# The region consisting of the most C-terminal 30 amino acid residues of Cockayne syndrome group B protein plays critical roles for functions in transcription-coupled repair and SUMOylaton

（CSB の C 末端領域 30 アミノ酸は転写と共役した修復における機能と SUMO 化に対し決定的な役割を担う）

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## Contents

## Abstract

The damage on DNA by UV is repaired by nucleotide excision repair (NER), and transcription-coupled repair (TCR) is a sub-pathway of NER. Cockayne syndrome (CS) is a recessive disorders and cells from CS patients exhibit hypersensitivity to UV. CSB is the gene product of one of the responsible genes for CS. It works in TCR and has ubiquitin binding domain (UBD) in its C-terminal region. The C-terminal region containing UBD is essential for the functions of CSB. In this study, I generated several CSB deletion mutants and analyzed the functions of the C-terminal region of CSB in TCR. I revealed that not only UBD but also the C-terminal 30 amino acid residues are required for UV-resistance and TCR. This region was involved in the interaction with RNA polymerase II, CSA translocation to nuclear matrix and association with chromatin of CSB after UV irradiation. I also found that CSB is modified by SUMO-2/3 in a UV dependent manner and that the modification was abolished in the CSB mutant deleted C-terminal 30 amino acid residues or by introducing an substitution of Lys-205 to Arg. This substitution lead cells to defect TCR. These results indicate that deletion of only 30 amino acid residues in the C-terminal region causes some dysfunctions of CSB and leads cells to UV hypersensitive and SUMOylation to CSB might contribute to TCR.

## Introduction

### 1. Nucleotide excision repair

All genetic information of us is retained in DNA so that it must be needed to be stable. However, DNA in cells is continuously threatened with various DNA damage, for example ultraviolet light (UV), some chemical compounds, active oxygen and so on. Damage on DNA causes mutation and apoptosis, leading to aging and cancer. To avoid these crises, cells have some DNA repair mechanisms. Nucleotide excision repair (NER), one of the repair mechanisms, works to deal with DNA lesions that distort double helix structure of DNA, for example, cyclobutane pyrimidine dimers and (6-4) pyrimidine-pyrimidone photoproducts formed by UV irradiation. NER consists of three steps (Fig. 1).

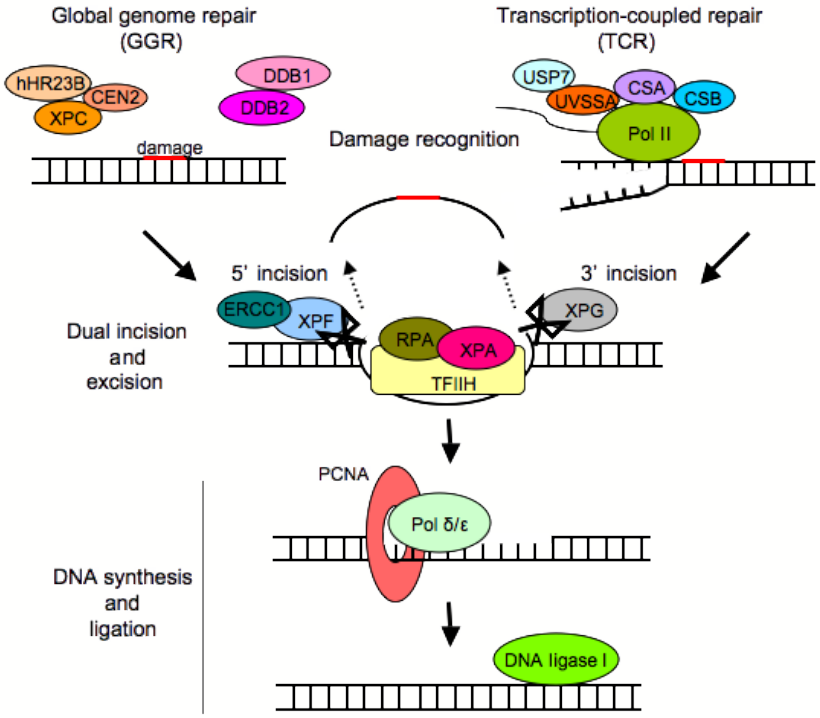


**Figure 1. Schematic representation of nucleotide excision repair (NER).**
NER consists of three steps: (1) damage recognition (2) damage incision and excision (3) DNA synthesis and ligation.
In damage recognition step, there are two subpathways; Global genome repair (GGR) and Transcription-coupled repair (TCR).

First, damage on DNA is recognized. Second, both sides of the lesion are incised and the oligonucleotide containing the lesion is removed. Finally, the gap is filled by DNA synthesis. There are two sub-pathways in NER. One is global genome repair (GGR) and the other is transcription-coupled repair (TCR). GGR works through whole genome. On the other hand, TCR specifically removes lesions on the transcribed strands of actively transcribed genes (1-4). About 99% of DNA damage are identified by GGR and the rest of damage are identified by TCR (5). In GGR, the hetero-trimer complex including xeroderma pigmentosum group C protein (XPC) and DNA damage-binding protein 1 and 2 (DDB1-DDB2) complex are involved in damage recognition (6, 7). By contrast, in TCR, RNA polymerase II (Pol II) that is arrested at the damage site is considered to be a signal for damage recognition. Once Pol II is arrested, other DNA repair factors, for example, Cockayne syndrome group B protein (CSB) and Cockayne syndrome group A protein (CSA) are gathered at the site (8, 9). UVSSA identified recently as a TCR factor (10-12) is also recruited to the site. Subsequent incision step is common both in GGR and TCR. Transcription factor IIH (TFIIH), one of general transcription factors, unwinds DNA double strand near the damage into single strand, and then xeroderma pigmentosum group A protein (XPA) and replication protein A (RPA) stabilize the bubble structure of locally unwound DNA. Excision repair cross-complementation group 1 protein (ERCC1)-xeroderma pigmentosum group F protein (XPF) complex and xeroderma pigmentosum group G protein (XPG) recognize the structure and cleave the damage strand on 5' and 3' sides of the lesion, respectively, resulting

in excision of about 30 nucleotides fragment containing the lesion. This removal makes a single-stranded gap on DNA, and DNA polymerases fill the gap by DNA synthesis in a proliferating cell nuclear antigen (PCNA) dependent manner. Subsequently, DNA ligase connects DNA strand (8, 9, 13, 14).

## 2. NER-deficient diseases

NER is necessary to keep DNA stable, but some autosomal recessive diseases including xeroderma pigmentosum (XP), Cockayne syndrome (CS), and UV-sensitive syndrome ($UV^{S}S$) have defects in NER (1,15, 16).

There are 8 complementation groups in XP; XP-A to XP-G and a variant, XP-V (15). In Japan, the frequency of XP is about one 10-thousandths. In XP-A to XP-G, the primary defect is in NER. In XP-V, NER is normal, but translesion DNA synthesis is defective. Clinical symptoms are different between complementation groups (Fig. 2A and 2B). All XP patients have photosensitivity, an extremely high risk of cancer on sun-exposed skin, and freckling. On the

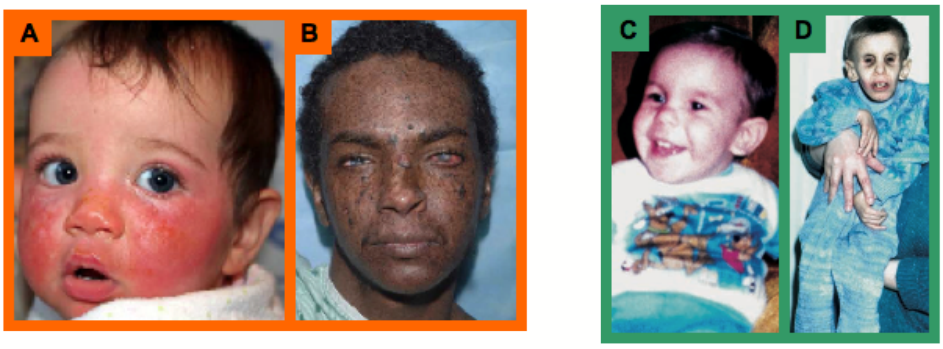


**Figure 2. XP and XP-CS patients.**
(A) A XP-D patient at 6 months old (XP420BE). Heavy blistering can be seen at sun exposure area on her face (5).
(B) A XP-C patient at 23 years old (XP393BE). He has many skin pigmentations and several skin cancers on his face. In addition, an cicatrix of cornea is seen on his each eye(5).
(C)(D) A XP-CS patient (XP20BE) at 1 year old (C) and 6 years old (D). As he grew up, typical symptoms of CS appeared. Loss of a subcutaneous fat, hallow eyes and a growth failure are significant (17).

other hand, patients of XP-A, -B, -D and XP-G have a tendency to have blistering of skin by only a little sun exposure, but patients of the remaining groups do not exhibit that symptom. Some patients of XP-A, -B, -D, and XP-G exhibit progressive neurological denaturation (5).

Clinical symptoms of CS are different from those of XP (Fig. 3A and 3B). In CS, photosensitivity, neurologic dysfunctions, premature aging and developmental disorder are main symptoms. XP patients are prone to develop skin cancer, but there is no report of CS patients with sunlight-induced skin cancer (16). The frequency of CS is about a millionth. There are 2 complementation groups, CS-A and CS-B (18,19). In CS, TCR is defective. CS patients exhibit photosensitivity almost at the same level to the patients of XP-A,


**Figure 3. CS, COFS and UV$^S$S patients.**
(A)(B) A CS patient at 2 years old (A) and 9 years old (B). The complementation group is not reported. Typical symptoms of CS, for example, loss of subcutaneous fat and beaked nose, appears markedly in (B), whereas no apparent symptoms of CS are not confirmed in (A). At 9 years old, he could not stand up by himself and mental retardation was observed (20).
(C)(D) A COFS patient at 3 weeks old (C) and 9 months old (D). He was born with several symptoms of COFS; hallow small eyes, cataract, spasticity of fingers and so on. His growth was dramatically delayed, and there are little change in growth between (C) and (D) (21).
(E)(F) A UV$^S$S patient (UV$^S$1KO) at 8 years old (E) and 33 years old (F). He had numerous freckles on his face and telangiectasia, but no growth failure and neurologic symptoms at 8 years old. At 33 years old, he exhibited only photosensitivity. Except of that, he has stayed been healthy (22).

who lacks both GGR and TCR (23-25). Cells from CS patients exhibit hypersensitivity to UV, but GGR functions normally, so that RNA synthesis is not recovered after UV irradiation (26, 27). CS patients are classified into three types (28-31). Type I is the major and classical one, and the CS patients of this type exhibit their symptoms as they get older. That is, they are born with little symptoms of CS, but at the early stage of their life, several symptoms begin to appear and get severe as they grow up. In type II, CS patients are born with noted symptoms of CS. Type II is regarded as a severe group. In contrast, type III is late-onset, so that this type is considered to be a mild group. Some patients exhibit symptoms of both CS and XP that are very severe photosensitivity and symptoms of CS type II (Fig. 2C and 2D). No skin cancer has reported in CS-XP patients like CS (17, 32). They have mutation in *XPD*, *XPB* or *XPG*.

Additionally, Cerebro-oculo-facio-skeletral (COFS) syndrome is similar to CS type II (Fig. 3C and 3D). Arthrogryposis, cataracts and microcephaly are typical symptoms and the patients have mutation in *CSB*, *XPD*, *XPG* or *ERCC1* (33, 34).

Clinical symptoms of UV$^{S}$S are photosensitivity and mild freckling (Fig. 3E and 3F). No neurologic symptoms, premature aging, and developmental disorders have been reported. However, cells from UV$^{S}$S patients exhibit UV hypersensitivity and have defects in TCR. Recovery of RNA synthesis after UV irradiation is not observed in the cells, like cells from CS patients. There are three complementation groups in UV$^{S}$S, which caused by mutation in *CSA*, *CSB*, or *UVSSA*. *UVSSA* was identified recently (10-12).

## 3. CSB

More than 80% of CS patients belong to CS-B, which is caused by mutations in *CSB* gene. CSB is the product of *CSB* gene and consists of 1493 amino acid residues. CSB has ATPase domain in the central region and belongs to SWI2/SNF2 family (35). Most proteins in this family have helicase activity and involved in transcription and chromatin remodeling. CSB does not have helicase activity, but has DNA-dependent ATPase and chromatin remodeling activities (36, 37). In C-terminal region, CSB has ubiquitin binding domain (UBD). This domain is essential for CSB function in TCR (38), but ubiqutinated protein that interacts with CSB via UBD is still unknown. It has been reported that CSB interacts with Pol II (39), CSA (40), p53 (41), XPG (42), XPB and XPD in TFIIH complex (43, 44). CSB interacts with Pol II in transcription elongation, suggesting that CSB is involved in not only TCR, but also transcription (39). That is supposed to be a reason why mutation of CSB affects severity of the clinical symptoms of CS patient and its characteristic features.

$CSB_{1\text{-}1220}$, which lacks C-terminal 273 amino acid residues containing UBD, had ATPase activity at almost the same level of that of wild type CSB and was able to interact with Pol II after UV irradiation in cross-linking condition, but cells expressing $CSB_{1\text{-}1220}$ exhibited hypersensitivity to UV and reduced recovery of RNA synthesis after UV irradiation (38)

In CSB knock out mice, phenotype is partially different from CS patients. UV hypersensitivity, the lack of the ability of RNA synthesis after UV irradiation,

and normal GGR are found in CSB deficient mice. But CSB deficient mice have only mild neurological symptoms and show high risk to skin cancer (45).

## 4. CSA

CSA is the product of *CSA* gene that is responsible for CS-A. It consists of 396 amino acid residues and has seven WD40-repeats (46, 47). The WD-40 repeats are found in many proteins that work in several mechanisms, for example, transcription, RNA processing and cell division. Despite of various protein functions, the structures of WD40-repeats are conserved (48). Eight missense mutations in *CSA* gene were reported, and all of the mutations occur within the WD40-repeats (49, 50). This motif has a key role for CSA structure to interaction with other proteins (50). CSA forms a complex containing DDB1, Cul4A, Roc1, and COP9 signalosome. This complex has ubiquitin ligase activity that depends on UV irradiation (51). CSA also interacts with CSB, p44 in TFIIH, XAB2, and UVSSA (10-12, 46, 52-54). CSB might be one of the substrate of this ubiquitin ligase because CSB is reported to be ubiquitinated and degraded after UV irradiation in a CSA dependent manner (55, 56). CSA is translocated to nuclear matrix following UV irradiation depending on CSB and TFIIH (57, 58).

## 5. UVSSA

UVSSA is the product of *UVSSA* gene that is responsible for UV$^{S}$S-A. It consists of 709 amino acid residues and has VHS/ENTH domain in its N-terminal and DUF2043 domain in its C-terminal (10, 11, 12, 59). The

functions of these domains are still unknown, but it was reported that VHS/ENTH domain of RBM16/SCAF8 is involved in the interaction with the C-terminal domain of the largest subunit of Pol II (60, 61). UVSSA interacts with Pol II, CSA and CSB. There are two forms of Pol II; hyperphosphorylated and hypophosphorylated forms. UVSSA specifically binds to hyperphosphorylated Pol II and might promote ubiquitination of it (12). UVSSA is associated with chromatin depending on CSA (53). In addition, UVSSA makes a complex with USP7, one of a deubiquitinating enzyme, and the complex makes CSB stable by preventing from degradation by proteasome (10-12). It is expected that analysis of the function of UVSSA reveals new aspect of TCR.

## 6. Posttranslational modifications of CSB

Posttranslational modifications play important roles for CSB function in TCR. For example, CSA-dependent ubiquitination to CSB is involved in the degradation of CSB and it might contribute to resumption of RNA synthesis (56). It was reported that CSB is also ubiquitinated by BRCA1-BIRD1, even in the absent of CSA (62). UVSSA-USP7 complex is assumed to deubiquitinate CSB. That event is needed for TCR to proceed properly (11). Not only ubiqutination, but also phosphorylation is essential for CSB in TCR. CSB is phosphorylated by c-Abl tyrosine kinase and this modification is related to the localization of CSB (63). Posttranslational modifications deserve to be considered to analyze the function of CSB in TCR.

## 7. SUMO modification

Small ubiquitin-like modifiers (SUMOs) were first reported in 1996 and 1997 (64, 65). Four isoforms of SUMO are found in mammals; SUMO-1, SUMO-2, SUMO-3 and SUMO-4. SUMO-1 is attached to its substrates mainly as mono-SUMOylation form, while SUMO-2 and SUMO-3 form poly-SUMO chain (66). SUMO-4 has been little analyzed yet. The difference of the function between SUMOylations by each isoform is still unknown. Because the amino acid sequences between SUMO-2 and SUMO-3 are 97% identical in human and their character is very similar, they are usually treated as SUMO-2/3 (67). To perform SUMOylation, SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2) and SUMO-ligase (E3) are needed (Fig. 4). In eukaryotes,

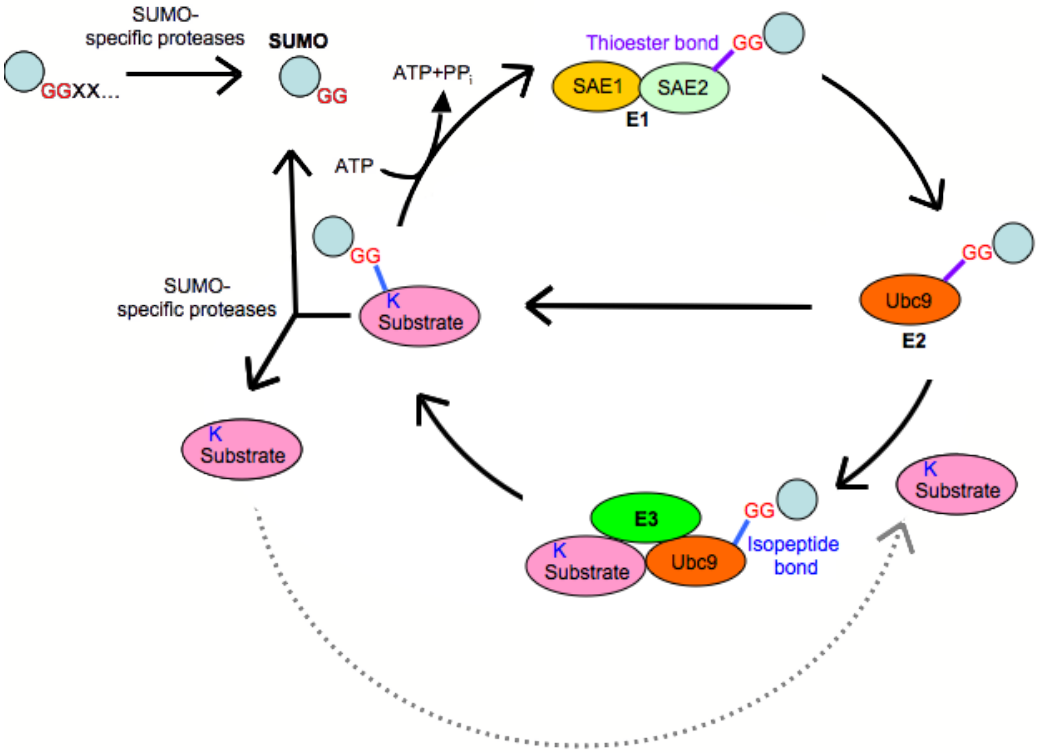


**Figure 4. Schematic diagram of SUMOylation pathway.**
In eukaryotes, SUMO-specific proteases cleave C-terminal of SUMO and Gly-Gly residues are exposed. This processed SUMO is activated by SAE1/SAE2 (E1) in ATP dependently and transferred to Ubc9 (E2). The SUMO binds to Lys residure in a substrate protein by isopeptide bond in dependent or independent manner on E3. SUMO is released from the substrate by SUMO-specific proteases and recycled (68).

SUMO-specific proteases cleave C-terminal of SUMO and Gly-Gly residues are exposed. SAE1/SAE2 works as E1 and the processed SUMO is adenylated and bound to SAE2 by thioester bond. Then, it transferred to Ubc9, the only E2 of SUMO. Ubc9 contributes to target selection of SUMO. In human, six E3s of SUMO are reported. In ubiquitination, E3 plays a role to translate ubiquitin from E2 to the lysine residue in the substrate, but in SUMOylation, E2 has the activity to perform the translation, so that E3 is only needed in some substrates (69-77). SUMO modification is intimately related to protein function in several situation, for example, translation, protein stability, protein interaction, localization and so on. In NER, some proteins are modified by SUMO. In *Saccharomyces cerevisiae*, Rad4, Rad16, Rad7, and Rad1 are SUMOylated (78). In human, XPC is modified by SUMO and the modification is involved in the stabilization of XPC (79).

In this study, I revealed that not only UBD, but also the C-terminal 30 amino acid residues of CSB is essential for the function of CSB in TCR. CSB mutants with deletion of this region exhibit some functional abnormality and cells expressing the mutant CSB are hypersensitive to UV. Moreover, I found that CSB is modified by SUMO-2/3 followed by UV irradiation. No other protein in TCR has been reported to be SUMOylated. Interestingly, CSB that lacks 30 amino acid residues in the C-terminal are not SUMOylated, but there is no lysine that is responsible for SUMOylation in the region. On the other hand, SUMOylation of CSB is repressed by substitution of Lys-205 to Arg and

$CSB_{K205R}$ expressing cells are hypersensitive to UV and lack TCR. This study provides new clues to solve the molecular mechanism of TCR.

## Results

### UV sensitivity and recovery of RNA synthesis after UV irradiation of cells expressing mutant CSB proteins with deletions of C-terminal region

To analyze the functions of C-terminal region of CSB in TCR, I generated several C-terminal truncated CSB mutants including $CSB_{1\text{-}1220}$ (Fig. 1A). $CSB_{1\text{-}1400}$ has a deletion from just N-terminal of UBD to the C-terminal. $CSB_{1\text{-}1445}$ and $CSB_{1\text{-}1463}$ have UBD and $CSB_{1\text{-}1463}$ lacks only C-terminal 30 amino acid residues. There is no report about functional domains and amino acid residues in the region corresponding to 1221-1400, 1446-1463 and 1464-1493 amino acid residues in CSB, except that Ser-1461 is a target site of phosphorylation. $CSB_{\Delta UBD}$ lacks 49 amino acids residues (1385-1433) containing UBD. For convenience, I designate regions in CSB as follows; 1221-1493 is the C-terminal region, 1221-1400 is the region just before UBD, 1401-1428 is UBD, 1429-1463 is the region just after UBD and 1464-1493 is the most C-terminal region. Besides expression constructs for the CSB mutants, an expression construct for wild type CSB ($CSB_{WT}$) was also generated. All the CSB proteins have FLAG-HA epitope tags on their N-terminal. Those epitope tagged CSBs were stably expressed in CS1ANSV cells. CS1ANSV cells were derived from a CS-B patient, theoretically express only short CSB (49), and are deemed not to have functional CSB. The transfectants expressing each CSB protein were for convenience designated as $CSB_{WT}$ cells, $CSB_{1\text{-}1220}$ cells, $CSB_{1\text{-}1400}$ cells, $CSB_{1\text{-}1445}$ cells, $CSB_{1\text{-}1463}$ cells, and $CSB_{\Delta UBD}$ cells, respectively.

First, the colony-forming ability of the UV-irradiated transfectants was measured (Fig. 1B). $CSB_{WT}$ cells exhibited almost the same level of UV sensitivity as normal (WI38VA13) cells. $CSB_{1\text{-}1220}$ cells showed UV hypersensitivity comparable to that of CS1ANSV cells. The cells expressing other deletion mutants were also more sensitive to UV than $CSB_{WT}$ cells but were less sensitive than CS1ANSV cells irrespective of whether CSB mutants have UBD or not. Thus, all the cell lines expressing CSB deletion mutants exhibited hypersensitivity to UV.

Next, the recovery of RNA synthesis after UV irradiation was measured. This becomes an index of TCR. RNA synthesis decreases after UV irradiation, but that in WI38VA13 cells and $CSB_{WT}$ cells was recovered to the level in non-irradiated cells at 24 h after UV irradiation (Fig. 1C). No such recovery was observed in CS1ANSV cells. All the cell lines expressing CSB deletion mutants had little ability to recover RNA synthesis after UV irradiation. $CSB_{1\text{-}1220}$ cells showed most severe reduction of RNA synthesis. On the other hand, the reduction of RNA synthesis in $CSB_{\Delta UBD}$ cells was slightly less than that of the cells expressing other deletion mutants, but was almost the same level to that in CS1ANSV cells. Anyway, all the cell lines expressing CSB deletion mutants were deficient in TCR.

These results indicate that not only UBD, but also the most C-terminal region affected TCR. If this region plays an important role for the function of CSB, this region should be conserved among species. Hence, I compared C-terminal amino acid sequences of mammalian CSB homologs (Fig. 1D upper). Approximately

80% of amino acid residues in the most C-terminal region were conserved between 6 mammals, and the amino acid sequences of *Homo sapiens* and *Macaca mulatta* were identical. When I focused on non-conserved amino acid residues, there was not large dispersion. Among 6 animals, only two amino acid residues were used at most non-conserved residues and three at one non-conserved residue. Only approximately 43% were common including other animals (Fig. 1D lower), but amino acid residues in this region are relatively well conserved. In frog (*Xenopus tropicalis*) CSB homolog and Rad 26 (*Saccharomyces cerevisiae* CSB homolog), there were no amino acid residues corresponding to this region.

The most C-terminal region is conserved enough among mammals, and modestly among other animals.

## The effects of C-terminal deletion of CSB on the function in TCR

The results that C-terminal regions of CSB were indispensable for UV resistance and the recovery of RNA synthesis after UV irradiation brought me the interest what functions in TCR do the regions have. It is reported that CSB interacts with Pol II in a UV dependent manner (40, 80, 81) and hypothesized that CSB works as a DNA translocase of Pol II (82). Therefore, I examined whether the mutant CSB proteins interact with Pol II after UV irradiation. N-terminal FLAG-HA epitope tagged CSB was immunoprecipitated from solubilized chromatin fractions using FLAG-M2 agarose and western blotting was performed with anti-Pol II antibody. The procedure is described in Fig. 2A.

In $CSB_{WT}$ cells, the amount of Pol II co-precipitated with CSB was increased after UV irradiation (Fig. 2B, lanes 1 and 2). Whereas, in all the CSB mutant cells, irrespective of whether they have UBD or not, the amount of co-precipitated Pol II was not increased after UV irradiation unlike in $CSB_{WT}$ cells (lanes 3-12). In $CSB_{1\text{-}1220}$ or $CSB_{1\text{-}1400}$ cells, which lack UBD, the amount of co-precipitated Pol II after UV irradiation stayed at the same as that before UV irradiation (lanes 3-6). However, in $CSB_{1\text{-}1445}$, $CSB_{1\text{-}1463}$ and $CSB_{\Delta UBD}$ cell, which have UBD or more C-terminal region than UBD, the amount of co-precipitated Pol II was slightly increased after UV irradiation (lanes 7-12). These results indicated that the deletion of C-terminal of CSB affected the UV-induced interaction with Pol II.

CSB is also needed for translocation of CSA to nuclear matrix after UV irradiation (57, 58). The translocation is relevant to TCR. So next, I investigated the ability of each CSB mutants to translocate CSA to nuclear matrix. Fig. 3A is the procedure for a cell-free method to detect UV-induced translocation of CSA (58). The mutant CSB cells were irradiated with UV or not irradiated and incubated for 1 h. CSK-Triton buffer-insoluble (CSK-ppt) fractions were prepared from the cells. CSK-Triton buffer-soluble (CSK-sup) fractions were prepared from CS-A (CS3BESV) cells expressing FLAG and HA epitope-tagged CSA. This fraction contains FLAG-HA tagged CSA. Each CSK-ppt fraction was incubated with the CSK-sup fraction. After DNase I treatment, the CSK-ppt were analyzed by western blotting with anti-HA antibody (Fig. 3B). When CSA translocation occurs, HA-tagged CSA is detected in a UV dependent manner. As

reported previously, CSA was detected when the CSK-ppt was prepared from $CSB_{WT}$ cells after UV irradiation (lanes 4 and 5) and not detected when CS1ANSV cells were used (lanes 2 and 3). When the CSK-ppt fractions were prepared from the mutant CSB cells, CSA was not detected (lanes 6-15). These results indicate that the C-terminal region of CSB is responsible for the CSA translocation to nuclear matrix. Furthermore, only the deletion of UBD or the most C-terminal region made the translocation attenuate.

## Association of the mutant CSB proteins with chromatin after UV irradiation

Because TCR is carried out on chromatin, association of TCR factors with chromatin is essential to progress TCR. For that reason, it is expected that the C-terminal region of CSB is needed to associate with chromatin. To test the expectation, insoluble fractions containing chromatin were prepared from CSB mutant cells after UV irradiation. Procedure for the preparation of insoluble fractions with CSK-Triton buffer is shown in Fig. 4A. The insoluble fractions were analyzed by western blotting using anti-CSB antibody (Fig. 4B). More $CSB_{WT}$ was detected in the insoluble fractions after UV irradiation. Intensity of the bands was quantified and plotted in Fig. 4C. The amount of $CSB_{WT}$ in the insoluble fraction was increased until 2 h after UV irradiation, and decreased to the level of non-irradiated cells by 16 h after UV irradiation. $CSB_{1\text{-}1220}$ and $CSB_{1\text{-}1400}$ were also increased in the insoluble fraction in a UV dependent manner, but the increases of both mutant CSB proteins were gentle and those at

the peak were small compared with that of $CSB_{WT}$. $CSB_{1-1463}$ was slightly increased until 2 h after UV irradiation and then decreased. $CSB_{\Delta UBD}$ was not increased after UV irradiation. The amount of $CSB_{1-1445}$ in the insoluble fraction was decreased by UV irradiation. These data showed that the deletion of C-terminal of CSB affected its association with chromatin.

## UV-dependent posttranslational modification by SUMO-2/3 to CSB

In Fig. 4B, some band shifts of CSB after UV irradiation were observed in the panel of $CSB_{WT}$ (lanes 2-4, asterisk). In the panel of $CSB_{\Delta UBD}$, the band shifts were very faint but also seen (lanes 27-29). In the panels of other CSB mutants, only single band of CSB (i.e. no band shift) was detected. It has been already reported that CSB is modified by ubiquitin and phosphate and that these posttranslational modifications affect the function of CSB in TCR (62, 63, 83). Therefore, the band shifts shown in Fig. 4B were presumed to reflect modifications of CSB. Given the above, I hypothesized that there are some relationships between C-terminal deletion of CSB and certain posttranslational modifications. To demonstrate this hypothesis, I first examined what modifications were detected to CSB after UV irradiation. The procedure is summarized in Fig. 5A. To exclude modifications to CSB-interacting proteins and to detect modifications to CSB itself, cells were treated with SDS lysis buffer, which disrupts protein interactions, and then CSB was immnopresipitated from the lysate. Modifications by ubiquitin and SUMO were examined by western blotting using specific antibodies. In this study, I could not detect ubiquitinated

CSB regardless of UV irradiation (Fig. 5B, lanes 1-8). It is reported that XPC, a GGR factor, is modified by SUMO-1 following UV irradiation (79), so I tested whether CSB was modified by SUMO using antibodies corresponding to three isoforms of SUMO; SUMO-1, SUMO-2 and SUMO-3 (Fig. 5B, lanes 9-12, 13-16, and 17-20, respectively). No bands were detected with anti-SUMO-1 antibody, whereas remarkable three bands more than 250 kDa and some upper faint bands were detected with anti-SUMO-2, and -3 antibodies only after UV irradiation.

Amino acid sequences of SUMO-2 and SUMO-3 are 97% identical and differences of function between them have not been found as yet, thereby they are usually written together as SUMO-2/3. Almost same band patterns were detected with anti-SUMO-2 and anti-SUMO-3 antibodies in Fig. 5B (lanes 16 and 20), so that I hereafter used only anti-SUMO-3 antibody to detect the band shifts.

## Analysis of CSB SUMOylation

Ubc9 is the only SUMO-conjugating enzyme and SUMO protein cannot be conjugated to substrate proteins without Ubc9. When Ubc9 was knocked down by the transfection of siRNA, the bands detected with anti-SUMO-3 antibody became faint (Fig. 6A). These data indicated that the bands are due to SUMO modification.

Soluble and insoluble fractions were prepared with CSK-Triton buffer and SUMOylation of CSB was analyzed. The bands were detected with

anti-SUMO-3 antibody only in the insoluble fraction chromatin and not in the soluble fraction (Fig. 6B).

I tested whether SUMO modification to CSB is affected in TCR-deficient cells (Fig. 6C). UVSSA-deficient Kps3 cells and CSA-deficient CS3BE cells were used. In both cells, CSB SUMOylation following UV irradiation was detected (lanes 1, 2, 5, and 6) as with in corrected cells transfected the responsible cDNAs (lanes 3, 4, 7, and 8).

ATPase activity of CSB is essential for its function in TCR. One amino acid substitution in ATPase domain (K538R) of CSB brings CSB dysfunctional in TCR by disrupting ATPase activity and UV hypersensitivity to cells expressing the mutant CSB protein (37). In the cells expressing ATPase-defective CSB, such a dysfunctional CSB was also SUMOylated after UV irradiation (Fig. 6D).

I next examined whether the CSB deletion mutants described above are SUMOylated (Fig. 7). $CSB_{\Delta UBD}$ was SUMOylated after UV irradiation as with $CSB_{WT}$ (lanes 11-12, and 1-2), but no other CSB mutants were SUMOylated.

Taken together, UV-dependent SUMO modification of CSB occurred independently of UVSSA, CSA and ATPase activity of itself. In addition, the C-terminal region of CSB excluding UBD was involved in SUMOylation of CSB.

## Effects of substitution of lysine to arginine in the C-terminal region of CSB

In Fig. 7, the C-terminal truncated CSB mutants ($CSB_{1-1220}$, $CSB_{1-1400}$, $CSB_{1-1445}$ and $CSB_{1-1463}$) were not modified by SUMO after UV irradiation.

$CSB_{1\text{-}1463}$, which has the smallest deletion, lacks only the most C-terminal region. Thus, it was assumed that lysine residues of SUMO acceptor exist in the most C-terminal 30 amino acid residues of CSB. In this region, there were two lysine residues, Lys-1487 and Lys-1489 (Fig. 8A). To investigate whether these lysine residues were responsible for SUMOylation to CSB, I generated two CSB mutants with FLAG-HA epitope tags on their N-terminal; $CSB_{K1487,\ 1489R\ (2K\rightarrow R)}$ and $CSB_{K1457,\ 1487,\ 1489R\ (3K\rightarrow R)}$. Substitution of SUMO acceptor lysine to arginine abolishes SUMOylation at the site. Lys-1457 is located out of the 30 amino acid residues but just N-terminal side of this region. It is known that transition of a SUMO-modified site to nearby lysine residue can be happen when an authentic lysine for SUMOylation is substituted. Thus, Lys-1457 was substituted to arginine in conjunction with the substitutions at Lys-1487 and Lys-1489. Lys-1457 is also reported to be modified by ubiquitin independently of UV irradiation (55).

SUMOylation was examined in CS1ANSV cells stably expressing the substitution mutants as described above. Substitutions of Lys-1457, Lys-1487 and Lys-1489 to arginine did not abolish SUMOylation of CSB after UV irradiation (Fig. 8B). I noted that the band pattern of $CSB_{3K\rightarrow R}$ in non-UV irradiated cells was different from that of the other CSB mutants. Single band was detected in $CSB_{3K\rightarrow R}$ cells (lane 3), but no bands were in the other mutant cells. In addition, the abilities of colony-forming and recovering RNA synthesis after UV irradiation of the CSB mutants were examined. It was revealed that both the substitutions did not affect UV sensitivity (Fig. 8C) and the ability of

recovering RNA synthesis following UV irradiation (Fig. 8D).

These results indicated that the three lysine residues in the C-terminal region are not SUMO acceptor sites after UV irradiation and that the substitution of the lysine residues to arginine have no effects on the function of CSB in TCR.

## Effects of inhibition of CSB SUMOylation were not equal to those of the C-terminal deletion

I found that CSB is modified by SUMO-2/3 following UV irradiation in this study, and no other proteins in TCR have been reported to be modified by SUMO yet. The C-terminal deletion mutants of CSB in this study, except for $CSB_{\Delta UBD}$, were not SUMOylated, but the relation between SUMOylation of CSB and some dysfunctions in the cells expressing CSB mutants (Fig. 1-4) is not clear.

To elucidate whether SUMOylation of CSB is involved in functions of CSB in TCR, I performed Ubc9 knock down by the transfection of siRNA. Ubc9 is the only SUMO-conjugating enzyme (E2) in eukaryotes, so that Ubc9 knock down leads to the absence of SUMOylation. Ubc9 was decreased with siRNA to Ubc9 (Fig. 9A). In Fig. 9B, interaction of CSB with Pol II were examined. FLAG-HA epitope tagged CSB was immunoprecipitated from the solubilized chromatin fraction and Pol II co-precipitated with CSB was detected as shown in Fig. 2A. The amount of immnoprecipitated CSB was similar between the cells transfected with control siRNA and siRNA to Ubc9, but CSB was slightly SUMOylated after UV irradiation in Ubc9 knocked down cells, whereas the

amount of co-precipitated Pol II was increased after UV irradiation irrespective of whether Ubc9 was knocked down or not (compare lanes 1 and 2 with lanes 3 and 4).

Next, the translocation of CSA to nuclear matrix was examined as shown in Fig. 3A. HA epitope tagged CSA was detected in the DNase I-digested CSK-ppt fraction containing nuclear matrix prepared from UV-irradiated CS1ANSV cells expressing CSB irrespective of whether Ubc9 was knocked down or not (Fig. 9C, lanes 5 and 7).

Association of CSB with chromatin after UV irradiation was also examined as shown in Fig. 4A. When Ubc9 was knocked down, the amount of CSB in the insoluble fraction including chromatin was increased at 1 and 2 h after UV irradiation and decreased to the level of the non-irradiated cells by 12 h after UV irradiation (Fig. 9D and E). Little difference was detected compared with the cells transfected with control siRNA except 1 h after UV irradiation, when the amount of CSB from the Ubc9 knocked down cells was slightly lower than that from the cells transfected with control siRNA.

### Ubc9 knock down led CSB to be relatively stable in Kps3 cells

It was reported that UVSSA has the function to stabilize CSB (10-12). In UVSSA-deficient cells, CSB is degraded by proteasome after UV irradiation. This is a very characteristic event and I examined whether SUMOylation of CSB is engaged in the event. UVSSA-deficient Kps3 cells were transfected with control siRNA or siRNA to Ubc9 and whole cell extracts from these cells after

UV irradiation were analyzed by western blotting using anti-CSB antibody (Fig. 10A). Intensity of the bands was quantified and plotted in Fig. 10B. CSB was decreased gradually after UV irradiation in the Ubc9 knock down cells, but the degree of decrease was less than that in the control cells. These results suggested that CSB is more stable unless it is SUMOylated even if UVSSA is deficient.

The above data brought a new interest to me whether the stabilization of CSB makes Kps3 cells TCR-proficient. However, the recovery of RNA synthesis after UV irradiation was not observed in Ubc9 knock down Kps3 cells as well as in the control Kps3 cells (Fig. 10C).

In normal cell, two types of Pol II are detected; hypophosphorylated one and hyperphosphorylated one. Hypophosphorylated Pol II disappears by UV irradiation and appears again after DNA damage is repaired and transcription is resumed, so that the recovery of hypophosphorylated form of Pol II can be used as an index of the resuming transcription. In Ubc9 knock down Kps3 cells, both hypo- and hyperphosphorylated forms of Pol II were observed without UV irradiation but hypophosphorylated form was not observed again after UV irradiation (Fig. 10A), in agreement with the loss of recovery of RNA synthesis in the cells.

Ubc9 knock down made CSB resistant to UV-dependent degradation in Kps3 cell, but it was not enough to recover RNA synthesis after UV irradiation.

## SUMOylation played some important role in TCR in human cells

In *Saccharomyces cerevisiae*, SUMO E3 ligases Siz1 and Siz2 are needed for NER (78). I found that CSB was modified by SUMO-2/3 in a UV-dependent manner in this study, but the role of SUMOylation to CSB in TCR is unknown. There are no reports that any TCR factors are SUMOylated in eukaryotes. CSB works not only as a TCR factor but also as a transcription factor, and it was reported that SUMOylation is essential for the function of transcription factors. For example, forkhead box protein M1 (FOXM1) is SUMOylated and SUMOylated FOXM1 is needed to regulate its target gene activity (84). Therefore, I investigated whether SUMO plays some roles in transcription and TCR. First, transcription in Ubc9 knocked down cells was compared with that in the control cells (Fig. 11A). Incorporation of [$^3$H]-uridine without UV-irradiation was equally in Ubc9 knocked down cells and the control cells. Next, the recovery of RNA synthesis after UV irradiation in both the cells was compared (Fig. 11B). 2 h after UV irradiation, incorporation of [$^3$H]-uridine was decreased in both the cells. 24 h after UV irradiation, incorporation of [$^3$H]-uridine was recovered in the control cells, while that was only 70% in Ubc9 knocked down cells. These results indicate that TCR is hampered by Ubc9 knocked down.

To clarify roles of SUMOylation in TCR, it was examined whether CSA and UVSSA are SUMOylated (Fig. 11C). Cells expressing FLAG-HA epitope tagged CSA and UVSSA were treated with SDS lysis buffer, and immuprecipitation was performed with anti-FLAG antibody as shown in Fig. 5A. These samples were analyzed by western blotting with antibodies against each SUMO isoform. Regardless of UV irradiation, neither CSA nor UVSSA was modified by any

SUMO isoforms. Among some TCR factors, only CSB was modified by SUMO and is expected to be affected by Ubc9 knock down.

## Substitution of Lys-205 to Arg represses SUMOylation of CSB

The possibility that SUMOylation of CSB affects TCR brought me to search responsible lysine residues for ~~of~~ SUMOylation in CSB again. As described above, there is no responsible lysine for SUMOylation in the most C-terminal region. I used SUMO plot™ Analysis Program (Abgent) to search candidate lysine residues, and 6 residues showed high score for SUMOylation (Fig. 12A). Among these lysine residues, Lys-884 and Lys-481 are in ATPase domain. Substitution of them might abolish not only SUMOylation but also ATPase activity, and defect in ATPase activity brings dysfunctions of CSB. It is impossible to distinguish dysfunctions ascribed to defect in SUMOylation from that in ATPase activity. Therefore I removed the two residues from candidates of SUMOylation site. A search by PhosphoSitePlus (Cell signaling) showed that posttranslational modifications in CSB concentrate ~~on~~ in mainly two regions (Fig. 12B). Among 4 candidate lysine residues, only Lys-205 is located in the region. Thus, an expression construct for a substitution mutant of Lys-205 to Arg ($CSB_{K205R}$) with FLAG-HA epitope tags on its N-terminal (Fig. 12C) was generated, and it was stably expressed in CS1ANSV cells. Then, SUMOylation of $CSB_{K205R}$ was examined as described in Fig. 5A (Fig. 12D). No band was detected with anti-SUMO3 antibody regardless of UV irradiation (lanes 3 and 4). When a cell line over expressing $CSB_{K205R}$ was used, some faint bands were

detected upon UV irradiation, but the band pattern was different from that of $CSB_{WT}$ (lanes 5 and 6). $CSB_{K205R}$ cells were more sensitive to UV than $CSB_{WT}$ cells but were less sensitive than CS1ANSV cells similarly to $CSB_{1\text{-}1400}$, $CSB_{1\text{-}1445}$, $CSB_{1\text{-}1463}$ and $CSB_{\Delta UBD}$ cells (Fig. 12E). In addition, the recovery of RNA synthesis after UV irradiation was not observed in $CSB_{K205R}$ cells as well as all the cell lines expressing CSB deletion mutants in Fig. 1C (Fig. 12F).

Substitution of K205R caused repression of CSB SUMOylation and defect in TCR, suggesting that SUMOylation of CSB is relevant to mechanism of TCR.

## Discussion

CSB plays a critical role in TCR and its deficiency causes CS (49). The cells from CS-B patients exhibit hypersensitivity to UV and the defect of TCR (26, 27). In this study, I identified the most C-terminal region of CSB ($CSB_{1464-1493}$) as a responsible region for those cellular phenotypes. As deletion of this region affected some functions of CSB, the region is essential for CSB in TCR, as well as UBD. I also found that CSB is modified by SUMO-2/3 in a UV-dependent manner and that the most C-terminal region is needed for this modification. This is the first report on SUMOylation of TCR specific factors in mammalian cells. SUMOylation of CSB was repressed by substitution of Lys-205 to Arg, and $CSB_{K205R}$ cells showed hypersensitivity to UV and TCR defect. These results suggest that SUMOylation of CSB is relevant to mechanism of TCR.

SUMOylation has various effects on functions of substrate proteins. It has been reported that SUMOylation is likely to function in some diseases that have symptoms of neurologic dysfunctions or premature aging. This study provides a new clue to solve the mechanism of TCR and to understand symptoms of CS.

### The most C-terminal region of CSB is essential for the function

No CSB mutants with deletion in this study had the abilities to interact with Pol II (Fig. 2), to translocate CSA (Fig. 3) and to associate with chromatin (Fig. 4) after UV irradiation. The results are summarized in Table 1 and these dysfunctions are outlined in Fig. 13. Once DNA is damaged, Pol II is arrested at the damage site (85) and TCR is initiated. Several TCR factors including CSA,

CSB and UVSSA are gathered at that site (8, 9). CSB is SUMOylated on chromatin after UV irradiation. DNA repair is linked to nuclear matrix and CSA is translocated there in a CSB dependent manner (57, 58). It is considered that the CSB mutants are not recruited to the damage site, and that causes the defect of CSA translocation. UVSSA is recruited onto chromatin in a CSA dependent manner (53). I could not detect co-immunoprecipitation of UVSSA with CSB after UV irradiation even in the $CSB_{WT}$ cells (data not shown), but it is suspected that UVSSA is not recruited to chromatin in the cells expressing CSB mutants. Following the arrest of Pol II, CSB works in TCR. Thus, TCR might be stopped at that stage in the CSB mutant cells, and UV sensitivity and the recovery of RNA synthesis might be incurred.

In this study, CSB is found to be modified by SUMO-2/3 and the deletion mutants except for $CSB_{\Delta UBD}$ was not SUMOylated (Fig. 7). There is a report that protein phosphorylation is regulated by SUMOylation (86). In CSB, Tyr-932 is phosphorylated by c-Abl kinase and c-Abl kinase and CSB are co-localized to nucleus and nucleolus after exposure to $H_2O_2$. STI571, one of the tyrosine kinase inhibitors, represses this co-localization, suggesting that the phosphorylation of CSB affects its subcellular localization (63). It was also mentioned that some CSB phosphorylations might be related to subcellular localization (87). Based on these information, one hypothesis about the relationship between the most C-terminal region and subcellular localization of CSB arises; non-SUMOylation of the deletion mutants leads the defect of UV-induced phosphorylation and that disrupts the association of CSB with

chromatin. Because $CSB_{\Delta UBD}$ has the most C-terminal region and SUMOylated, there should be another reason of its dysfunction different from the above hypothesis.

We tried to identify lysine residues of SUMO acceptor in CSB, but amino acid substitutions of lysine residues in the most C-terminal region of CSB to arginine did not affect its SUMOylation (Fig. 8), indicating that SUMO acceptor do not exist in the region. The region is possibly required for interaction with SUMOylation machinery, for example Ubc9. An amino acid substitution of Lys-205 to Arg represses SUMOylation to CSB. Lys-205 locates in N-terminal region. The structure of CSB is still unknown, but C-terminal and N-terminal regions might locate closely and influence each other.

## Function of the C-terminal region of CSB in TCR

In the C-terminal region of CSB, there are several factors that can affect the function of CSB, in particular, [1] amino acid residues for phosphorylation; Ser-1142, Ser-1348 and Ser-1461 (88-91), and for ubiquitination; Lys-1457 (55), [2] UBD (38), and [3] the most C-terminal region; 1464-1493 (this study). It was reported that Ser-1461 did not affect UV-induced association with chromatin (87). In this study, I generated a CSB mutant including the substitution of Lys-1457 ($CSB_{3K\rightarrow R}$, Fig. 8), but it also has two more substitutions, so that it is difficult to distinguish the effect of Lys-1457 substitution from that of the others. When I performed the functional analysis of CSB with its deletion mutants, it was unavoidable to consider the possibility that there are some unknown effects

of phosphorylation and ubiquitination. In this study, all the cells expressing deletion mutants lack TCR (Fig. 1B and C). In Fig. 1B, the colony formation ability after UV irradiation of $CSB_{1\text{-}1220}$ cell is lower than those of the other mutant CSB cells. When I focus on [1]-[3] described above, only $CSB_{1\text{-}1220}$ does not have Ser-1348 among the mutants. Phosphorylaton of the serine residue might cause the difference between $CSB_{1\text{-}1220}$ cells and the other mutant CSB cells in this assay. Through this study, there is little difference of CSB function between $CSB_{1\text{-}1463}$ and $CSB_{\Delta UBD}$ besides SUMOylation following UV irradiation. $CSB_{\Delta UBD}$ was modified by SUMO-2/3 but $CSB_{1\text{-}1463}$ was not (Fig. 7). Anyway, both CSB mutants do not have normal function of CSB. In the previous report (38), the transplantation of UBA domain from *Saccharomyces cerevisiae* Rad23, the homolog of HR23B in *Homo sapiens*, to $CSB_{1\text{-}1220}$ ($CSB^{del}$ in the report) restored CSB function including the ability of colony formation and the recovery of RNA synthesis after UV irradiation. This UBA domain-transplanted CSB do not have the most C-terminal region of CSB, so that it is considered to be equivalent to $CSB_{1\text{-}1463}$ in this study. In spite of that, $CSB_{1\text{-}1463}$ exhibited dysfunction like $CSB_{\Delta UBD}$. This discordance might come from that they used UBA of Rad23. It is possible that UBA domain of Rad23 also has some functions that can be substituted for the most C-terminal region of human CSB. In *S. cerevisiae* Rad26, the homolog of CSB, there is no motif that corresponds to UBA (38). In *S. cerevisiae*, SUMO E3 ligase plays little or no role in Rad26-dependent TCR (78), so that it is conceivable that Rad26 does not modified by SUMO. All essential functions of human CSB are not necessarily

conserved in *S. cerevisiae* Rad26. In mammals, CSB knock out mice slightly exhibit growth failure and do not have neurological dysfunctions, unlike CS patients (92). CSB function might be different slightly among species. At least in human cells, both UBD and the most C-terminal region, and some posttranslational modifications are necessary for functions of CSB in TCR.

It was reported that $CSB_{1-1220}$ is able to interact with Pol II after UV irradiation (38), and our data does not correspond to that. It is likely due to the difference of the experimental conditions. They performed the interaction assay using a cross-linker and caught the weak interaction. On the other hand, we did not use it. Taken together, $CSB_{1-1220}$ might interact with Pol II after UV irradiation, but the interaction is too weak to detect in the condition we used.

There are no SNPs that correspond to the mutations in this study; amino acid substitution of Lys-205 and nonsense mutation at 1220, 1400, 1445 or 1463, but C-terminal deletions of CSB are found in CS cells.

## Contribution of SUMOylation to CSB function

Ubc9 knock down revealed that there were almost no marked connections between SUMOylation and some functions of CSB examined in this study (Fig. 9). Only contribution to the association of CSB with chromatin remains obscure (Fig. 9E). It is probably caused by the fact that Ubc9 was not eliminated completely by the knock down experiments and SUMOylation of CSB occurred to some extent by residual Ubc9. I mentioned above about the relationship between SUMOylation and association of CSB with chromatin.

In UVSSA-deficient cells, Ubc9 knock down made CSB comparatively stable (Fig. 10). It was reported that SUMO-2 or SUMO-3 chains act as a signal recruiting E3 ubiquitin ligase and lead SUMOylated substrate to proteasomal degradation (93). Without UVSSA, I could detect SUMO-3 modification to CSB after UV irradiation (Fig. 6C). SUMOylation might works as a landmark for ubiquitination that links to proteasomal degradation in UVSSA-deficient cells. There is another idea that why Ubc9 knock down caused stabilization of CSB. BRCA1 is one of the candidates of ubiquitin ligase to CSB (62). BRCA1 is modified by SUMO, and ubiquitin ligase activity of BRCA1 is increased by the SUMOylation (94). Ubs9 knock down might decrease ubiquitin ligase activity of BRCA1 and that induces CSB stability. The purpose of CSB degradation is unclear, but it is likely that stability of CSB is controlled by a mechanism performed by ubiquitination and SUMOylation.

I discovered that CSB is modified by SUMO-2/3 in this study. In GGR pathway, it was reported that XPC is modified by SUMO-1 after UV irradiation (79). In XPA deficient cells, SUMOylation of XPC does not occur and XPC become unstable after UV irradiation. Little or no SUMOylation of XPC also leads to the reduction. Therefore, UV-induced SUMOylation of XPC is assumed to work to protect XPC from the degradation by proteasome (79). In Kps3 cell, modification of CSB by SUMO-2/3 works opposite to that of XPC by SUMO-1. The functional difference between SUMO-1 and SUMO-2/3 is still unclear. There could be a reason that CSB is modified by SUMO-2/3, but not by SUMO-1.

## Function of SUMOylated CSB in TCR

I have not find what roles SUMOylated CSB have in TCR yet. SUMOylation is reported to have several roles. Flap endonuclease 1 (FEN1) cleaves 5' flap and 5' end of Okazaki fragments in DNA repair and replication, respectively. Inhibition of FEN1 SUMOylation suppresses its degradation and affects cell cycle (95). Bloom syndrome protein (BLM) has DNA helicase activity. BLM is SUMOylated and has SUMO-interacting motif (SIM). Both SUMOylation of BLM and interaction with some SUMOylated proteins via SIM mediate its localization to PML nuclear bodies (96). SUMOylation also involved in protein-protein interaction. Psmd1 is a subunit of 19S regulatory lid of 26S proteasome SUMOylation of Psmd1 regulates the association with Adrm1, which is another proteasome subunit, suggesting that proteasome composition and function are changed by SUMOylation (97).

I have not find what roles SUMOylated CSB have in TCR yet. $CSB_{1\text{-}1220}$ cell did not exhibit apparent defects in cell cycle (data not shown). In Ubs9 knock down cells, CSB interacts with Pol II and has ability to translate CSA to nuclear matrix (Fig. 12B and C). These results suggest that SUMOylation of CSB plays a role after damage recognition step. It has been reported that CSB interact with xeroderma pigmentosum group G protein (XPG) (8). Because XPG has SIM, SUMOylated CSB might interact with SIM of XPG (Fig. 13).

## Contribution of SUMOylation to TCR in human cells

In Fig. 11, Ubc9 knock down brought cells to the reduction of the recovery of

RNA synthesis after UV irradiation. Ubc9 knock down affects all SUMOylation in the cells, so that I cannot exclude the possibility that some other proteins affected this reduction. But even under the Ubc9 knocked down situation, RNA synthesis without UV irradiation was the similar level to that in the control cells (Fig. 11A), and only the recovery of RNA synthesis after UV irradiation was reduced (Fig. 11B). These results suggest that inhibition of SUMOylation of TCR factors or the proteins involved in resume of RNA synthesis results in the reduction. CSA and UVSSA are excluded from the candidates of SUMO target TCR factors, because I could not detect SUMOylation form of them (Fig. 11C). Three SUMO-modified proteins were reported to be involved in RNA synthesis in mammals (98). HsTAF5 and hsTAF12 are the subunits of human TFIID and modified by SUMO-1 both *in vitro* and *in vivo*, and only the SUMOylation to hsTAF5 prevents TFIID from binding to promoter DNA *in vitro* (99). Transcription Elongation Regulator 1 (TCERG1) is involved in transcriptional elongation and pre-mRNA splicing. Non-SUMOylation mutant of TCRG1 enhanced its activity for transcription (100). Among the three proteins, only hsTAF5 is considered as the candidate that affects the recovery of RNA synthesis under Ubc9 knocked down condition, but *in vivo*, there is no report about the function of SUMOylated hsTAF5. Thus, it is conceivable enough that SUMOylation to TCR factors including CSB is critical to the recovery of RNA synthesis after UV irradiation.

## Implications of SUMO for diseases

There are some diseases that SUMO is deeply engaged in. Patients of Alzheimer's disease (AD) exhibit dementia and have degenerative lesion in their cerebral cortex. In a mouse model of AD, alterations in equilibrium between SUMOylation and de-SUMOylation occur from early phase of AD (101). SUMO contribution has been also reported in Parkinson's disease (PD). Patients of PD exhibit the shaking in a limb, the stiffness of the muscle and so on, and some abnormalities are occurred in their substantia nigra in midbrain and striata in basal ganglia. In PD, SUMO is considered to be a key factor to analyze some disease-associated proteins and subcellular processes (102). SUMO presumably plays an important role in CS.

## Materials and Methods

### Expression constructs and stable cell lines

To generate epitope-tagged CSB expression constructs, wild type and each mutant CSB cDNA fragments were amplified by PCR using each primer set (Table 1) and cut by *Xho* I (5' end) and *Xba* I (3' end). The fragments were cloned in-frame and downstream of the sequence encoding FLAG epitope followed by HA epitope in pcDNA3.1 vector (provided by Dr Masafumi Saijo, Osaka University). $CSB_{2K\rightarrow R}$, $CSB_{3K\rightarrow R}$ and $CSB_{K205R}$ cDNAs were generated using QuikChange II-E Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. Primer sets for the mutants are shown in Table 1. DNA sequencing of the plasmids ruled out the presence of PCR-derived mistakes. An expression construct for C-terminal FLAG and HA tagged wild type CSB was also generated. CSB-FLAG-HA cDNA was cut out by *Xho* I for 5' end and *Not* I for 3' end from pCAGGS-CSB-FLAG-HA construct (provided by Dr Masafumi Saijo, Osaka university) and inserted into pcDNA3.1 vector. Each expression construct was transformed into HIT-DH5α *E. Coli* (SciTrove). After culture in LB medium with 500 μg/ml carbenicillin, plasmid DNAs were prepared using QIAprep Miniprep kit (QIAGEN).

To isolate stable transfectants, CS1ANSV cells were transfected with the CSB expression constructs using Effectene Transfection Regent (QIAGEN) according to the manufacturer's procedure. Stable transfectants were selected in the presence of 500 μg/ml G418 (Nakarai Tesque).

### Cell culture

The cell lines used in this study were SV40 immortalized human fibroblasts; WI38VA13 (normal), CS1ANSV (CS-B), Kps3 ($UV^{S}S$-A), CS3BESV (CS-A) and CS1ANSV cells stably expressing wild type or each mutant CSB. Kps3 cells stably expressing FLAG-HA-UVSSA and CS3BESV cells stably expressing CSA-FLAG-HA were generated previously in preceding studies (26, 27). CS1ANSV cells stably expressing FLAG-HA-$CSB_{K538R}$ were provided by Dr Masafumi Saijo, Osaka University. All cell lines were cultured in DMEM containing 10% fetal bovine serum and 100 U/ml penicillin-100 μg/ml streptomycin at 37℃ in 5% $CO_2$ incubator.

## UV irradiation

Cells were washed once with PBS and 20 J/$m^2$ of UV-C was irradiated to the cell. The culture medium was added immediately after UV irradiation.

## Whole cell extract

Cells ($1.0\times10^6$) were washed once with PBS and lysed with 100 μl of 1× SDS-PAGE sample buffer (62.5 mM Tris-HCl pH6.8, 2% SDS, 10% glycerol, Bromophenol blue and 10 mM mercaptoethanol) by boiling for 5 minutes.

## UV survival

$1.0\times10^3$ cells were inoculated into 10-cm dishes and incubated overnight. Cells were washed once with PBS and irradiated with UV-C at various dosages. The cells were cultured for 10 days, fixed with formalin solution (10% formalin

in PBS) and stained with crystal violet solution (2.5 mM crystal violet and 5% ethanol). Colonies were counted using a binocular microscope.

### Recovery of RNA synthesis after UV irradiation

$1.0\times10^5$ cells were inoculated into 35-mm dishes and incubated overnight. Cells were washed once with PBS, either irradiated with UV-C at 10 J/m$^2$ or not irradiated, and incubated in 1 ml of the culture medium. To measure RNA synthesis, [$^3$H]uridine (Perkin Elmer) was added to each dish to 370 kBq/ml at each time points. After incubation for 1h, the labeling was terminated by adding $NaN_3$ at a final concentration of 200 μg/ml. Cells were solubilized with 0.8% SDS. Then, an equal volume of 10% trichloroacetic acid containing 0.1M sodium diphosphate was added to the lysates and incubated on ice. Acid-insoluble materials were collected on Glass Microfilters GF/C (Whatman). The radioactivity was measured in Insta-Fluor Plus cocktail (Perkin Elmer) with a liquid scintillation counter Tri-Carb 2810 TR (Perkin Elmer). The ratio of the radioactivity of UV-irradiated cells to that of non-irradiated cells was considered as recovery of RNA synthesis after UV irradiation.

### Interaction of CSB with RNA polymerase II (Pol II)

After 20 J/m$^2$ of UV irradiation, cells were incubated for 30 minutes and harvested. $1.0\times10^6$ cells were resuspended in Micrococcal nuclease (MNase) buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 300 mM sucrose, 0.1% Triton X-100, 1 mM DTT and protease inhibitors) and

incubated on ice for 10 minutes. The lysates were fractionated by centrifugation (5000 rpm, 5 minutes, 4℃) and the pellet was resuspended in MNase buffer including 15 U/ml of MNase and incubated at 25℃ for 15 minutes. The reaction was terminated by adding EDTA to 5 mM and centrifuged at 5000 rpm for 5 minutes at 4℃. The supernatant was recovered. The pellet was washed with MNase buffer and the supernatant was combined with the above supernatant and used as the solubilized chromatin fraction. The solubilized chromatin fraction was incubated with 20 μl of anti-FLAG M2 agarose beads (SIGMA) overnight at 4℃. The beads were washed 5 times with NETN buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 1% NP-40, 1 mM EDTA and 0.5 mM PMSF) and then boiled in an equal volume of 2×SDS-PAGE sample buffer (125 mM Tris-HCl pH6.8, 4% SDS, 20% glycerol, Bromophenol blue and 10 mM mercaptoethanol). SDS-PAGE was performed and separated proteins were transferred to a PVDF membrane. Immunoblot detection was performed with anti-Pol II and anti-CSB antibodies using Pierce Western Blotting Substrate Plus (Thermo Scientific).

### Subcellular fractionation

After 20 J/m$^2$ of UV irradiation, cells were harvested at each time point. $1.0\times10^6$ (Fig. 4 and 9) or $4.0\times10^6$ (Fig. 6) cells were resuspended in CSK-Triton buffer (10 mM PIPES pH6.8, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 0.5% Triton-X 100, 1 mM EGTA, 1 mM DTT and protease inhibitors) with gently vortexing and incubated on ice for 10 minutes. The lysates were

fractionated by centrifugation (6000 rpm, 3 minutes, 4℃). The supernatants were mixed with an equal volume of 2×SDS-PAGE sample buffer, boiled for 5 minutes, and used as soluble fraction. The pellets were washed once with CSK-Triton buffer, boiled in 1×SDS-PAGE sample buffer, and used as insoluble fraction. SDS-PAGE was performed and separated proteins were transferred to a PVDF membrane. Immunoblot detection was performed with anti-CSB, anti-lamin B and anti-XPA antibodies using Pierce Western Blotting Substrate Plus.

### Translocation of CSA to nuclear matrix after UV irradiation

UV-induced translocation of CSA in cell-free system was examined as described previously (58). CS1ANSV cells expressing CSB were irradiated with 20 J/m$^2$ of UV and incubated for 1 h, and then treated with CSK-Triton buffer to prepare the insoluble fractions (CSK-ppt fraction). The soluble fractions (CSK-sup fraction) were prepared from CS3BESV cells stably expressing CSA tagged with FLAG and HA by the treatment with CSK-Triton buffer. The CSK-sup fraction containing FLAG-HA epitope tagged CSA was incubated with the CSK-ppt fraction on ice for 1 hour. After centrifugation (6000rpm, 3 minutes, 4℃), the pellet was washed twice with CSK-Triton buffer and incubated with 5 U of recombinant DNase I (Takara) in CSK-Triton buffer at 30℃ for 10 minutes, and centrifuged (6000rpm, 3 minutes, 4℃). After washing 3 times with CSK-Triton buffer, 1×SDS-PAGE sample buffer was added to the pellet and boiled for 5 minutes. The CSA retained in the DNase I-insoluble fractions was

detected by immunoblotting with anti-HA antibody.

## Detection of SUMOylated proteins

SUMOylated proteins were detected as described previously (79) with some modifications. $2.0\times10^6$ cells were solubilized by addition of SDS lysis buffer (62.5 mM Tris-HCl, pH6.8, 10% glycerol, 2% SDS and protease inhibitors) and boiling for 10 minutes. The lysates were passed through a 25G needle (TERUMO) to shear DNA and diluted 10-folds with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl and 1 mM EDTA). 20 mM N-ethylmaleimide, 20 μM PR-619, 1 mM sodium fluoride, 1mM sodium orthovanadate (V), and 2 mM β-glycerophosphoric acid disodium salt ware added into RIPA immediately before use. 20 μl of Protein G Sepharose 4 Fast Flow agarose beads (GE Healthcare) and 2 μg anti-CSB antibody (Fig. 4, 5, 6, 7, and 8) or 20 μl of anti-FLAG M2 agarose beads (SIGMA) (Fig. 10) were incubated overnight with the diluted lysates at 4℃. The beads were washed 5 times with RIPA buffer and then boiled in an equal volume of 2×SDS-PAGE sample buffer. SDS-PAGE was performed and separated proteins were transferred to a PVDF membrane. Immunoblot detection was performed with anti-ubiquitin, anti-SUMO-1,-2,-3, and anti-CSB antibodies using Pierce Western Blotting Substrate Plus.

## RNA interference

Ubc9 silencing was performed by transfection of siRNA (sc-36773 from Santa

cruz) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's procedure. Reverse transfection was done first, and forward transfection was carried out subsequently 24 h after the reverse transfection. Cells were irradiated with UV 24-48 h after forward transfection, incubated for the indicated time, and then harvested.

## Antibodies

Polyclonal anti-CSB (E-18), anti-SUMO-1 (FL-101), anti-ubquitin (FL-76), anti-lamin B (C-20), anti-CSA (W-16), anti-XPA (FL-273), anti-Ubc9 (H-81) and monoclonal anti-SUMO-3 (sc-130884), anti-Pol II (sc-17798) antibodies were from Santa Cruz. Polyclonal anti-SUMO2 antibody was from Gene Tex. Monoclonal anti-ubiquitin (FK2) antibody was from MBL. Monoclonal anti-DDDDK(FLAG)-tag (FLA-1) antibody was from MBL. Monoclonal anti-HA-tag (3F10) antibody was from Roche.

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## Figure Legends

### Fig. 1. C-terminal 30 amino acid residues of CSB are essential for TCR.

(A) Schematic representation of CSB mutants. ATPase domain and UBD is indicated by dark gray box and black box, respectively. Each CSB mutant was tagged with FLAG and HA epitopes at the N-terminal and expressed in CS1ANSV cells. The regions indicated by black horizontal bars are designated as follows; 1221-1493 is the C-terminal region, 1221-1400 is the region just before UBD, 1401-1428 is UBD, 1429-1463 is the region just after UBD, and 1464-1493 is the most C-terminal region.

(B) Colony-forming ability of cells expressing each CSB mutant after UV irradiation. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

(C) RNA synthesis of cells expressing each CSB mutant after UV irradiation. Cells were irradiated with 10 J/m$^2$ of UV and incorporation of [$^3$H]-uridine was measured at 24 h after UV irradiation. The relative incorporation of [$^3$H]-uridine in the UV-irradiated cells was compared with that of non-irradiated cells. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

(D) Alignment of amino acid sequences of CSB homologs in vertebrates. Sequences after UBD are shown. Sequence information was obtained from NCBI site. Accession numbers are NP_000115.1 (Homo sapiens), AFH31457.1 (Macaca mulatta), AAI32448.1 (Mus musculus), NP_001100766.1 (Rattus

norvegicus), XP_005637540.1 (Canis lupus familiaris), XP_008268053 (Oryctolagus cuniculus), XP_009085572 (Serinus canaria), XP_008115961.1 (Anolis carolinensis), and XP_688972 (Danio rerio). The asterisk (*) means the conserved amino acid among species.

**Fig. 2. Interaction of CSB mutant with Pol II in the solubilized chromatin fractionation.**

(A) Procedures of the interaction of CSB with Pol II.

(B) Co-immunoprecipitation (IP) of Pol II with epitope-tagged CSB. Solubilized chromatin fractions were prepared from UV-irradiated and non-irradiated cells and CSB was immunoprecipitated with anti-FLAG-M2 agarose. The IP samples were analyzed by western blotting using anti-Pol II and anti-CSB antibodies. 25% and 75% of IP sample were used to detect CSB and Pol II, respectively.

**Fig. 3. Effect of CSB mutation on translocation of CSA to nuclear matrix.**

(A) Procedures of the cell-free system used for CSA translocation to nuclear matrix.

(B) CSA translocation assay using CSK-ppt fractions prepared from cells expressing mutant CSB. The CSK pellet fractions were prepared from UV-irradiated and non-irradiated cells and incubated with the CSK supernatants containing FLAG-HA epitope tagged CSA, and treated with DNase I. The CSA retained in the DNase I-insoluble fractions was detected by

western blotting with anti-HA antibody. Lamin B is a loading control. HA (CSA) and non-specific bands are indicated by arrow with asterisk (*) and double asterisk (**), respectively.

**Fig. 4. Association of CSB with chromatin induced by UV irradiation.**

(A) Procedures of subcellular fractionation with CSK-Triton buffer.

(B) Association of CSB with chromatin. The insoluble fractions were prepared from the cells at indicated time after UV irradiation and analyzed by western blotting with anti-CSB antibody. The samples from cells with the same cell numbers were applied to each lane of the gel. Lamin B is a loading control. XPA is known to be bound to chromatin after UV irradiation. Asterisk represents shifted bands of CSB.

(C) Quantification of (B). Intensity of the CSB bands was quantified and the intensity in the UV-irradiated cells was compared with that of non-irradiated cells. Points are averages of two independent experiments.

**Fig. 5. Detection of UV-induced modification of CSB.**

(A) Procedure of the detection of modification of CSB.

(B) Detection of ubiquitination and SUMOylation of CSB. CSB immunoprecipitated from the cell lysates was analyzed by western blotting with two anti-ubiquitin antibodies and three anti-SUMO antibodies. After detection of the modifications, the membrane was re-blotted with anti-CSB antibody. IP

samples from cells with the same cell numbers were applied into each lane of the gel.

**Fig. 6. Analysis of UV-induced SUMOylation of CSB.**

(A) Effects of Ubc9 knock down by siRNA. Upper panels: WI38 cells were transfected with siRNA to Ubc9 or control siRNA. To assess Ubc9 knock down, whole cell lysates were analyzed by western blotting with anti-Ubc9 antibody. Lamin B is a loading control. Lower panel: IP samples were prepared from the knock down cell lysates as described in Fig. 5A and were analyzed by western blotting with anti-SUMO-3 antibody.

(B) Detection of SUMOylation of CSB in the CSK-fractionated samples. Soluble and insoluble fractions were prepared as described in Fig. 4A. CSB was immunoprecipitated from the fractions and analyzed by western blotting with anti-SUMO-3 antibody. After detection of SUMO-3, the membrane was re-blotted with anti-CSB antibody.

(C) SUMO-3 modification of CSB after UV irradiation in Kps3 and CS3BE cells. Kps3 and CS3BE cells are deficient in UVSSA and CSA, respectively. The cell lysates were prepared from both the cells and each corrected cells. CSB was immuprecipitated from the lysates and analyzed by western blotting with anti-SUMO-3 antibody. After detection of SUMO-3, the membrane was re-blotted with anti-CSB antibody.

(D) SUMO-3 modification of ATPase-deficient CSB after UV irradiation. $CSB_{K538R}$ has single amino acid substitution in ATPase domain and lacks

ATPase activity. The cell lysates were prepared from cells expressing C-terminally FLAG-HA epitope tagged $CSA_{WT}$ and $CSA_{K538R}$. CSB was immunoprecipitated from the lysates and analyzed by western blotting with anti-SUMO-3 antibody. After detection of SUMO-3, the membrane was re-blotted with anti-CSB antibody. All IP samples were prepared from cells with the same cell numbers.

**Fig. 7. Analysis of SUMOylation of CSB mutant.**

SUMO-3 modification of CSB deletion mutants after UV irradiation. The cell lysates were prepared from the cells expressing CSB mutants. CSB was immunoprecipitated from the lysates and analyzed by western blotting with anti-SUMO-3 antibody. After detection of SUMO-3, the membrane was re-blotted with anti-CSB antibody. IP samples from cells with the same cell numbers were applied into each lane of the gel.

**Fig. 8. Effects of amino acid substitutions in the C-terminal region of CSB.**

(A) Schematic representation of the position of lysine in the C-terminal 43 amino acid residues of CSB. Lysine residues (red) were substituted to arginine. Two and three amino acid substituted CSB ($K_{1487,\ 1489}R$ and $K_{1457,\ 1487,\ 1489}R$, respectively) were generated. Each mutant CSB was FLAG-HA epitope tagged at the N-terminal and expressed in CS1ANSV cells.

(B) SUMO-3 modification of the lysine-substituted CSB mutants after UV

irradiation. The cell lysates were prepared from the cells expressing CSB mutants. CSB was immunoprecipitated from the lysates and analyzed by western blotting with anti-SUMO-3 antibody. After detection of SUMO-3, the membrane was re-blotted with anti-CSB antibody. IP samples from cells with the same cell numbers were applied into each lane of the gel.

(C) Colony-forming ability of cells expressing the lysine-substituted CSB mutants after UV irradiation. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

(D) RNA synthesis of cells expressing the lysine-substituted CSB mutants after UV irradiation. Cells were irradiated with 10 J/m$^2$ of UV and incorporation of [$^3$H]-uridine was measured at 24 h after UV irradiation. The incorporation of [$^3$H]-uridine in the UV-irradiated cells was compared with that of non-irradiated cells. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

**Fig. 9. Effects of Ubc9 knock down on CSB functions.**

(A) Ubc9 knock down by siRNA. Cells expressing N-terminally FLAG-HA tagged CSB were transfected with siRNA to Ubc9 or control siRNA. To assess Ubc9 knock down, whole cell extracts were prepared from the Ubc9 knock down and control cells and analyzed by western blotting with anti-Ubc9 antibody. Lamin B is a lording control.

(B) Interaction of CSB with Pol II. The solubilized chromatin fractions were prepared from the Ubc9 knock down and control cells as described in Fig. 2A.

N-terminally FLAG tagged CSB was immunoprecipitated from the fractions with anti-FLAG-M2 agarose. The IP samples were analyzed by western blotting with anti-Pol II and anti-CSB antibodies. 25% and 75% of the IP sample were used to detect CSB and Pol II, respectively. The bottom panel shows Ubc9 knock down by siRNA. IP samples were prepared from the knock down and control cell lysates as described in Fig. 5A and were analyzed by western blotting with anti-SUMO-3 antibody.

(C) Translocation of CSA to nuclear matrix. The CSK pellet fractions were prepared from the Ubc9 knock down and control cells, and the cell-free CSA translocation assay was performed as described in Fig. 3A. The CSA retained in the DNase I-insoluble fractions was detected by western blotting with anti-HA antibody. Epitope tagged CSB was also detected with anti-HA antibody. Lamin B is a loading control.

(D) Association of CSB with chromatin. WI38 cells were transfected with siRNA to Ubc9 or control siRNA. The insoluble fractions were prepared as described in Fig. 4A and analyzed by western blotting with anti-CSB antibody. Lamin B is a loading control. XPA is known to be bound to chromatin after UV irradiation.

(E) Quantification of (D). Intensity of the CSB bands was quantified, and the relative amount of CSB was calculated as the intensity in non-irradiated cells is 100%. Points are averages of two independent experiments.

**Fig. 10. Effects of Ubc9 knock down in UVSSA-deficient cells.**

(A) UV-induced degradation of CSB. UVSSA-deficient Kps3 cells were

transfected with siRNA to Ubc9 or control siRNA. Whole cell extracts were prepared from the cells at indicated time after UV irradiation and analyzed by western blotting. Lamin B is a loading control.

(B) Quantification of (A). Intensity of the CSB bands was quantified, and the relative amount of CSB was calculated as the intensity in non-irradiated cells is 100%. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

(C) RNA synthesis of the knocked down Kps3 cells after UV irradiation. The cells were irradiated with10 J/m$^2$ of UV and incorporation of [$^3$H]-uridine was measured at 2 h and 24 h after UV irradiation. The incorporation of [$^3$H]-uridine in the UV-irradiated cells was compared with that of non-irradiated cells. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

**Fig. 11. Evaluation of the contribution of SUMOylation to TCR.**

(A) Effects of Ubc9 knock down on RNA synthesis. WI38 cells were transfected with siRNA to Ubc9 or control siRNA and incorporation of [$^3$H]-uridine was measured. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

(B) Effects of Ubc9 knock down on the recovery of RNA synthesis after UV irradiation. The knock down cells were irradiated with 10 J/m$^2$ of UV and incorporation of [$^3$H]-uridine was measured at 2 h and 24 h after UV irradiation. The incorporation of [$^3$H]-uridine in the UV-irradiated cells was compared with

that of non-irradiated cells. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

(C) Analysis of SUMOylation of CSA and UVSSA. CS3BE cells stably expressing C-terminally FLAG-HA epitope tagged CSA and Kps3 cells stably expressing N-terminally FLAG-HA epitope tagged UVSSA were UV- or non-irradiated. The cell lysates were prepared from the cells and immunoprecipitation was performed with the lysates using anti-FLAG agarose as described in Fig. 5A. SUMOylation of CSA and UVSSA was analyzed by western blotting with anti-SUMO-1, anti-SUMO-2, and anti-SUMO-3 antibodies. After detection of SUMOylation, the membrane was re-blotted with anti-FLAG antibody. All IP samples were prepared from cells with the same cell numbers. Asterisk (*) and double asterisk (**) indicate UVSSA and CSA bands, respectively.

## Fig. 12. Searching for SUMOylation site of CSB

(A) SUMOylation sites predicted by SUMOplot™ Analysis Program (Abgent). SUMOylation candidate sites (red) were shown in amino acid sequence of CSB. The sites with high score were indicated by underline in the sequence and listed on the right. (B) Map of posttranslational modifications in human cell. Ubiquitylation (Lys-1295 and Lys-1457), methylation (Lys-170, Lys-297, Lys-448 and Lys-1054), and phosphorylation (other Lys residues that are described in red character) sites provided by PhosphoSitePlus (Cell Signaling) were shown. Many posttranslational modifications were located in two regions

(red rectangles). (C) Schematic representation of $CSB_{K205R}$ mutant. Lys-205 residues (red) were substituted to Arg.

(D) SUMO-3 modification of $CSB_{K205R}$ after UV irradiation. The cell lysates were prepared from $CSB_{K205R}$ cells. CSB was immunoprecipitated from the lysates and analyzed by western blotting with anti-SUMO-3 antibody. After detection of SUMO-3, the membrane was re-blotted with anti-CSB antibody. IP samples from cells with the same cell numbers were applied into each lane of the gel.

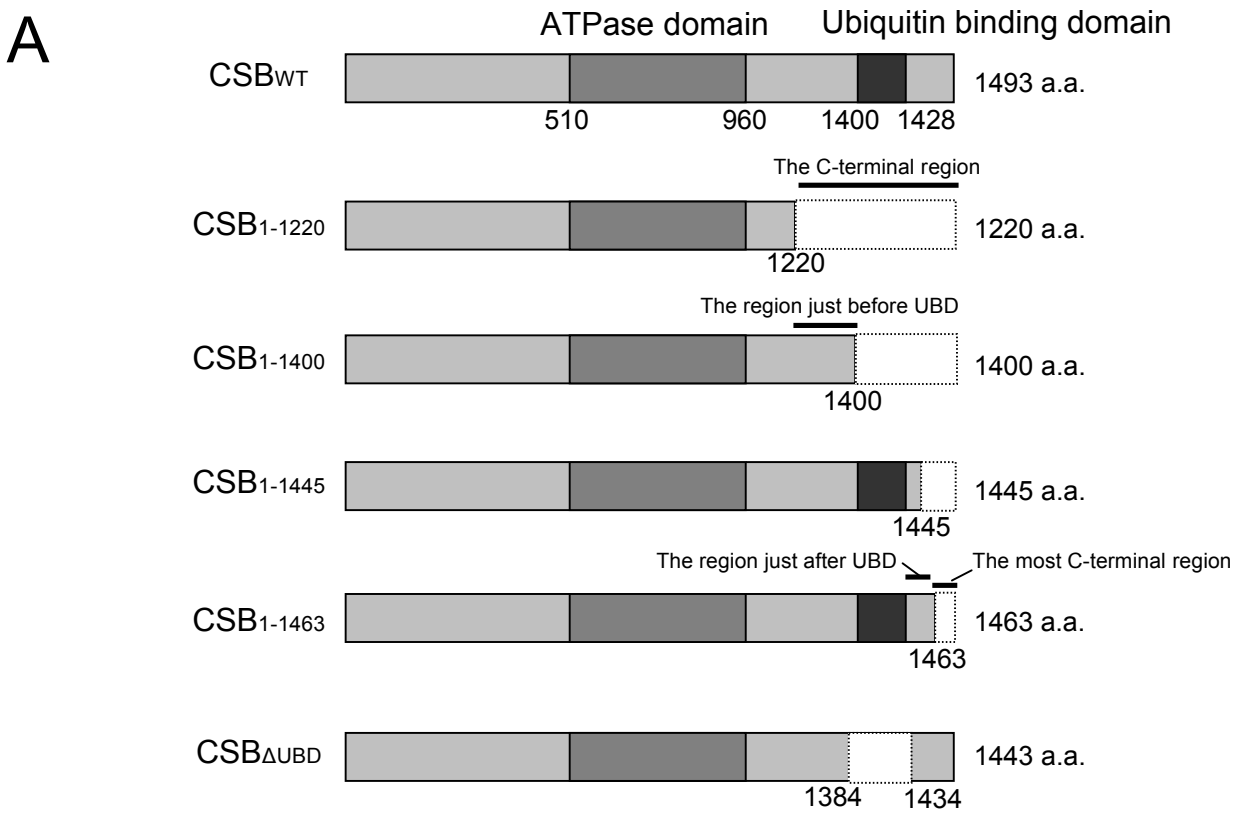
(E) Colony-forming ability of $CSB_{K205R}$ cells after UV irradiation. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

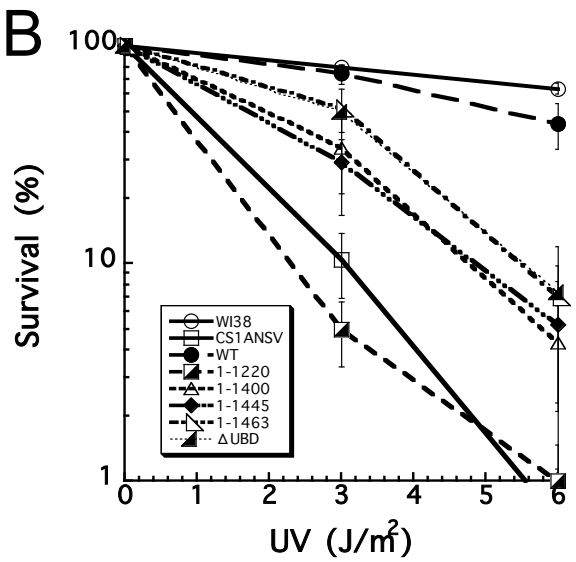
(F) RNA synthesis of $CSB_{K205R}$ cells after UV irradiation. Cells were irradiated with 10 J/m$^2$ of UV and incorporation of [$^3$H]-uridine was measured at 2 h and 24 h after UV irradiation. The incorporation of [$^3$H]-uridine in the UV-irradiated cells was compared with that of non-irradiated cells. The points are average for at least three independent experiments, and vertical bars indicate standard errors.

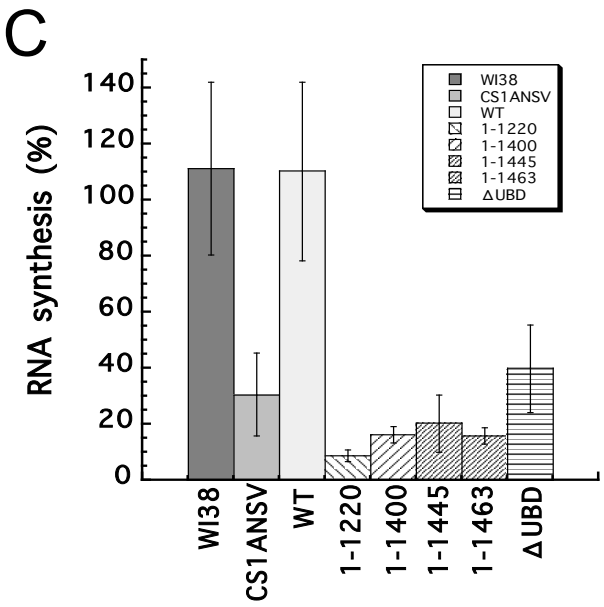
**Fig. 13. Schematic diagram of functions of the C-terminal region of CSB in TCR.**

Normally, CSB is associated with chromatin after UV irradiation and interacts with arrested Pol II at the damage site. CSB is modified by SUMO-2/3 at chromatin in a UV dependent manner. On the other hand, C-terminal deleted CSB is little associated with chromatin and cannot interact with Pol II. As a

result, those CSB mutant cells exhibit deficiency in TCR and hypersensitivity to UV. CSB mutants with C-terminal deletion is not modified by SUMO-2/3.




D

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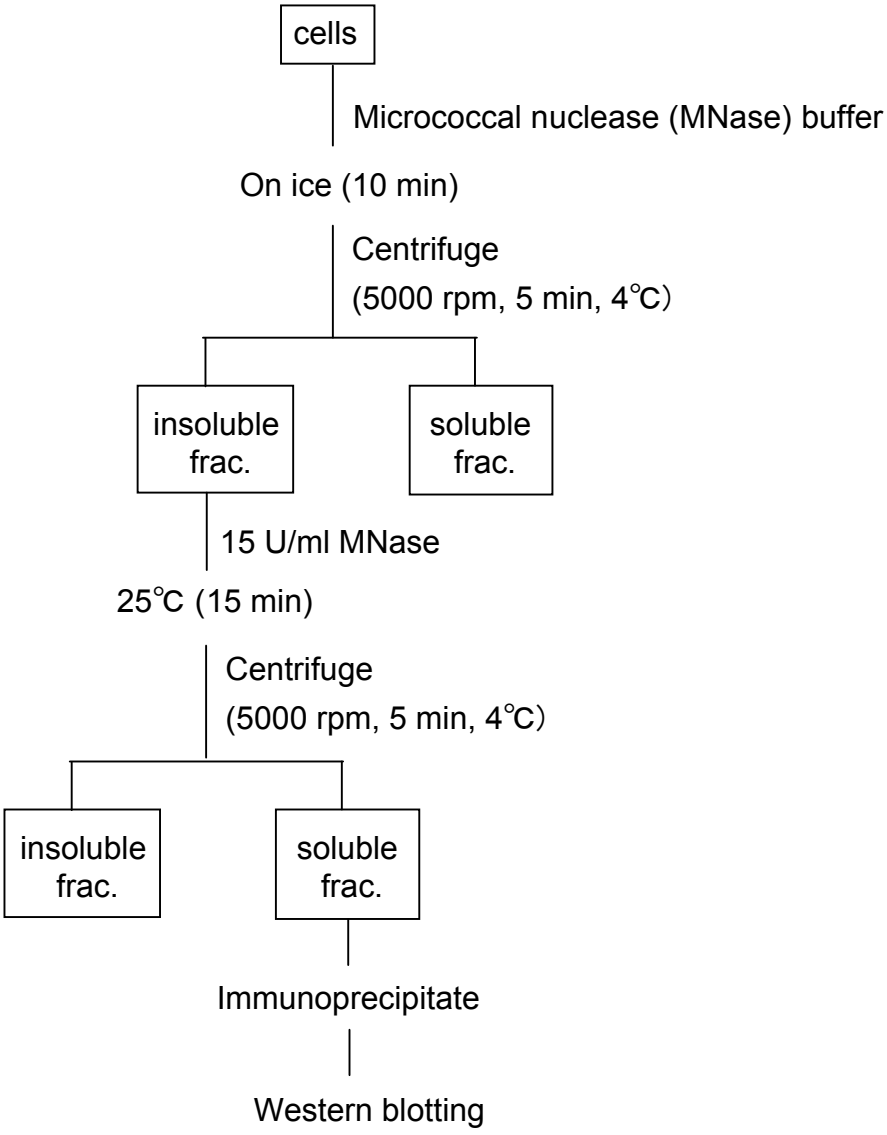
Homo sapiens          L I LPERL ESESGHQ- - - EASALL PT TEHDDLLVEMRNFI AFQAHTDGQASTREI LQEFESKLSASQSCVFRELLRNLCTFHRTSGGEGI WKLKPEYC
Macaca mulatta        L I LPERL ESESGHQ- - - EASALL PT TEHDDLLVEMRNFI AFQAHTDGQASTREI LQEFESKLSASQSCVFRELLRNLCTFHRTSGGEGI WKLKPEYC
Mus musculus          MI LPERL ESDSEHA- -E AAAVPPCGTEHDDLLVDMRNFI AFQAQVDGQASTQEI LQEFESKLSVAQSCVFRELLRNLCNFHRTPGGEGI WKLKPEYC
Rattus norvegicus     MI LPERL ESDGEQAGAA AAAAPPCSTEHDALLVDMRNFI AFQAQVDGQASTQEI LQAFESKLSVAQSCVFRELLRNLCNFHRTPGGEGI WKLKPEYC
Canis lupus familliaris L I LPQRL ESENAHE- - - APAPPPPT TEHDDLLVEMRNFI AFQARVDGQASTQEI LQEFESKLSASQSCVFRQLLRNLCTFHRTSSGEGI WKLKPEYC
Oryctolagus cuniculus L I VPERL ESEDGHLGAA GPAPAPSATEHDELLVEVRNFI AFQARVDGQASTQEI LREFESRLSASQSCVFRELLRNLCTFRRAAGGEGI WKLKPEYC
                      *    ****                    **** ***  ******** ****** *** *** **  ****** ****** * *    **** ********

Serinus canaria       L VLPQQTRDEGDEH-QP APAPVQGSTEYDELLVDL RNFLAFQARVDGEASTRELLQEFESKLPAEHSCVFRELLRNI CTFHRSPNGEGVWRLKPEFR
Anolis carolinensis   L PHRTDGQTDRRVQ- - - AVAALPVAREHEELLMDI QNFVAFRVRVPGQASTQEI LQEYESKLSVEQSCI FRELLRNI CNFHRHSSGEGI WKLKPEFS
Danio rerio           L KKPQSQEDEEEET- -Q SVAVNAAPTEHDELLVDL RNFVAFQAQVDGQASTKEI LEYFTPRLTSTQMPVFRELLNNI CEFHRLPGKEGMWKLKADYR
                                          *    **    ** **      *  ** ***      *      ** ** * * * *    ** * **
```

The most C-terminal region

Figure 1

A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soluble frac.
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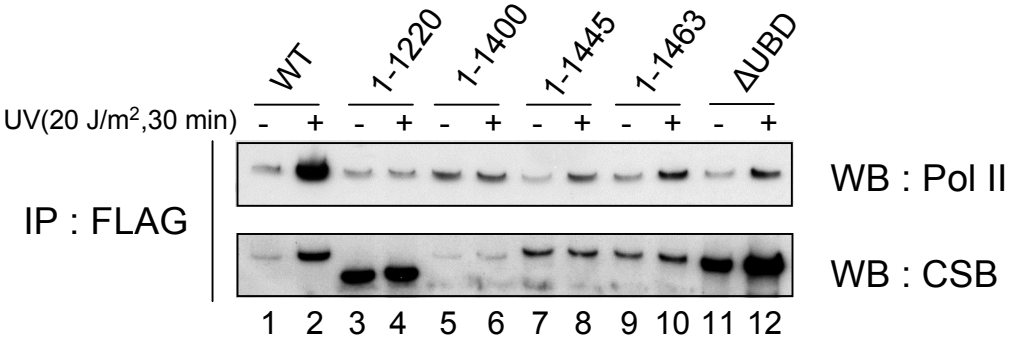
25℃ (15 min)
Centrifuge
(5000 rpm, 5 min, 4℃)
insoluble frac.
soluble frac.
Immunoprecipitate
Western blotting

B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1-1220
1-1400
1-1445
1-1463
ΔUBD
UV(20 J/m2,30 min) - + - + - + - + - + - +
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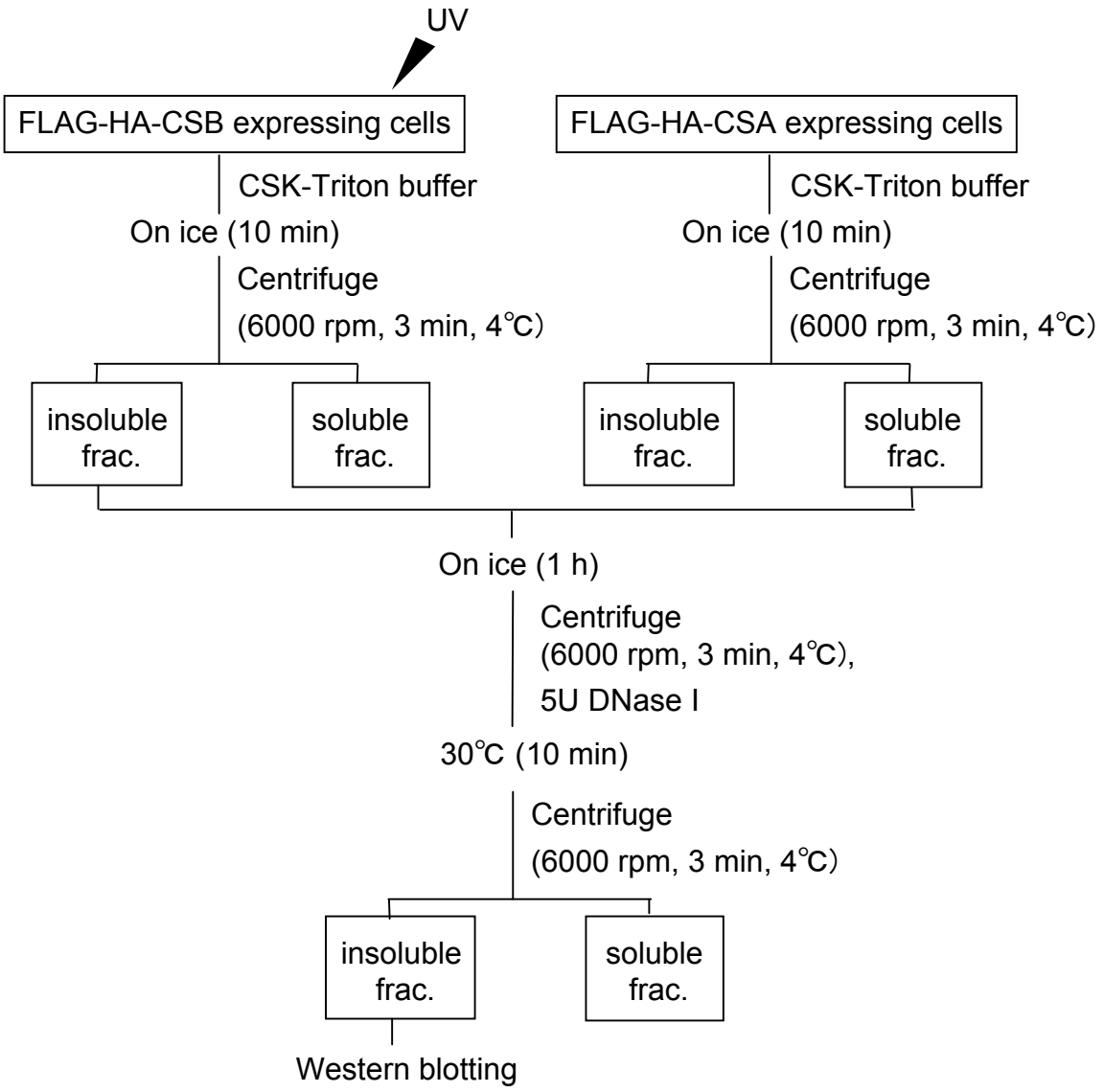
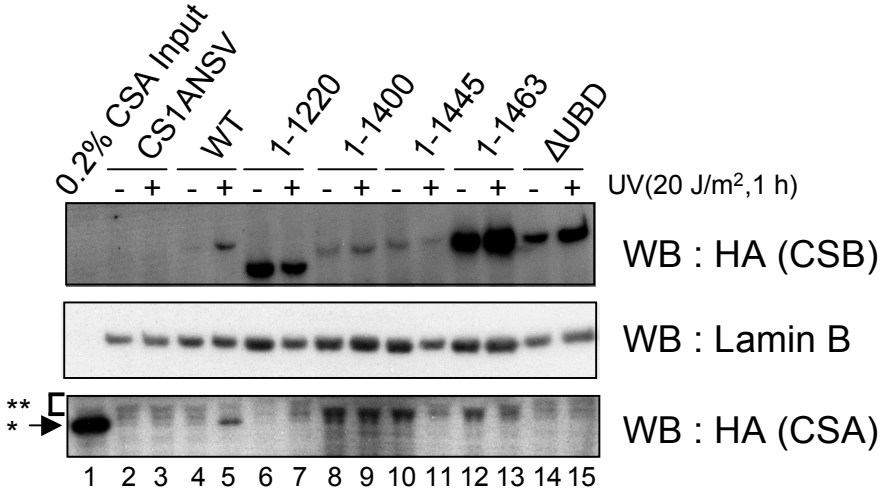
WB : Pol II
WB : CSB
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2

A


UV
FLAG-HA-CSB expressing cells
FLAG-HA-CSA expressing cells
CSK-Triton buffer
On ice (10 min)
Centrifuge
(6000 rpm, 3 min, 4℃)
insoluble frac.
soluble frac.
On ice (1 h)
Centrifuge
(6000 rpm, 3 min, 4℃),
5U DNase I
30℃ (10 min)
Western blotting

B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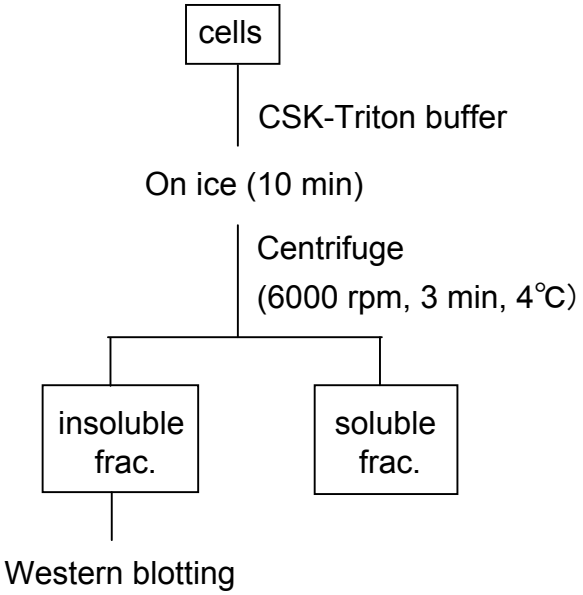
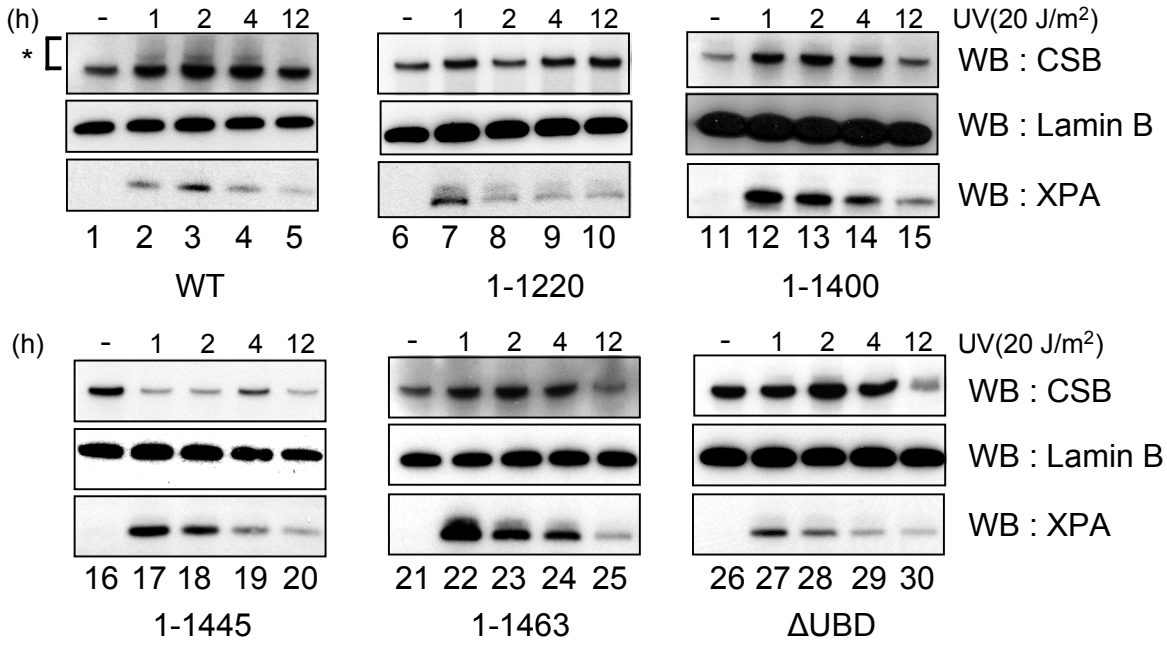
WT
1-1220
1-1400
1-1445
1-1463
ΔUBD
UV(20 J/m2,1 h)
WB : HA (CSB)
WB : Lamin B
WB : HA (CSA)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3

A


cells
CSK-Triton buffer
On ice (10 min)
Centrifuge
(6000 rpm, 3 min, 4℃)
insoluble frac.
soluble frac.
Western blotting

B


(h) - 1 2 4 12
UV(20 J/m2)
WB : CSB
WB : Lamin B
WB : XPA
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
WT
1-1220
1-1400
16 17 18 19 20
21 22 23 24 25
26 27 28 29 30
1-1445
1-1463
ΔUBD

C

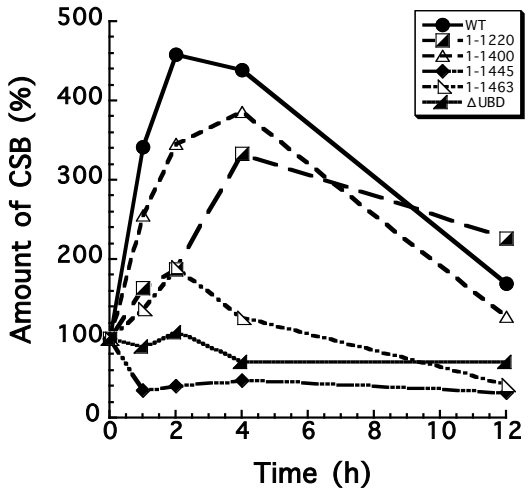

Amount of CSB (%)
Time (h)
WT
1-1220
1-1400
1-1445
1-1463
ΔUBD

Figure 4

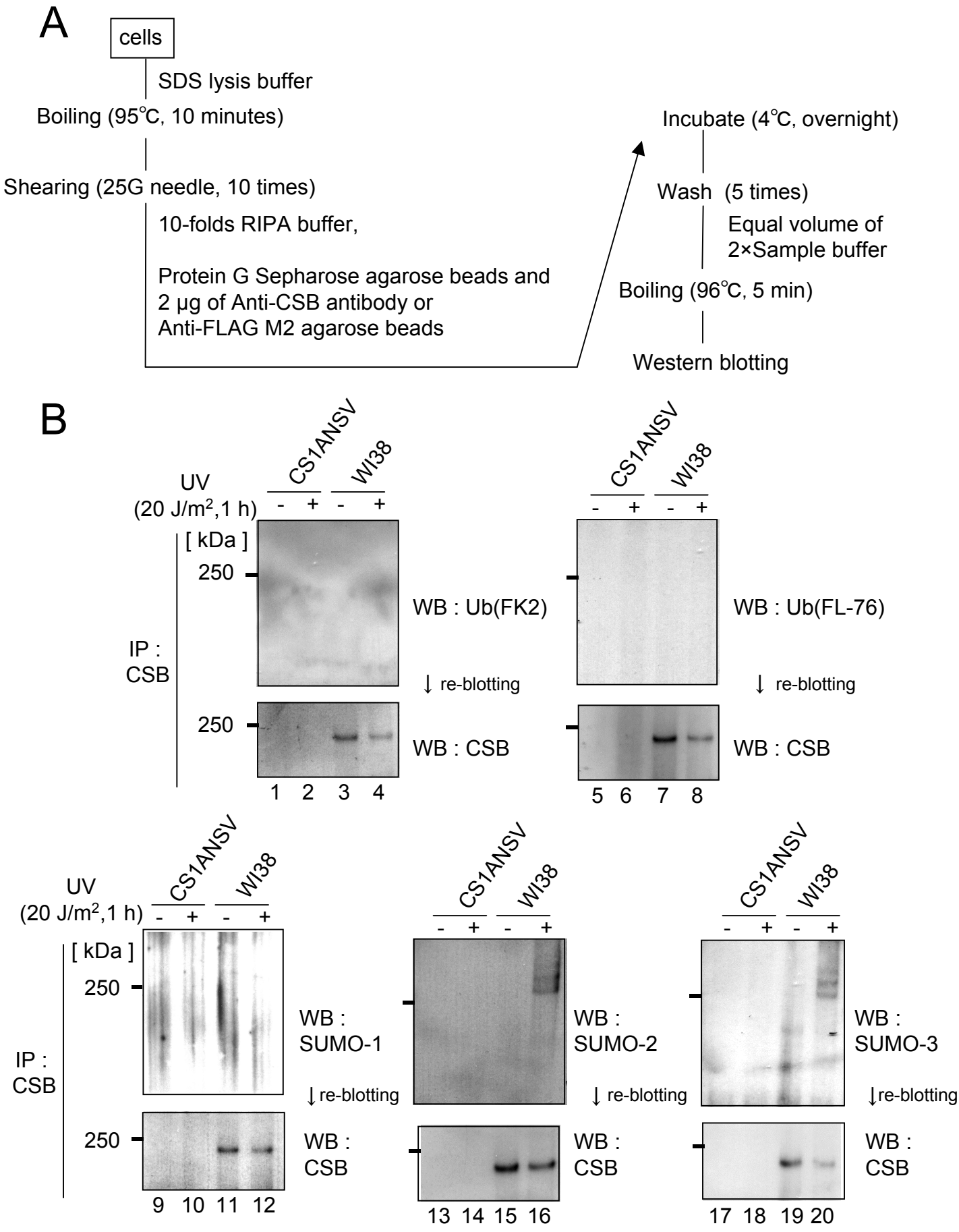
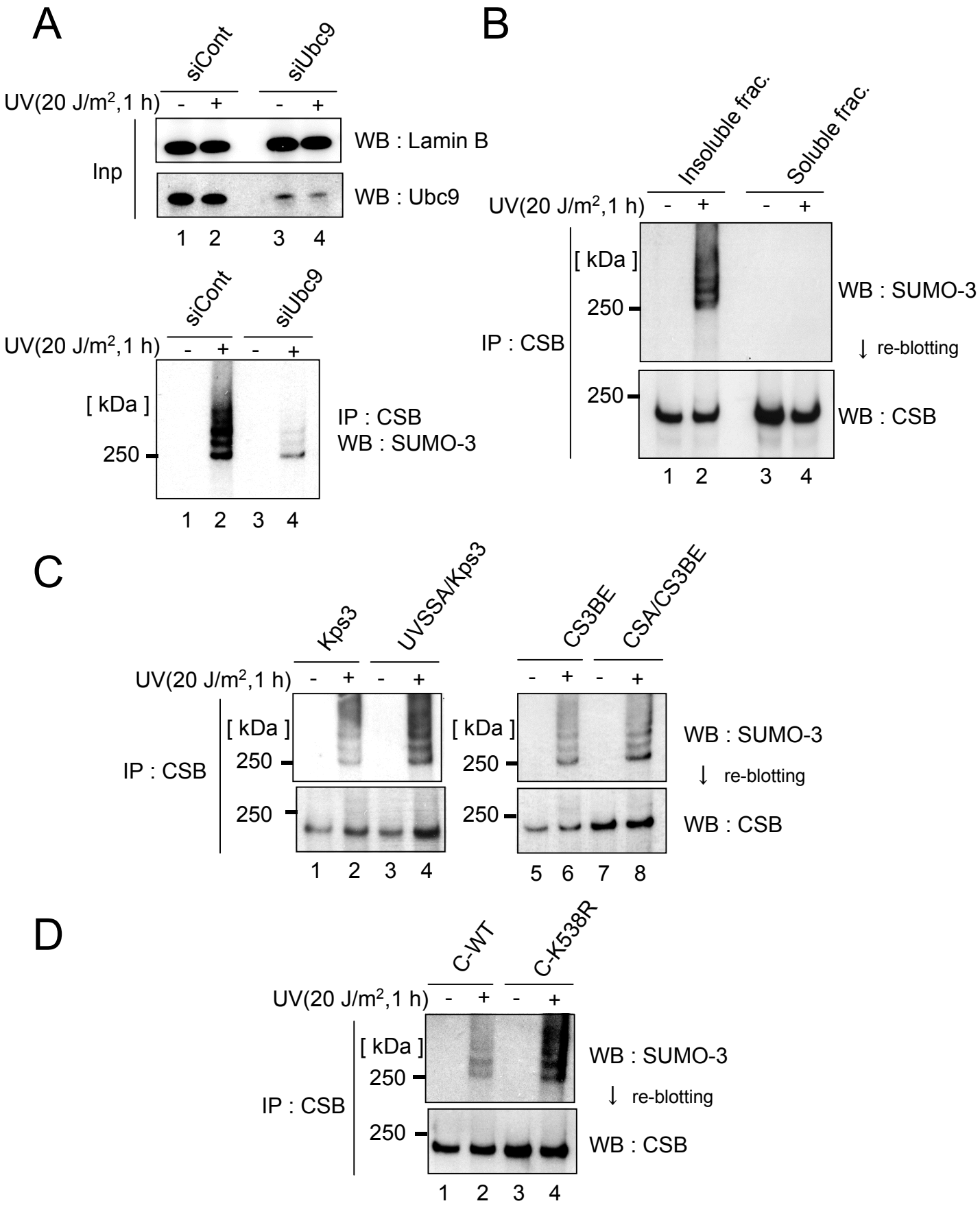

A
cells
SDS lysis buffer
Boiling (95℃, 10 minutes)
Shearing (25G needle, 10 times)
10-folds RIPA buffer,
Protein G Sepharose agarose beads and
2 μg of Anti-CSB antibody or
Anti-FLAG M2 agarose beads
Incubate (4℃, overnight)
Wash (5 times)
Equal volume of
2×Sample buffer
Boiling (96℃, 5 min)
Western blotting
B
UV
(20 J/m2,1 h)
CS1ANSV
WI38
- + - +
[ kDa ]
250
IP :
CSB
WB : Ub(FK2)
↓ re-blotting
WB : CSB
1 2 3 4
WB : Ub(FL-76)
5 6 7 8
WB :
SUMO-1
WB :
SUMO-2
WB :
SUMO-3
9 10 11 12
13 14 15 16
17 18 19 20

Figure 5


A
siCont
siUbc9
UV(20 J/m2,1 h)
- + - +
Inp
WB : Lamin B
WB : Ubc9
1 2 3 4
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siUbc9
UV(20 J/m2,1 h)
- + - +
[ kDa ]
250
IP : CSB
WB : SUMO-3
1 2 3 4
B
Insoluble frac.
Soluble frac.
UV(20 J/m2,1 h)
- + - +
[ kDa ]
250
IP : CSB
WB : SUMO-3
↓ re-blotting
250
WB : CSB
1 2 3 4
C
Kps3
UVSSA/Kps3
CS3BE
CSA/CS3BE
UV(20 J/m2,1 h)
- + - + - + - +
IP : CSB
[ kDa ]
250
250
[ kDa ]
250
250
WB : SUMO-3
↓ re-blotting
WB : CSB
1 2 3 4 5 6 7 8
D
C-WT
C-K538R
UV(20 J/m2,1 h)
- + - +
IP : CSB
[ kDa ]
250
250
WB : SUMO-3
↓ re-blotting
WB : CSB
1 2 3 4

Figure 6

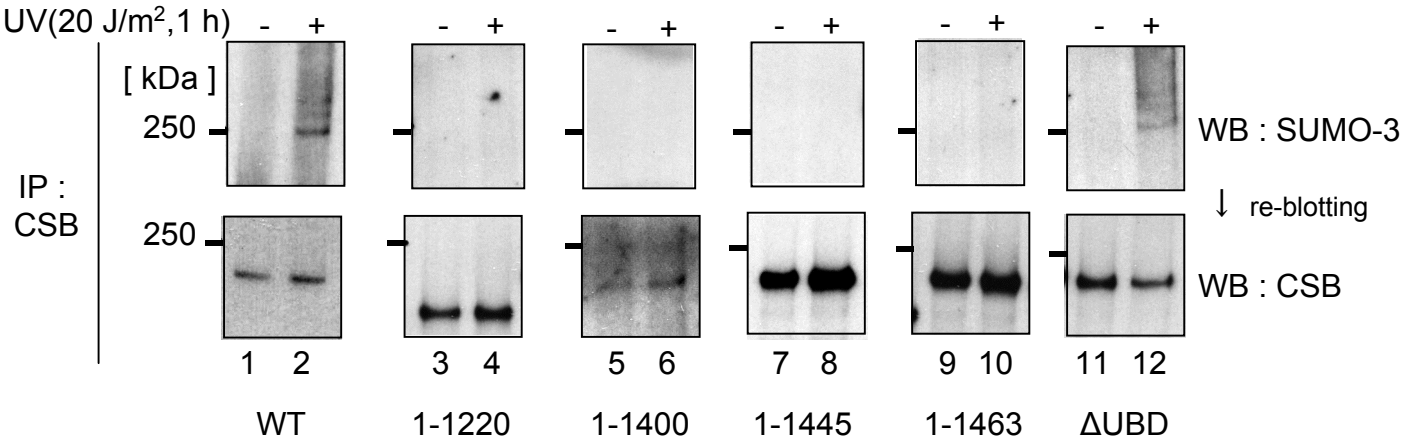

UV(20 J/m2,1 h)
- + - + - + - + - + - +
[ kDa ]
250
250
IP :
CSB
WB : SUMO-3
↓ re-blotting
WB : CSB
1 2 3 4 5 6 7 8 9 10 11 12
WT
1-1220
1-1400
1-1445
1-1463
ΔUBD

Figure 7

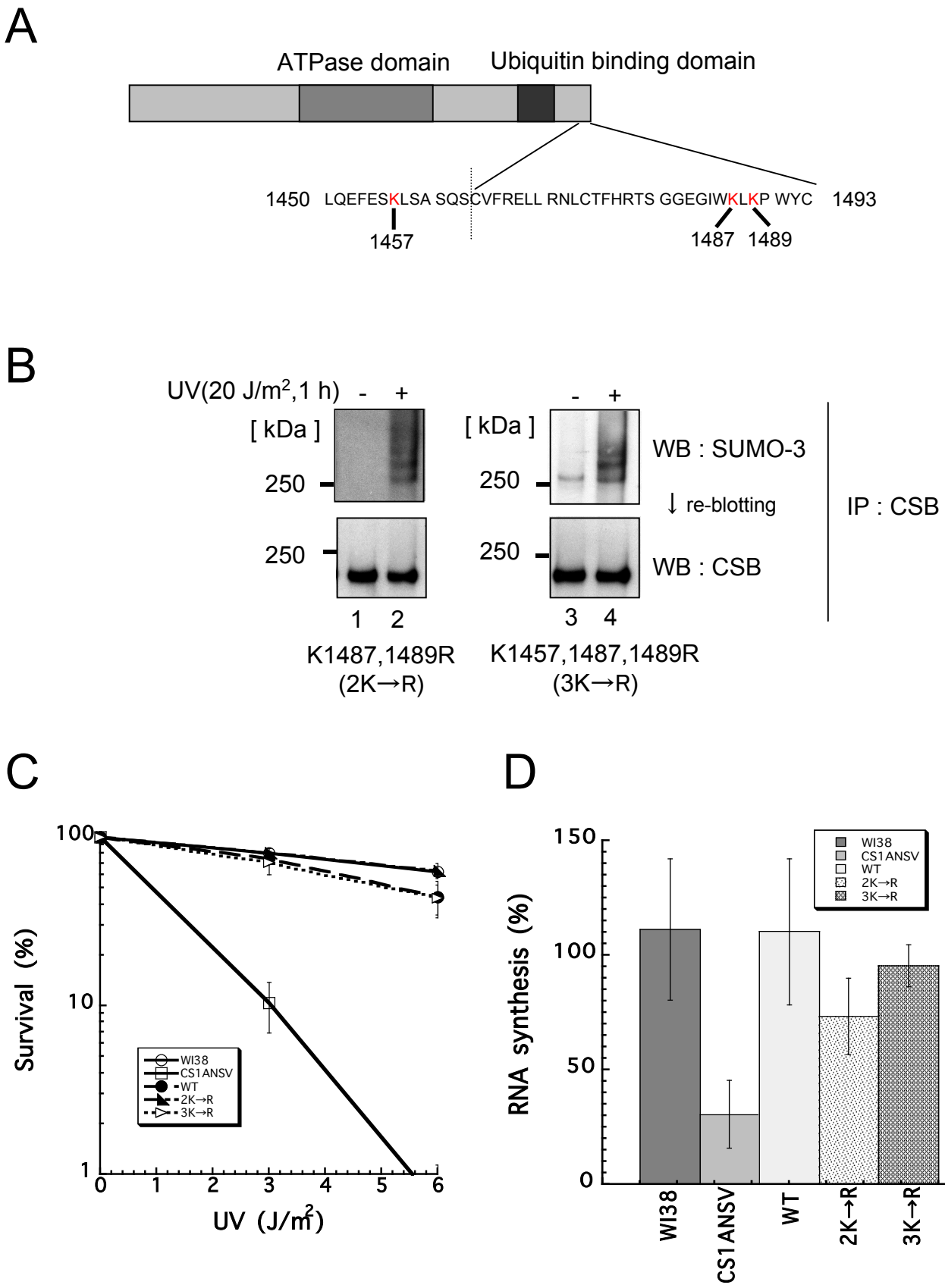

A
ATPase domain
Ubiquitin binding domain
1450 LQEFESKLSA SQSCVFRELL RNLCTFHRTS GGEGIWKLKP WYC 1493
1457
1487 1489
B
UV(20 J/m2,1 h) - +
[ kDa ]
250
250
1 2
K1487,1489R
(2K→R)
- +
[ kDa ]
250
250
3 4
K1457,1487,1489R
(3K→R)
WB : SUMO-3
↓ re-blotting
WB : CSB
IP : CSB
C
Survival (%)
100
10
1
0 1 2 3 4 5 6
UV (J/m2)
WI38
CS1ANSV
WT
2K→R
3K→R
D
RNA synthesis (%)
150
100
50
0
WI38
CS1ANSV
WT
2K→R
3K→R

Figure 8

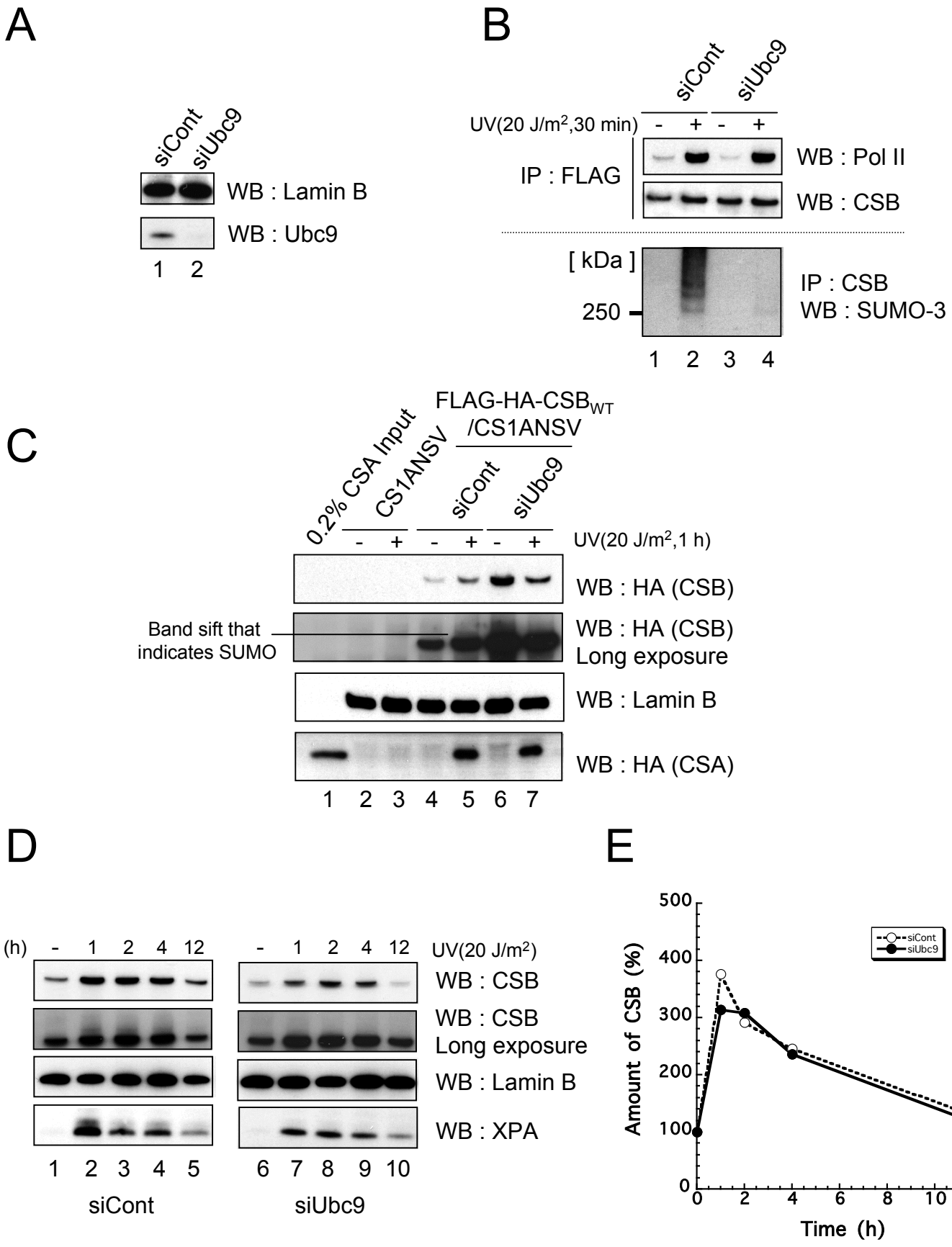

A
siCont
siUbc9
WB : Lamin B
WB : Ubc9
1 2
B
siCont
siUbc9
UV(20 J/m2,30 min) - + - +
IP : FLAG
WB : Pol II
WB : CSB
[ kDa ]
250
IP : CSB
WB : SUMO-3
1 2 3 4
C
FLAG-HA-CSBWT
/CS1ANSV
0.2% CSA Input
CS1ANSV
siCont
siUbc9
UV(20 J/m2,1 h)
- + - + - +
WB : HA (CSB)
Band sift that
indicates SUMO
WB : HA (CSB)
Long exposure
WB : Lamin B
WB : HA (CSA)
1 2 3 4 5 6 7
D
(h) - 1 2 4 12 - 1 2 4 12
UV(20 J/m2)
WB : CSB
WB : CSB
Long exposure
WB : Lamin B
WB : XPA
1 2 3 4 5 6 7 8 9 10
siCont
siUbc9
E
Amount of CSB (%)
500
400
300
200
100
0
siCont
siUbc9
0 2 4 6 8 10 12
Time (h)

Figure 9

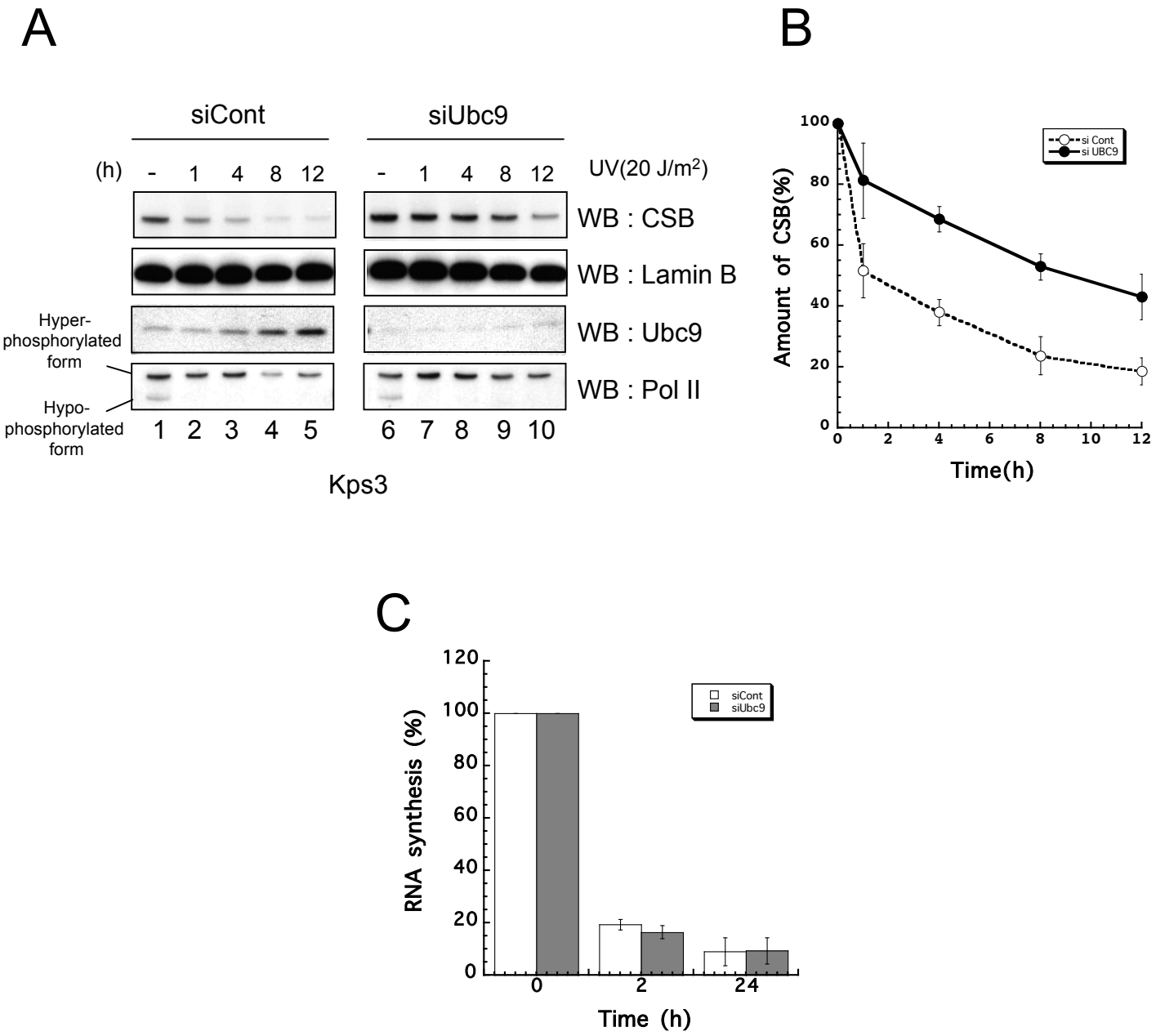

A
siCont
siUbc9
(h) - 1 4 8 12 - 1 4 8 12
UV(20 J/m2)
WB : CSB
WB : Lamin B
WB : Ubc9
WB : Pol II
Hyper-phosphorylated form
Hypo-phosphorylated form
1 2 3 4 5 6 7 8 9 10
Kps3
B
si Cont
si UBC9
Amount of CSB(%)
Time(h)
C
siCont
siUbc9
RNA synthesis (%)
Time (h)

Figure 10

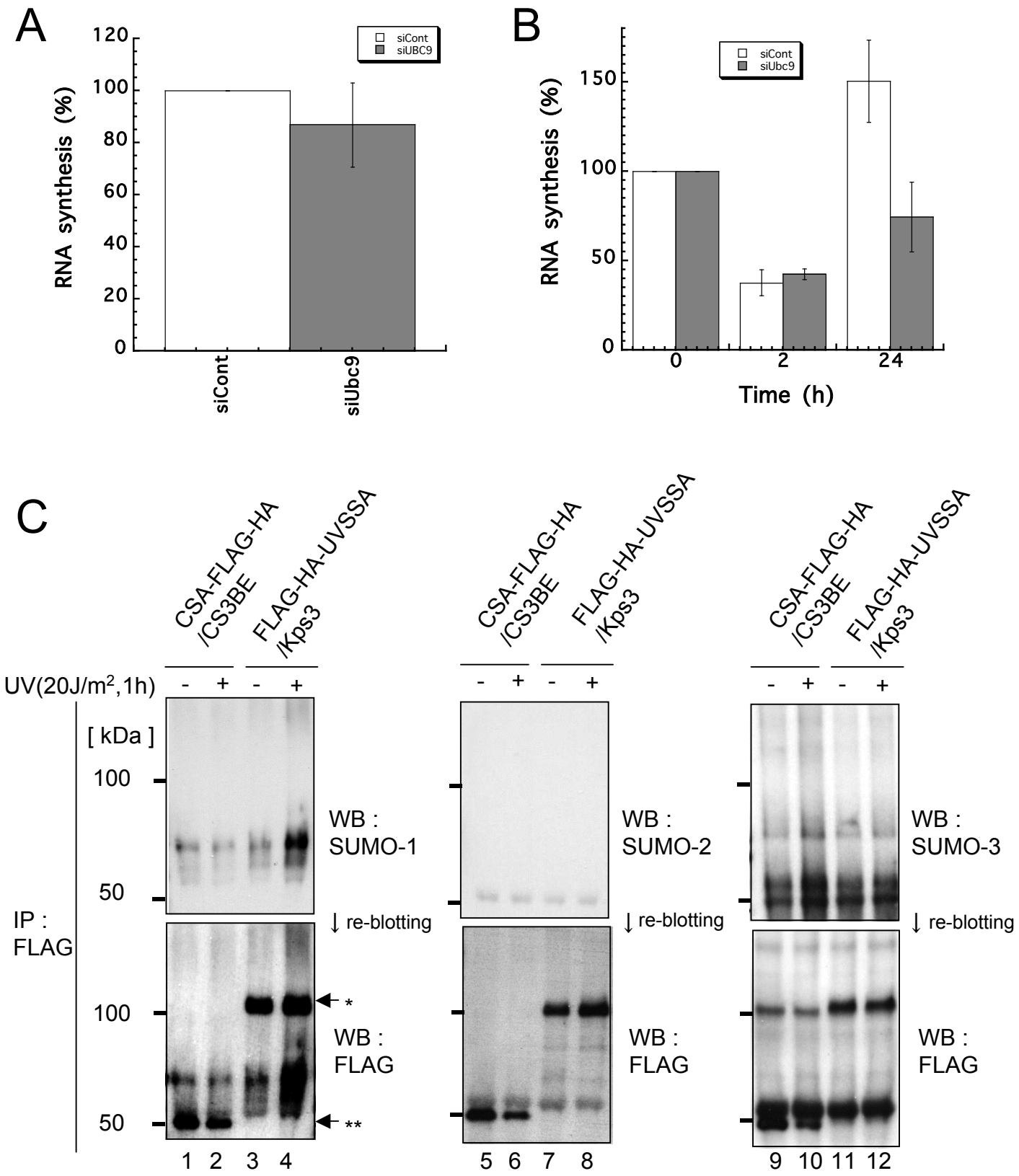

A
RNA synthesis (%)
120
100
80
60
40
20
0
siCont
siUBC9
siCont
siUbc9
B
RNA synthesis (%)
150
100
50
0
siCont
siUbc9
0
2
24
Time (h)
C
CSA-FLAG-HA /CS3BE
FLAG-HA-UVSSA /Kps3
UV(20J/m2,1h)
- + - +
[ kDa ]
100
50
IP : FLAG
WB : SUMO-1
↓ re-blotting
WB : FLAG
*
**
WB : SUMO-2
WB : SUMO-3
1 2 3 4
5 6 7 8
9 10 11 12

Figure 11

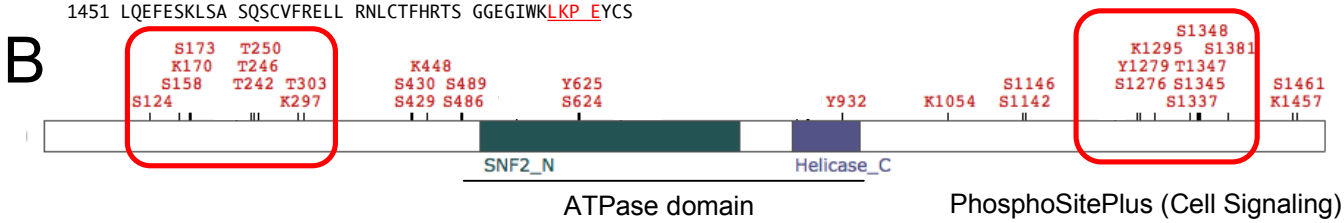
A

```
  1 MPNEGIPHSS QTQEQDCLQS QPVSNNEEMA IKQESGGDGE VEEYLSFRSV
 51 GDGLSTSAVG CASAAPRRGP ALLHIDRHQI QAVEPSAQAL ELQGLGVDVY
101 DQDVLEQGVL QQVDNAIHEA SRASQLVDVE KEYRSVLDDL TSCTTSLRQI
151 NKIIEQLSPQ AATSRDINRK LDSVKRQKYN KEQQLKKITA KQKHLQAILG
201 GAEVKIELDH ASLEEDAEPG PSSLGSMLMP VQETAWEELI RTGQMTPFGT
251 QIPQKQEKKP RKIMLNEASG FEKYLADQAK LSFERKKQGC NKRAARKAPA
301 PVTPPAPVQN KNKPNKKARV LSKKEERLKK HIKKLQKRAL QFQGKVGLPK
351 ARRPWESDMR PEAEGDSEGE ESEYFPTEEE EEEEDDEVEG AEADLSGDGT
401 DYELKPLPKG GKRQKKVPVQ EIDDDFFPSS GEEAEAASVG EGGGGGRKVG
451 RYRDDGDEDY YKQRLRRWNK LRLQDKEKRL KLEDDSEESD AEFDEGFKVP
501 GFLFKKLFKY QQTGVRWLWE LHCQQAGGIL GDEMGLGKTI QIIAFLAGLS
551 YSKIRTRGSN YRFEGLGPTV IVCPTTVMHQ WVKEFHTWWP PFRVAILHET
601 GSYTHKKEKL IRDVAHCHGI LITSYSYIRL MQDDISRYDW HYVILDEGHK
651 IRNPNAAVTL ACKQFRTPHR IILSGSPMQN NLRELWSLFD FIFPGKLGTL
701 PVFMEQFSVP ITMGGYSNAS PVQVKTAYKC ACVLRDTINP YLLRRMKSDV
751 KMSLSLPDKN EQVLFCRLTD EQHKVYQNFV DSKEVYRILN GEMQIFSGLI
801 ALRKICNHPD LFSGGPKNLK GLPDDELEED QFGYWKRSGK MIVVESLLKI
851 WHKQGQRVLL FSQSRQMLDI LEVFLRAQKY TYLKMDGTTT IASRQPLITR
901 YNEDTSIFVF LLTTRVGGLG VNLTGANRVV IYDPDWNPST DTQARERAWR
951 IGQKKQVTVY RLLTAGTIEE KIYHRQIFKQ FLTNRVLKDP KQRRFFKSND
1001 LYELFTLTSP DASQSTETSA IFAGTGSDVQ TPKCHLKRRI QPAFGADHDV
1051 PKRKKFPASN ISVNDATSSE EKSEAKGAEV NAVTSNRSDP LKDDPHMSSN
1101 VTSNDRLGEE TNAVSGPEEL SVISGNGECS NSSGTGKTSM PSGDESIDEK
1151 LGLSYKRERP SQAQTEAFWE NKQMENNFYK HKSKTKHHSV AEEETLEKHL
1201 RPKQKPKNSK HCRDAKFEGT RIPHLVKKRR YQKQDSENKS EAKEQSNDDY
1251 VLEKLFKKSV GVHSVMKHDA IMDGASPDYV LVEAEANRVA QDALKALRLS
1301 RQRCLGAVSG VPTWTGHRGI SGAPAGKKSR FGKKRNSNFS VQHPSSTSPT
1351 EKCQDGIMKK EGKDNVPEHF SGRAEDADSS SGPLASSSLL AKMRARNHLI
1401 LPERLESESG HLQEASALLP TTEHDDLLVE MRNFIAFQAH TDGQASTREI
1451 LQEFESKLSA SQSCVFRELL RNLCTFHRTS GGEGIWKLKP EYCS
```

| No. | Pos. | Group | Score |
|---|---|---|---|
| 1 | K32 | NEEMA IKQE SGGDG | 0.94 |
| 2 | K205 | LGGAE VKIE LDHAS | 0.93 |
| 3 | K1489 | EGIWK LKPE YC | 0.91 |
| 4 | K1092 | NRSDP LKDD PHMSS | 0.91 |
| 5 | K884 | QKYTY LKMD GTTTI | 0.91 |
| 6 | K481 | DKEKR LKLE DDSEE | 0.91 |

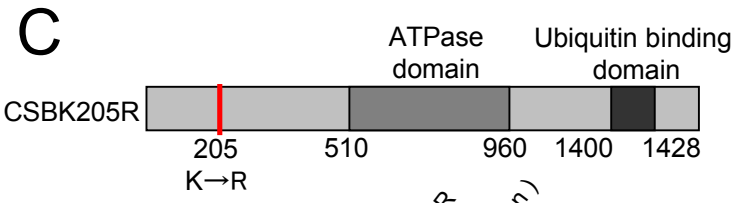
SUMOplot™ Analysis Program (Abgent)

B

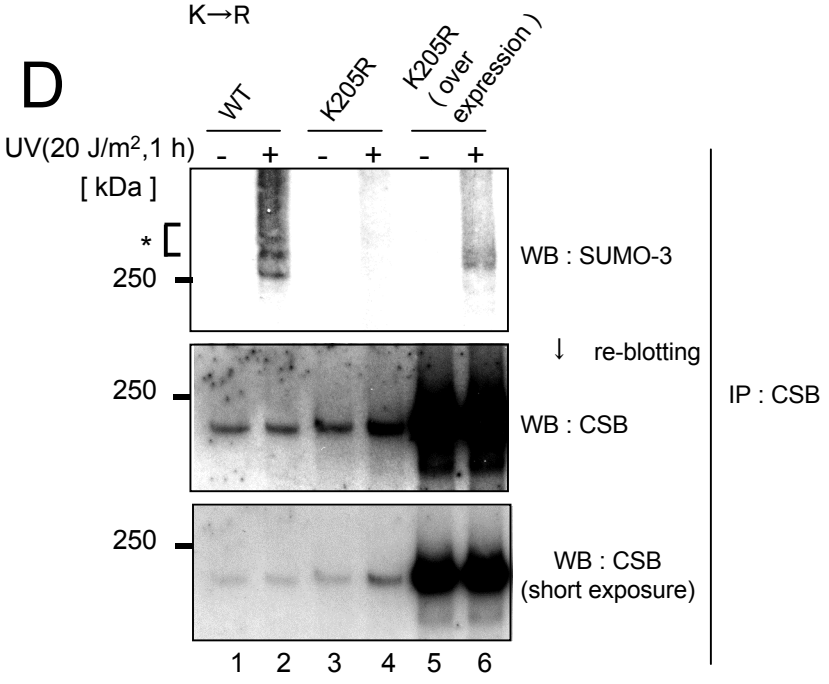



PhosphoSitePlus (Cell Signaling)

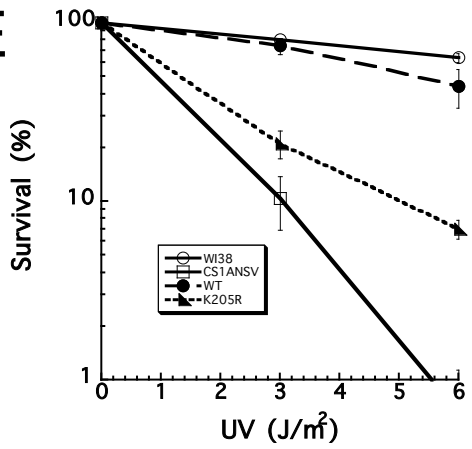
C




D




E




F

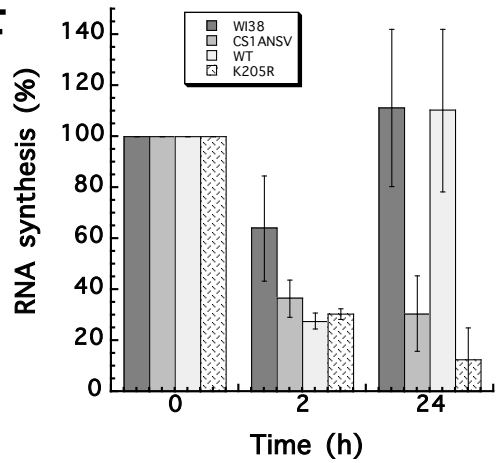



Figure 12

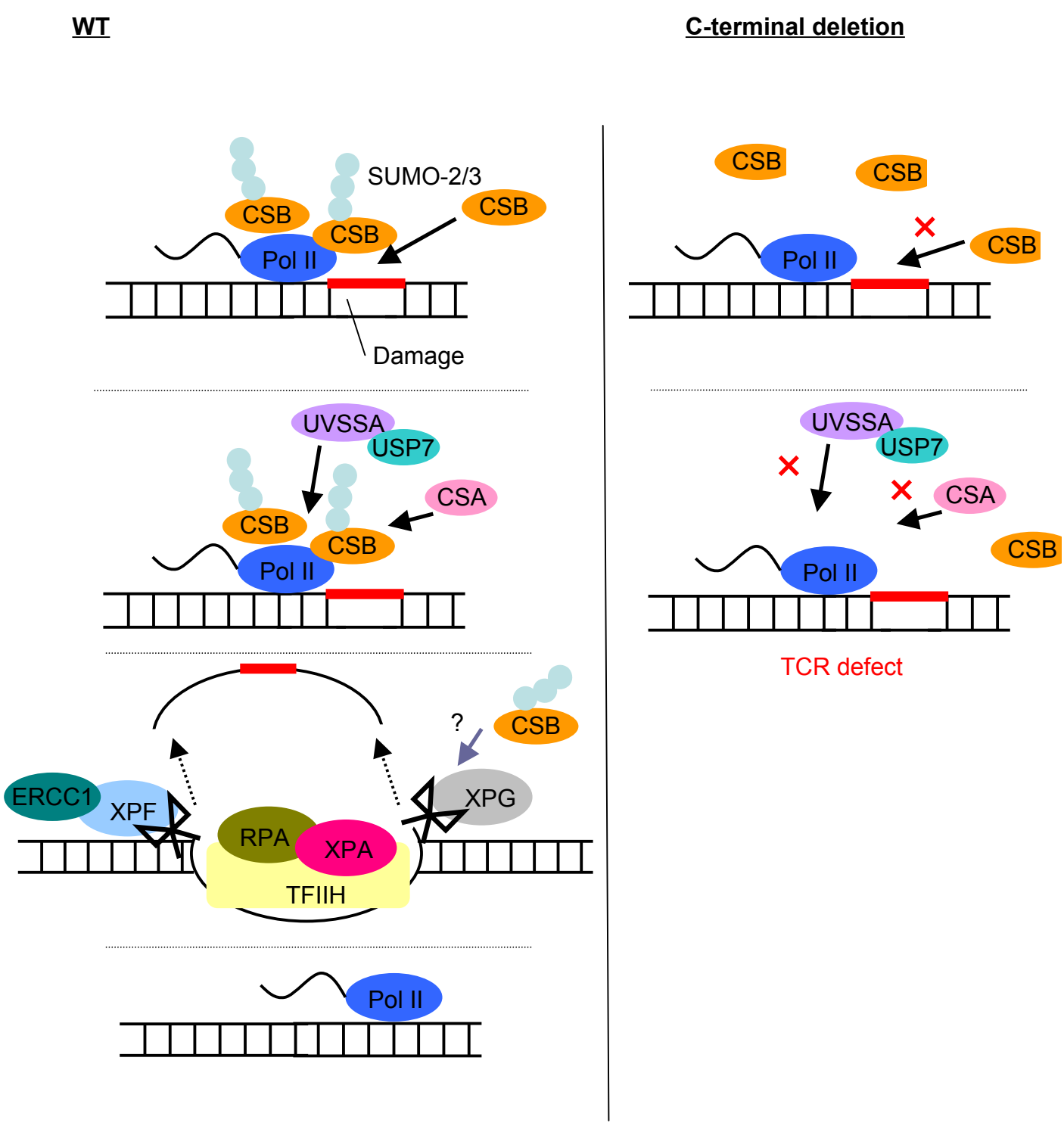

WT
C-terminal deletion
SUMO-2/3
CSB
CSB
CSB
Pol II
Damage
CSB
CSB
CSB
Pol II
UVSSA
USP7
CSA
CSB
CSB
Pol II
UVSSA
USP7
CSA
CSB
Pol II
TCR defect
?
CSB
ERCC1
XPF
XPG
RPA
XPA
TFIIH
Pol II

Figure 13

**Table 1. Primer sets that were used in this study.**
Forward primers basically have Kozak sequence and *XhoI* site, and reverse primers basically have *XbaI* site. Underlines indicate cDNA sequence of CSB.

| | | Primer | |
|---|---|---|---|
| | | Forward | Reverse |
| pcDNA-FLAG-HA-$CSB_{WT}$ | | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-GACTCTAGATTAGCAGTATTCTGGCTTGAGTTTCCAAATTCCTTCACC-3' |
| pcDNA3-FLAG-HA-$CSB_{1-1220}$ | | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-GACTCTAGATTAAGTTCCTTCAAACTTGGCGTCTCTGCAATGC-3' |
| pcDNA3-FLAG-HA-$CSB_{1-1400}$ | | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-GACTCTAGATTAAATCAGGTGGTTTCTAGCTCTCATTTTAGCC-3' |
| pcDNA3-FLAG-HA-$CSB_{1-1445}$ | | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-GACTCTAGATTAGGCCTGGCCATCAGTGTGGG-3' |
| pcDNA3-FLAG-HA-$CSB_{1-1463}$ | | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-GACTCTAGATTAAGACTGTGATGCAGATAACTTGG-3' |
| pcDNA3-FLAG-HA-$CSB_{\Delta UBD}$ | 1-1484 | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-PHO-GAGGGGCCCGGATGAAGAGTCTGCATCTTCTGC-3' |
| | 1434-1493 | 5'-PHO-TTCATCGCTTTCCAGGCCCACACTGATGGCCAGGCC-3' | 5'-GACTCTAGATTAGCAGTATTCTGGCTTGAGTTTCCAAATTCCTTCACC-3' |
| pcDNA3-FLAG-HA-$CSB_{2K\rightarrow R}$ | | 5'-GACCTCGAGCACCATGCCAAATGAGGGAATCCCCC-3' | 5'-GACTCTAGATTAGCAGTATTCTGGCCTGAGTCTCCAAATTCCTTCACCACC-3' |
| pcDNA3-FLAG-HA-$CSB_{3K\rightarrow R}$ | | 5'-CTGCAGGAGTTTGAATCCAGGTTATCTGCATCACAGTCTTG-3' | 5'-CAAGACTGTGATGCAGATAACCTGGATTCAAACTCCTGCAG-3' |
| pcDNA3-FLAG-HA-$CSB_{K205R}$ | | 5'-GGAGCAGAGGTGAGAATTGAACTAGATCACGCC-3' | 5'-GGCGTGATCTAGTTCAATTCTCACCTCTGCTCC-3' |

**Table 2. Summary of the difference between each CSB mutant.**
The results from figure 1 to figure 8 are collected in this table.
Triangles in the content of association with chromatin means that these CSB mutant can associate with chromatin after UV irradiation, but these associations are weaker than that of CSB $_{WT}$.
ND means not done.

| | WT | 1220 | 1400 | 1445 | 1463 | ΔUBD | 2K→R | 3K→R |
|---|---|---|---|---|---|---|---|---|
| UBD | + | - | - | + | + | - | + | + |
| UV sensitivity | - | + | + | + | + | + | - | - |
| Recovery of RNA synthesis | + | - | - | - | - | - | + | + |
| Interaction with Pol II | + | - | - | - | - | - | ND | ND |
| Translocation of CSA | + | - | - | - | - | - | ND | ND |
| Association with chromatin | + | △ | △ | - | - | - | ND | ND |
| SUMO-2/3 modification | + | - | - | - | - | + | + | + |

## Acknowledgement

I sincerely appreciate to Prof. Tanaka for his useful advice and support to my study in his lab. And I also express my sincere gratitude to Dr. Masafumi Saijo, who gave me a direct instruction during my lab life. I started my lab work without any knowledge of experiment technique of biochemical and methodical mind, so he must have a hard time teaching to me. Nevertheless, he taught tirelessly for me. Owing to him, I could continue my work. Dr. Mineaki Seki and Dr. Takashi Narita, the associated professors, always support me and gave advices. I express cordial gratitude to them. I also give heartfelt thanks to the office administrators, Ms. Yoko Hirooka and Hiroko Kanemitsu, who support my lab life. Other graduates and students of Tanaka lab, whether senior or younger, provided much of a stimulus to me. I leaned a lot by their orientations to their work and way of life, so I owe my gratitude to them all.
I really appreciate meeting these people and having great experience in this lab.

In my study, myc-tagged SUMO-1, -2, -3 plasmids were provided by Prof. Hisato Saitoh in Kumamoto university, and HA-tagged SUMO-1, -2 plasmids were provided by Prof. Sugasawa in Kobe university. All materials were used in not-shown data in this study. I appreciate very much the support from them.

## Footnotes

### List of meetings (Japanese Institute)

(Verbal presentation)

1) 申育實、西條将文、田中亀代次、「転写と共役したヌクレオチド除去修復機構における CSB の機能解析」、『日本分子生物学会年会』、2W5II-9、福岡、2012 年 12 月

2) 申育實、西條将文、田中亀代次、「転写と共役したヌクレオチド除去修復機構における CSB の機能解析」、『超異分野若手シンポジウム』、大阪、2014 年 3 月

(Poster presentation)

1) 申育實、西條将文、 田中亀代次、「転写と共役したヌクレオチド除去修復機構における CSB の機能解析」、『日本分子生物学会年会』、2P0209、福岡、2012 年 12 月

## List of meetings (International Institute)

(Verbal presentation)

1) Yooksil Sin, Masafumi Saijo, Kiyoji Tanaka.「Functional analysis of CSB in transcription-coupled repair」,『The 6th Korea-China-Japan Graduate Student Forum』, OB-06, Daejeon, Korea (September 2013)

(Poster presentation)

1) Yooksil Sin, Masafumi Saijo, Kiyoji Tanaka. 「Functional analysis of CSB in transcription-coupled repair」,『The 8th 3R Symposium』, P-85, Hyogo, Japan (November 2012)

2) Yooksil Sin, Masafumi Saijo, Kiyoji Tanaka. 「Functional analysis of CSB in transcription-coupled repair」,『The 6th Korea-China-Japan Graduate Student Forum』, PB-21, Daejeon, Korea (September 2013)

3) Yooksil Sin, Masafumi Saijo, Kiyoji Tanaka. 「Functional analysis of CSB in transcription-coupled repair」,『Disorders of DNA Damage Response-Bench to Bedside-』, P-05, Hyogo, Japan (March 2014)

## Award

申育實、「超異分野若手シンポジウム　企業賞（東洋紡賞）」、2014 年 3 月