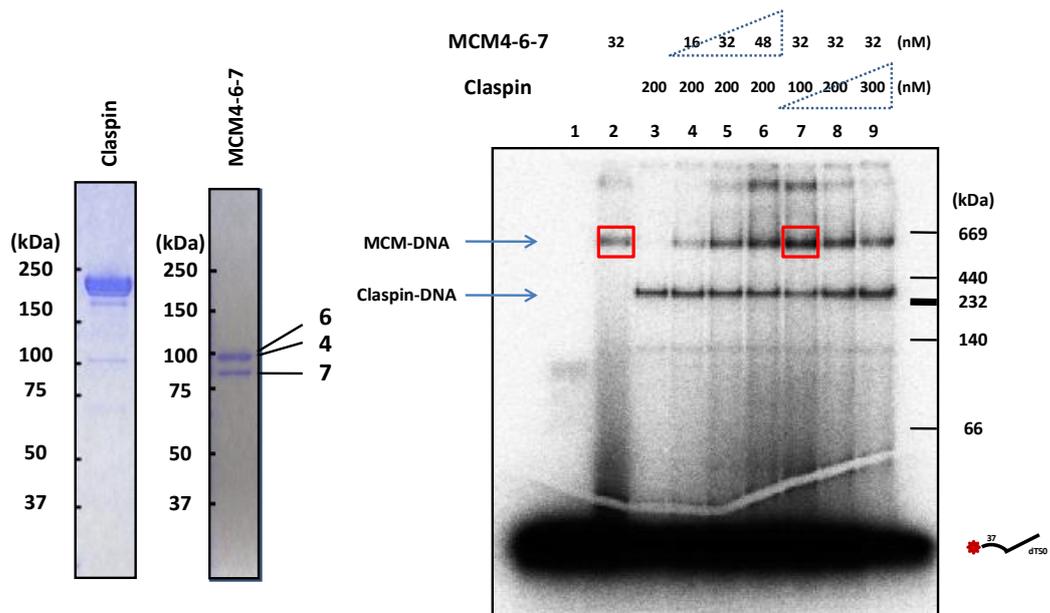
**B**



C



**Fig. 18 Interaction between Claspin and MCM**



**Fig. 19 DNA binding assays of Claspin and MCM**