The p150 subunit of CAF-1 Causes Association of SUMO2/3 with the DNA Replication Foci

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Abstract

The small ubiquitin-related modifier 2/3 (SUMO2/3) can be post-translationally conjugated to a wide variety of proteins constituting chromatin, the platform for genetic and epigenetic regulation. Nevertheless, it is unclear how SUMO2/3 and SUMO2/3 modified proteins are delivered to the chromatin fibers. Here we report that the largest subunit of chromatin assembly factor 1 (CAF-1), human p150, interacts directly and preferentially with SUMO2/3. Amino acid residue of 98-105 in p150 is essential and sufficient for SUMO2/3 interaction. p150-SUMO2/3 interaction coincided with regions that replicate chromatin fibers, because accumulation of the proliferating cell nuclear antigen (PCNA), and incorporation of bromodeoxyuridine (BrdU) were detected at foci colocalized with both p150 and SUMO2/3 during the S-phase in a cell line expressing epitope-tagged p150. Although inhibition of SUMO2/3 expression had only a small effect on p150 deposition on the replication sites, depletion of p150 led to delocalization of SUMO2/3 from the replication foci. Furthermore, p150 mutants deficient in SUMO2/3-interaction, caused a major reduction of SUMO2/3 at the replication foci. Thus, our findings suggest an expanded role of p150 as a SUMO2/3-interacting factor, and raise the intriguing possibility that p150 plays a role in promoting delivery of SUMO2/3 or SUMO2/3 modified proteins (or both) on chromatin fibers during replication.

Keywords: Posttranslational modification, SUMO, DNA replication, chromatin, CAF-1.

Introduction

Small ubiquitin-related modifiers (SUMOs) can be post-translationally conjugated to a wide variety of cellular proteins; this is termed SUMOylation [1]. A large proportion of SUMOs, but not all, localize in the nucleus, implying that the SUMO modification pathway has an important role in regulating nuclear structures and functions [2]. So far, at least three vertebrate paralogs have been reported: SUMO1, SUMO2 and SUMO3. SUMO2 and SUMO3 are more related to each other (95% amino acid identity) than they are to SUMO1 (~50% identity). Although SUMO1 and SUMO2/3 can be equally conjugated to a subset of proteins, several lines of evidence indicate that SUMO1 and SUMO2/3 are conjugated to different proteins, and represent unique signals regulating different cellular functions [3-6]. It is increasingly evident that SUMO2/3 modification plays a crucial role in controlling many aspects of genetic and epigenetic events via regulating assembly and disassembly of chromatin components in vertebrate cells. However, the mechanism by which different chromatin proteins are selectively modified or deposited (or both) on chromatin fibers by one paralog relative to another is unknown.

The dynamics of eukaryotic chromatin fibers during the cell cycle have proven to be important in regulating genetic and epigenetic stability of eukaryotic cells [7]. Spatiotemporally regulated

delivery/deposition of histone and non-histone proteins on to DNA during replication is the critical step in establishment and maintenance of epigenetic marks on chromatin fibers. Among the crucial factors for such regulation, it has been demonstrated that the chromatin assembly factor 1 (CAF-1), composed of three subunits (p150, p60 and p48), assists in loading histone H3-H4 to the newly replicated DNA strand [7]. Of particular interest, the CAF-1 chaperon complex is known to interact with not only histones, but also with multiple non-histone proteins, including the homotrimeric sliding clamp, proliferating cell nuclear antigen (PCNA) DNA polymerase accessory factor [8], methyl CpG DNA binding domain protein 1 (MBD1) [9, 10] and the specific heterochromatin protein 1 (HP1) [11, 12]. This implies that CAF-1 has a role in coordinating not only histone deposition, but also non-histone protein association with chromatin fibers during DNA synthesis. However, in terms of chromatin assembly during DNA synthesis, the molecular basis of how this spectrum of interaction of CAF-1 can be regulated remains largely unknown.

In this paper, we show that CAF-1 p150 is a SUMO-interacting protein and propose its role in the delivery of SUMO2/3 or SUMO2/3 modified proteins (or both) to chromatin fiber in the context of DNA replication.

Materials and Methods

Plasmids

To generate p150 expression vectors, human CAF-1 p150 was amplified by PCR using the oligonucleotides listed in the Supplemental Materials.

Antibodies

Two rat monoclonal antibodies and two rabbit polyclonal antibodies, against SUMO1 and against SUMO2/3 were used as described previously [13]. The other antibodies used in this study were anti-Flag M2 (Sigma), anti-His_6 (Roche), anti-HA (Clontech), anti-PCNA (PC-10, Santa Cruz Biotechnology), anti-p150 (ss1, abcam) and anti-BrdU (Ab-3, Thermo Scientific).

Recombinant protein expression and GST-pull-down assay

Expression and purification of recombinant proteins were carried out as previously described [13]. GST-pulldown assay was carried out according to Uchimura et al. [13].

Cell Cycle Synchronization, and Indirect Immunofluorescence Assay

Cells were synchronized with the double thymidine block protocol. In brief, cells were blocked

by replacing the normal culture medium with fresh culture medium containing 2.5 mM thymidine for 12 hr. Cells were released from the block by removing the thymidine-containing medium, washing the monolayer twice, and incubating for 10 hr in fresh normal culture medium. The cells were blocked again for 12 h, after that they were released to continue their cell cycle synchronously from the G_1/S border. Indirect immunofluorescence analysis was carried out according to Uchimura et al. [13]. For BrdU staining, cells were treated with 10 μM BrdU for 15 min. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100 followed by denaturing with 2N HCl for 30 min. For PCNA staining, cells were fixed in methanol at -20 °C for 5 min.

Immunoprecipitation

For the immunoprecipitation described in Figure 2B, cultured cells were treated with dimethyl 3,3-dithiobispropionimidate-2HCl (5 mM) (Pierce) on ice for 30 min. Then cells were rinsed with an ice-cold buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl), and lysed in the buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 300 mM NaCl, 10 mM $MgCl_2$, 2 mM NaF, 20mM N-ethyl maleimide, 10% glycerol, the protease inhibitors, followed by sonication. The lysate was subjected to immunoprecipitation as described previously [13]. The immunoprecipitated proteins were eluted with SDS-sample buffer containing 300 mM DTT to cleave completely the cross-linked S-S-bonds.

Results and Discussion

Identification of CAF-1 p150 as a SUMO2/3-interacting protein

To identify a chromatin protein(s) that interacts with SUMO2/3, we performed a yeast two-hybrid screening [13], and isolated the partial cDNA fragments of a mouse homologue of the large subunit of histone chaperone CAF-1 p150. As a step toward understanding the role of p150 in binding SUMO2/3, we first obtained the full-length human p150 cDNA (Fig 1A) and generated the recombinant protein to perform a glutathione S-transferase (GST) pull-down assay (Fig. 1B). His_6-p150 appeared to associate more efficiently with GST-SUMO2- and GST-SUMO3-beads than with GST-SUMO1-beads. When a lysate prepared from the 293S cells expressing Flag- and hemagglutinin (HA)-epitope-tagged human p150, designated as FH-p150, was used in the GST pull-down assay, the preferential interaction of FH-p150 with GST-SUMO2/3-beads was pronounced (Fig. 1C). Next, to determine the region responsible for binding to SUMO2/3, we generated a series of deletion mutants of recombinant p150. Eight amino acid residues of p150 ($p150_{98\text{-}105}$) were found to be essential and sufficient for binding to GST-SUMO2/3 (Fig. 1A and D). In addition, two kinds of p150 point mutants, designated His_6-p150-I99A and His_6-p150-D100A, eliminated the interaction with GST-SUMO3, confirming the importance of $p150_{98\text{-}105}$ for *in vitro* interaction between p150 and SUMO2/3 (Fig. 1E).

We investigated the evolutionary conservation of the amino acid sequence of $p150_{98\text{-}105}$. We performed a database search and revealed that $p150_{98\text{-}105}$ was highly conserved from yeast to humans (Supplemental Fig 1A). It should be noted that $p150_{98\text{-}105}$ does not overlap with the previously identified protein binding domains in p150, such as the binding domains for HP1 [11], PCNA [14], p60 [15, 16] and MBD1 [9] (Fig. 1A), and is not involved in the basic histone chaperone activity of CAF-1 [11, 14], suggesting that $p150_{98\text{-}105}$ has a (previously unknown) role among the CAF-1 p150 family. Furthermore, analysis of $p150_{98\text{-}105}$ polypeptide sequence revealed a sequence motif that closely matched the conserved SUMO-interacting/binding motif (SIM) found in other SUMO-binding proteins [17], indicating that $p150_{98\text{-}105}$ may represent a canonical SIM (Supplemental Fig 1B), and p150 is a SIM-containing SUMO2/3-interacting protein. To examine the affinity of $p150_{98\text{-}105}$ (SIM_{p150}) with SUMO2/3, we performed a "Bead Halo" assay, which is a simple and rapid procedure that was developed to detect low-affinity interactions in real-time under equilibrium binding conditions (Supplemental Fig. 1C and D) [18], and revealed that that the affinity of $p150_{98\text{-}105}$ with SUMO3 was almost similar to, although less pronounced, than that of SIM derived from MCAF1 (GST-SIM_{MCAF1}), a previously well-characterized SIM (Kd = ~1.3 μM) [19].

p150-SUMO2/3 interaction occurs in vivo

To assess whether p150 interacts preferentially with SUMO2/3 *in vivo*, we observed subcellular localization of transiently expressed Flag-tagged p150 and endogenous SUMO2/3 in HeLa cells. As shown in Fig 2A, a substantial number, but not all, of the cells showed multiple foci in which both Flag-p150 and endogenous SUMO2/3 were enriched. We detected few cells containing foci that were co-stained with both anti-SUMO1 and anti-Flag antibodies. In addition, an immunoprecipitation analysis revealed that multiple SUMO-modified proteins were co-precipitated with both anti-SUMO1 and -SUMO2/3 antibodies. However, the signals detected by anti-SUMO2/3 antibody appeared significantly greater than those by anti-SUMO1 antibody (Fig. 2B). Collectively, these results indicate the preferential binding of p150 to SUMO2/3 over SUMO1 *in vivo.*

It should be noted that, when proteins immunoprecipitated with anti-Flag antibodies were probed with anti-Flag antibody, we failed to detect the SUMOylated FH-p150 bands that were expected to migrate above the 150 kDa band of the non-modified full-length FH-p150 (Figure 2B and Supplemenrtal Fig. 2A). In addition, we tested SUMOylation of p150 using the *E. coli* SUMOylation system [20]; however, the results were negative (Supplemental Fig. 2B). Thus, these results indicate that p150 was not an efficient SUMOylation substrate. Rather, it is feasible that p150 has an important role in regulating the interaction with other chromatin proteins containing SUMO2/3 moiety.

p150-SUMO2/3 interaction appears in DNA replication foci

Considering that p150 is a component of histone delivery/deposition factor and is concentrated at the sites of DNA replication during the S-phase [15, 21], we expected that the p150-SUMO2/3 foci represented the regulated accumulation of SUMO2/3 at the sites of DNA replication via the interaction with p150 in cells undergoing S-phase progression. To investigate whether p150-SUMO2/3 interaction occurred at the replication sites, we established a cell line in which Flag-HA-tagged p150 (FH-p150) was stably expressed. As shown in Supplemental Fig. 3, the cell line, designated as NFH-p150 293S, expressed FH-p150 approximately 6-times more than did endogenous p150, and grew normally in comparison with the control 293S cell line. Because this cell line allowed us to detect the precise distribution of p150 as well as p150-SUMO2/3 interaction *in vivo*, we examined whether p150-SUMO2/3 interaction appeared during the S-phase in this cell line using a conventional method: pulse labeling with bromodeoxyuridine (BrdU) and immune-staining with the antibody specific for PCNA, a protein constituting the replication machinery at sites of ongoing replication, were combined [21, 22].

To identify cells undergoing S-phase, we labeled cells with BrdU for 15 min followed by immunofluorescence analysis using antibody against BrdU. Among the BrdU-positive cells (approximately 40% of the total cell population), we found multiple foci that were triple-stained with antibodies for BrdU, FH-p150 and SUMO2/3 antibodies, suggesting that p150-SUMO2/3 interaction

appeared in the cells undergoing S-phase (Fig. 3A). To determine whether FH-p150 and SUMO2/3 were located at the replication machinery, we used an antibody against PCNA and found that many of the PCNA-positive cells in which p150 and SUMO2/3 were co-localized with punctuate nuclear foci (Fig. 3A). These data indicate that both FH-p150 and SUMO2/3 accumulated at active sites for DNA synthesis, and suggest the existence of p150-SUMO interaction during the S-phase in NFH-p150 293S cells.

Furthermore, we demonstrated that the cells expressing either HA-p150-I99A (Fig. 3B) or HA-$p150_{101\text{-}938}$ (data not shown), two of the SUMO-interaction-deficient mutants, significantly impaired the accumulation of SUMO2/3 at the PCNA-foci; this provides further evidence that p150 is a necessary component for deposition of SUMO2/3 to sites of DNA replication, and confirms the importance of SIM_{p150} for interaction with SUMO2/3 at the replication foci.

Dynamics of p150-SUMO2/3 interaction during S-phase

The number, distribution and size of p150-SUMO2/3 foci varied from cell to cell (Fig. 3A and data not shown), implicating the spatiotemporal regulation of p150-SUMO2/3 interaction with replication machinery during the S-phase. Therefore, we aimed to locate sites of DNA replication more precisely by PCNA staining, and investigate the interaction dynamics of p150 and SUMO2/3 with

replication machinery. We first synchronized NFH-p150 293S cells at the beginning of the S-phase by thymidine block and then released them for various times to prepare cell populations at different stages of the S-phase. Based on the well defined spatiotemporal organization of sites of DNA replication, which were identified by either the time period after release from the thymidine block, PCNA staining or BrdU incorporation (or both) [21-25], we grouped the immunostaining patterns of the synchronized cell population during the S-phase progression into three categories (Fig. 3C): early S-phase patterns with a high density of small foci spread throughout the nucleus detected after 1~4 hr release; mid S-phase patterns with a scattered medium foci throughout the nucleus, and a faint concentration on the nuclear rim observed after 4~6 hr release; and late S-phase patterns with a few large dots located mainly at the nuclear and nucleolar periphery, detected after 8~10 hr release.

In early S-phase, the number and fluorescence intensity of PCNA dots rapidly increased as the cells were allowed to progress into S-phase and begin replication around the euchromatic regions. During this time, anti-HA and –SUMO2/3 antibodies detected many small dots spread throughout the nucleus and co-localized with the PCNA dots, suggesting that p150-SUMO2/3 interaction occurred at the PCNA-containing replication machinery (*upper panel*). The pattern of p150-SUMO2/3 interaction detected by anti-HA and –SUMO2/3 antibodies changed during the mid (*middle panel*) and late S-phase (*bottom panel*); that is, the nuclear interior at these stages appeared to be filled with p150-SUMO2/3 foci

coincident with PCNA foci. The small number of large, homogeneously stained PCNA foci detectable at the nuclear and nucleolar periphery during mid or late S-phase (or both), may correspond to constitutive heterochromatin [22-25]; the p150-SUMO2/3 foci co-localized with PCNA foci during mid and late S-phase in this cell line, would represent the constitutive heterochromatin regions. These results indicate dynamic associations of p150 and SUMO2/3 with PCNA-containing replication machinery, and imply that p150-SUMO interaction may be regulated spatiotemporally during the S-phase.

p150 is required for delivery/deposition of SUMO2/3 at the replication foci

To assess the role of p150 in SUMO2/3 delivery/deposition on chromatin fibers undergoing DNA replication, we transfected NFH-p150 293S cells with two independent pSilencer plasmids containing siRNAs against p150 (#1 and #2) to down regulate its expression. As shown in Fig. 4A, immunoblot analysis indicated efficient reduction of FH-p150 by both pSilencer plasmids. After 48 hr treatment with p150 pSilencer plasmids, we performed immunostaining with anti-SUMO2/3 antibody; there were many cells with a remarkable reduction in SUMO2/3 accumulation at the foci detected by both anti-FH-p150 and -PCNA antibodies (Fig. 4B and C). In contrast, when we transfected NFH-p150 293S cells with two independent siRNAs against SUMO2/3 to down regulate

their expression, many of the SUMO2/3-knockdown cells showed accumulation of the p150 foci co-stained with anti-PCNA antibody (Fig 4D). Taken together, these results indicate that SUMO2/3 was not a necessary component for p150 localization, but the level of p150 in the cells has significant effects on the deposition of SUMO2/3 to the sites of DNA replication, suggesting a p150-dependent mechanism to deliver preferentially SUMO2/3 or SUMO2/3 modified proteins (or both) to the chromatin fibers during the S-phase.

The exact target chromatin protein(s) of p150 (i.e. SUMOylation-dependent and replication-coupled) remain unexplained. However, several proteins are feasible. For instance, many studies have demonstrated that p150 may act to initiate heterochromatin assembly by interacting with heterochromatin associated proteins such as HP1, MBD1 and SETDB1 [10, 12, 22, 26, 27]; these appear to be efficient SUMOylation substrates or interacting partners for SUMO [13, 26, 28, 29]; thus, we assume that one or some of the components involved in the assembly of HP1-MBD1-SETDB1-containing heterochromatin are candidates for the SUMOylation-dependent delivery to replicating heterochromatin by p150. More work is needed to identify SUMO2/3 modified chromatin protein(s) which associates with p150 and explore the precise functional role of p150-SUMO2/3 interaction at the replication sites.

Conclusion

We demonstrated that p150 interacts directly with SUMO2/3, and found that the binding of p150 with SUMO2/3 is dependent on SIM located near its N-terminus. When p150 was over-expressed in cultured human cells, we found that SUMO2/3 foci dramatically changed their number, distribution and size as the S-phase progressed, and most of these foci, but not all, appeared at the regions where the DNA and chromatin fibers actively replicate. In addition, we revealed that reduced expression of p150 failed in the delivery of SUMO2/3 to the DNA replication foci. Thus, in the context of DNA /chromatin synthesis, our identification of CAF-1 p150 as a SIM-containing SUMO2/3-interacting protein shows that it is a novel regulatory component in the SUMO modification pathway, and should warrant continued studies on the functional significance of p150-SUMO2/3 interaction in the deposition of chromatin proteins.

Acknowledgements

We appreciate the helpful discussions with the members of the Saitoh Laboratory . This work was supported by grants to Hisato Saitoh from the Naito Foundation, the Novartis Foundation, the Ministry of Education, Culture, Sport, Science and Technology (MEXT), and the Japan Society for the Promotion of Science (JSPS). Junsuke Uwada is a Junior Research Associate of the global COE

Program at the Global Initiative Center for Pulsed Power Engineering.

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Figure Legends

Figure 1. CAF-1 p150 is a SUMO-interacting protein

(A) Schematic representation of human CAF-1 p150, and a summary of the GST pull-down assay. p150 deletion constructs are represented as *thin black lines*. The *Table* on the right summarizes the pull-down assay. + indicates sufficient binding, - indicates negligible binding.

(B) 500 μl of the lysate from *E. coli* expressing recombinant full-length His_6-p150 was incubated with beads bound to 5 μg of GST-only, GST-SUMO1, GST-SUMO2 and GST-SUMO3. Proteins associated with the beads were analyzed by immunoblot analysis using anti-His_6 antibody.

(C) 500 μl of the lysate prepared from the 293S cells expressing Flag-HA-tagged p150 (FH-p150), was incubated with beads bound to 5 μg of GST-only, GST-SUMO1, GST-SUMO2 and GST-SUMO3. Proteins associated with the beads were analyzed by immunoblot assay, using the anti-Flag antibody.

(**D**) 500 μl of the lysate from *E. coli* expressing recombinant full-length His_6-SUMO3 was incubated with beads bound to 5 μg of GST-only, GST-$150_{98\text{-}105}$ and GST-$150_{98\text{-}102}$. Proteins associated with the beads were analyzed by immunoblot analysis using anti-His_6 antibody.

(**E**) 500 μl of the lysate from *E. coli* expressing recombinant full-length His_6-p150-wild type, - I99A and -D100A mutants were incubated with beads containing GST-only or GST-SUMO3. Proteins

associated with the beads were analyzed by immunoblot analysis using anti-His_6 antibody.

Figure 2. SUMO2/3-p150 interaction is detectable *in vivo*.

(**A**) HeLa cells expressing Flag-p150 were fixed, and subjected to indirect immunofluorescence analysis using anti-Flag antibody either with anti-SUMO2/3 (*upper panel*) or anti-SUMO1 (*bottom panel*) antibody. *Insets* on the right side of each picture show 2.5-fold magnification of selected regions (*white boxes*). *Merged* images are shown on the right side. *Bar* = 10 μm.

(**B**) Exponentially growing NFH-p150 293S cells and 293S cells were subjected to immunoprecipitation analysis with anti-Flag antibody as described in Materials and Methods. Western-blot analyses using anti-Flag (*left panel*), anti-SUMO1 (*middle panel*) or anti-SUMO2/3 (*right panel*) antibody, are shown. *Arrowheads* indicate SUMO modified proteins that co-immunoprecipitated with FH-p150.

Figure 3. p150-SUMO2/3 interaction occurs at/around the sites of DNA synthesis during S-phase in NFH-p150 293S cells.

(**A**) Exponentially growing NFH-p150 293S cells in which FH-p150 was stably expressed were incubated in the presence of BrdU for 15 min followed by indirect immunofluorescence analysis

using antibodies as indicated. *Bar* = 10 μm.

(**B**) HeLa cells expressing HA-p150wt and HA-p150-I99A were expressed were subjected to indirect immunofluorescence analysis using antibodies as indicated. *Bar* = 10 μm.

(**C**) Exponentially growing NFH-p150 293S cells were arrested at the G1/S boundary by the double thymidine-bock procedure. After culturing in standard medium for 1 hr (*upper panel*), 6 hr (*middle panel*) and 8 hr (*bottom panel*), the cells were fixed and subjected to indirect immunofluorescence analysis using antibodies as indicated. *Bar* = 10 μm.

Figure 4. SUMO2/3 localization at replication foci is reduced in p150-depleted cells.

(**A**) NFH-p150 293S cells were transfected with pSilencer-p150 (#1 and #2) or control vector. 48 hours post-transfection, total cell lysates were subjected immunoblot analysis, indicating efficient reduction of p150 by both pSilencer plasmids.

(**B**) NFH-p150 293S cells were transfected with control (*upper panel*) or pSilencer-p150 #1 vector (*bottom panel*). 48 hours post-transfection, cells were triple-stained with antibodies as indicated. The *merged* image is indicated on the *right*. *Bar* = 10 μm.

(**C**) The result in (**B**) was quantified by scoring 50 cells from two independent experiments. *Bar chart* depicts the ratio of cells in which SUMO2/3 accumulated at the PCNA-containing replication foci.

(**D**) NFH-p150 293S cells were transfected with pSilencer-SUMO2 and pSilencer-SUMO3 vector. 48 hours post-transfection, cells were triple-stained with antibodies as indicated (*bottom panel*). The *merged* images are indicated on the *right*. A representative of the non-transfected cells is also shown (*upper panel)*. *Bar* = 10 μm.

Figure 1

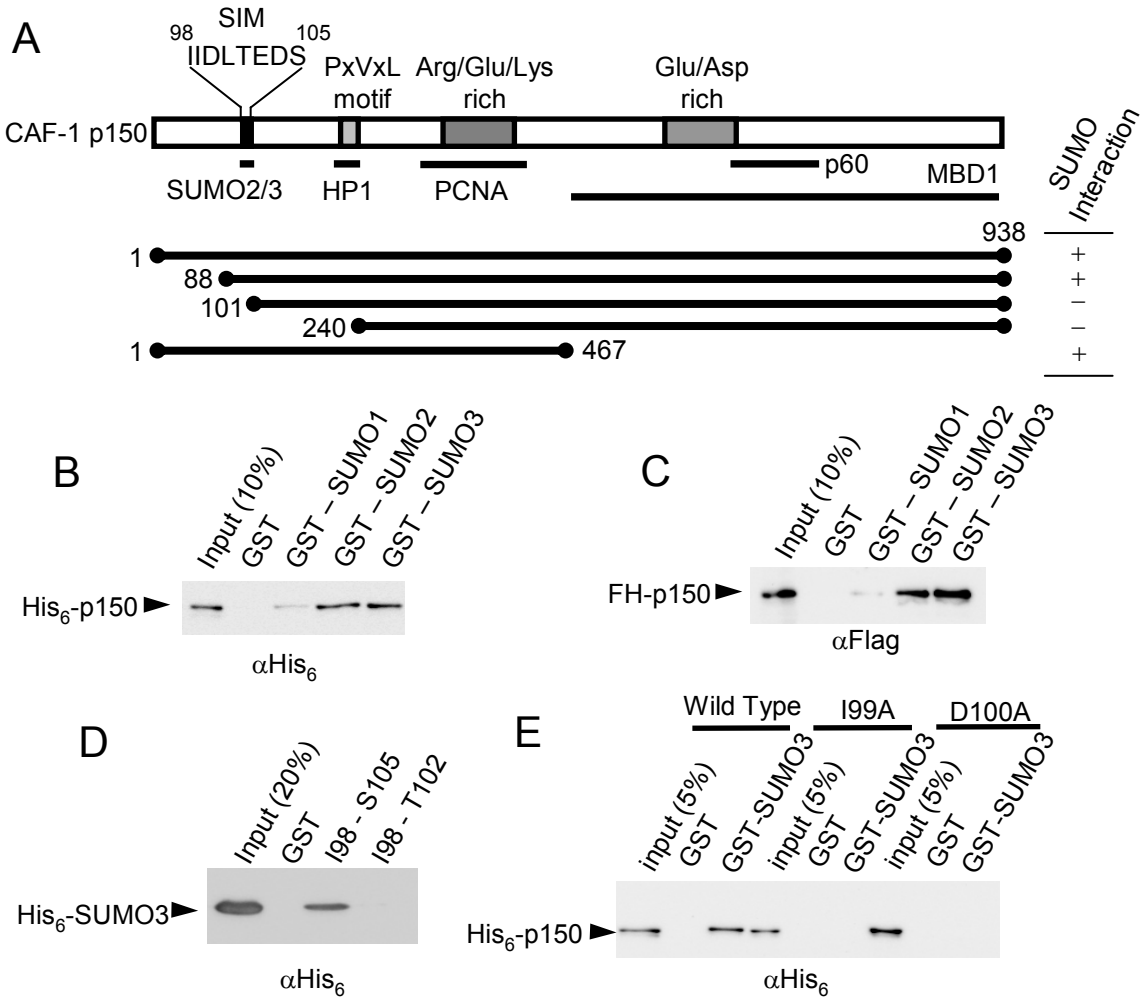

A
98
SIM
105
IIDLTEDS
PxVxL motif
Arg/Glu/Lys rich
Glu/Asp rich
CAF-1 p150
SUMO2/3
HP1
PCNA
p60
MBD1
SUMO Interaction
938
1
88
101
240
1
467
+
+
–
–
+
B
Input (10%)
GST
GST – SUMO1
GST – SUMO2
GST – SUMO3
His6-p150
αHis6
C
Input (10%)
GST
GST – SUMO1
GST – SUMO2
GST – SUMO3
FH-p150
αFlag
D
Input (20%)
GST
I98 - S105
I98 - T102
His6-SUMO3
αHis6
E
Wild Type
I99A
D100A
input (5%)
GST
GST-SUMO3
input (5%)
GST
GST-SUMO3
input (5%)
GST
GST-SUMO3
His6-p150
αHis6

Figure 2

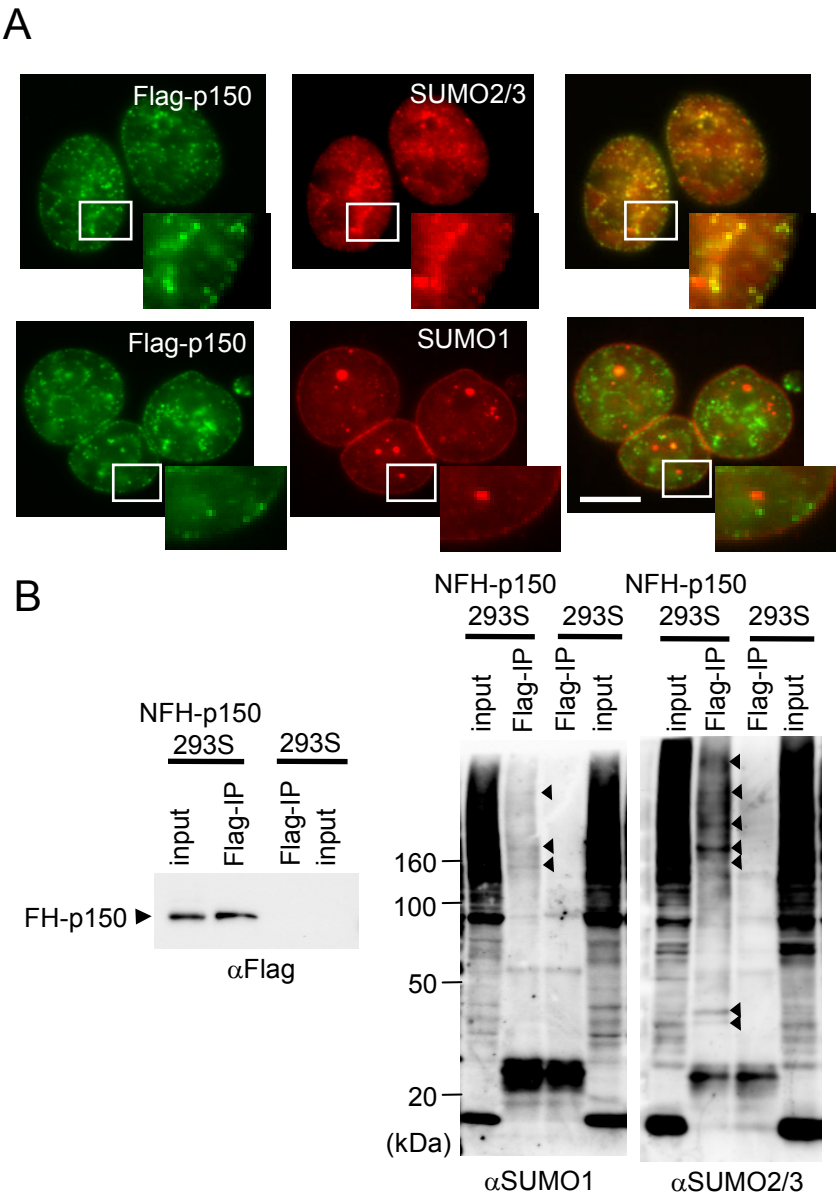

A
Flag-p150
SUMO2/3
Flag-p150
SUMO1
B
NFH-p150
293S
293S
input
Flag-IP
Flag-IP
input
FH-p150
αFlag
NFH-p150
NFH-p150
293S
293S
293S
293S
input
Flag-IP
Flag-IP
input
input
Flag-IP
Flag-IP
input
160
100
50
20
(kDa)
αSUMO1
αSUMO2/3

Figure 3

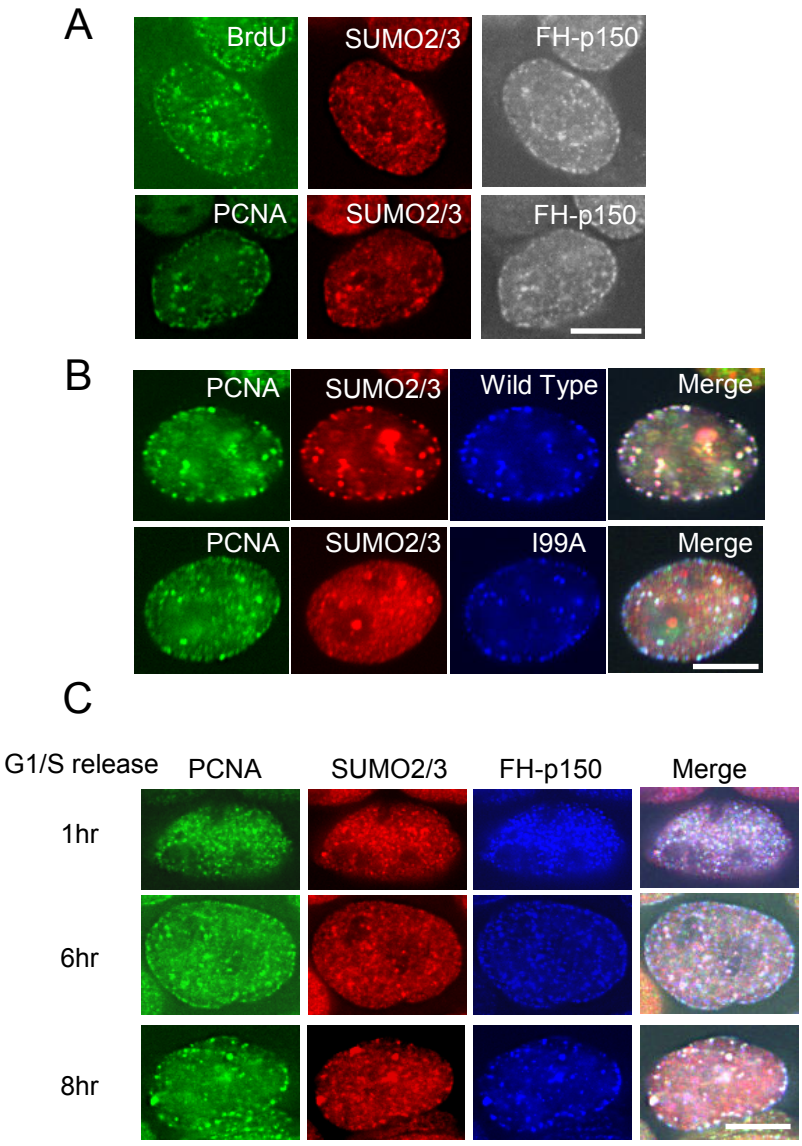
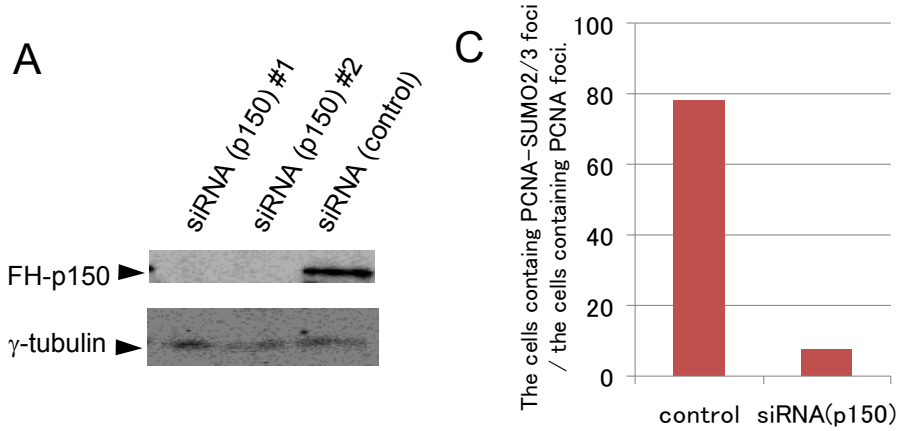
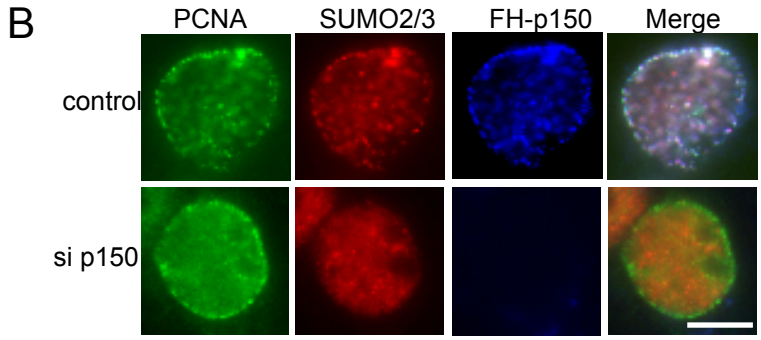
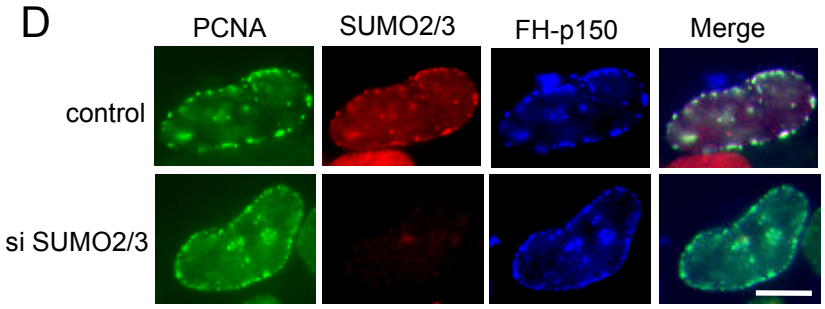

A
BrdU
SUMO2/3
FH-p150
PCNA
SUMO2/3
FH-p150
B
PCNA
SUMO2/3
Wild Type
Merge
PCNA
SUMO2/3
I99A
Merge
C
G1/S release
PCNA
SUMO2/3
FH-p150
Merge
1hr
6hr
8hr

Figure 4


A
siRNA (p150) #1
siRNA (p150) #2
siRNA (control)
FH-p150
γ-tubulin
C
The cells containg PCNA-SUMO2/3 foci / the cells containing PCNA foci.
100
80
60
40
20
0
control
siRNA(p150)


B
PCNA
SUMO2/3
FH-p150
Merge
control
si p150


D
PCNA
SUMO2/3
FH-p150
Merge
control
si SUMO2/3

Supplemental Methods

Plasmids

To generate p150 expression vectors, human CAF-1 p150 was amplified by PCR using the following oligonucleotides: forward primer, 5′-ATCTCTAGAGAATTCATGGATTGCAAAGATAGAC-3′ (an *EcoRI* site is underlined); reverse primer, 5′-ATCCTCGAGTCTAGATCAGGATGCACCCAGTGG-3′ (an *XhoI* site is underlined). The generated PCR fragments were digested by *Eco*RI and *Xho*I and cloned with pET28 (Novagen), pcDNA3-Flag and pcDNA-HA predigested with *Eco*RI and *Xho*I. The deletion mutants of p150 were generated with the following oligonucleotides: forward primer for 88aa-, 5′-CGGAATTCATCGAAACCAGTATTGGCCAG-3′; forward primer for 101aa-, 5′-CGGAATTCTTGACAGAGGACTCGAATGAG-3′; forward primer for 240aa-, 5′-CGGAATTCGCCACACCCCAAGGCAAGAAC-3′; reverse primer for -467aa, 5′-ATCCTCGAGTCTAGATCAGGTCCGACGCCGAGGGGC-3′. To generate pGEX 4T-1 p150-(I98-S105) and p150-(I98-T102), the following origonucleotides were used and subcloned into pGEX 4T-1(GE Healthcare): forward primer, 5′-CGGAATTCATCATTGATTTGACA-3′: reverse primer (I98-S105), 5′-CTCTCGAGCTAGTCCTCTGTCAAATCAAT-3′: reverse primer (I98-T102), 5′-CTCTCGAGCTATGTCAAATCAATGAT-3′. To construct plasmids expressing p150-I99A and

-D100A, site-directed mutagenesis was performed. SUMO-1/2/3 expression plasmids were used as described previously (Uchimura et al., 2006). A shRNA expression vector targeting SUMO2, SUMO3 and CAF-1 p150 were constructed by inserting duplex oligonucleotides into a pSilencer-3.1- H1-puro vector (Ambion). The oligonucleotides used were as follows: pSilencer SUMO2, 5′-GATCCGGCCTACTGCGAGAGGCAGGGCTTGTTCAAGAGACAAGCCCTGCCTCTCGCAGTA GGCCTTTTTTGGAAA-3′ and 5′-AGCTTTCCAAAAAAGGCCTACTGCGAGAGGCAGGGCTTGTCTCTTGAACAAGCCCTGCCTC TCGCAGTAGGCCG-3′; pSlencer SUMO-3, 5′-GATCCAGCCTATTGTGAACGACAGGGATTGTTCAAGAGACAATCCCTGTCGTTCACAATAG GCTTTTTTTGGAAA-3′ and 5′-AGCTTTCCAAAAAAAGCCTATTGTGAACGACAGGGATTGTCTCTTGAACAATCCCTGTCGTT CACAATAGGCTG-3′; pSilencer p150 #1, 5′-GATCCGCAACTGTCATGTGGGTTCTTTCAAGAGAAGAACCCACATGACAGTTGTTTTTTGGA AA-3′ and 5′-AGCTTTTCCAAAAAACAACTGTCATGTGGGTTCTTCTCTTGAAAGAACCCACATGACAGTT GCG-3′; pSilencer p150 #2, 5′-GATCCGAACAAGCTCAGACTGCAATTCAAGAGATTGCAGTCTGAGCTTGTTCTTTTTTGGA

AA-3′ and

5′-AGCTTTTCCAAAAAAGAACAAGCTCAGACTGCAATCTCTTGAATTGCAGTCTGAGCTTGTT

CG -3′.

Bead halo assay

This method is based on a modification of a low-affinity protein interaction procedure "Bead Hallo assay", described by Rexach and colleagues (Patel and Rexach, 2008). The detailed procedure is as follows. The SIM_{p150}(I98-S105), SIM_{MCAF1}(G965-E975) and SIM_{SETDB1}(T120-D131) were expressed in *E. coli* as a recombinant GST-fusion protein and were then incubated with glutathione-Sepharose 4B beads (GE Healthcare). We used 10 μl of the packed beads for bacterial lysate prepared from 50 ml of bacterial culture. This procedure results in GST-fusion proteins immobilized at high concentrations on the surface of Sepharose beads. (Approximately 80-90 μg of GST-fusion protein were bound to 10 μl of packed beads). The beads were resuspended as a 50% slurry in PBS and a 2.0 μl portion of the slurry, in which approximately 20 particles of beads were included, was mixed, on a 76 x 26 mm Micro Slide Glass, (Thikness 1.2 mm, Matsunami), with 1.0 μl of bacterial lysate containing recombinant GFP-SUMO1/3. As soon as the incubation started, the beads can be viewed in real-time under equilibrium conditions using a fluorescence microscope with 10x air objective (Eclipse E600, Nikon).

When a GFP-SUMO1/3 binds to an immobilized GST-fusion protein of interest, the interaction is visible through GFP filters as a halo of fluorescence around the dark beads.

Supplemental Figure 1. Comparison of SIM_{p150} with other SIMs.

(**A**) The amino acid sequence of human CAF-1 $p150_{98\text{-}105}$ is highly conserved among vertebrate and invertebrate species. The sequences within the *gray region* indicate amino acid residues similar to human $p150_{98\text{-}105}$. The corresponding Accession Nos. are human p150, AAA76736; mouse p150, NP_038761; chicken p150, NP_001073220; zebrafish p150, NP_001038478; fruit fly (*D. melanogaster*), NP_572495; and yeast (*S. cerevisiae*), NP_015343. The *numbers* on the *left side* indicate the position of the amino acid residue in each protein.

(**B**) Sequence comparison with previously identified SUMO-interacting polypeptides. The sequences the *gray region* indicate amino acid residues similar to the canonical SIM's hydrophobic core (V/I-X-V/I-V/I or V/I-V/I-X-V/I). The *black underline* represents the region similar to a negatively charged cluster of amino acids that often follows the hydrophobic core sequences of SIM. The corresponding Accession Nos. are p150, AAA76736; MCAF1, AAO91864; SETDB1, NP036564; PIAS1, NP057250; DAXX, NP001135441; PML, NP150241; SP100, NP001073860; TOPORS, NP005793. The *numbers* on the *left side* indicate the position of the amino acid residue in each protein.

(**C**) Bead Halo experiments for analyses of the SUMO3 interaction with SIM_{p150}, SIM_{MCAF1} or SIM_{SETDB1}. Bacterial lysate containing 0.1 μg/ml (*1st column; 100x*), 0.01 μg/ml (*2nd column; 10x*) and 0.001 μg/ml (*3rd column; 1x*) of EGFP-SUMO3 were mixed with the bead-immobilized GST-SIM_{p150} (*1st lane*), GST-SIM_{MCAF1} (*2nd lane*), GST-SIM_{SETDB1} (*3rd lane*) and GST-alone (*4th lane*). An aliquot of the immobilized protein was resolved by SDS-PAGE, stained with Coomassie blue, and is shown on the left. The mixtures were treated as described in the Materials and Methods, and then imaged under a fluorescent microscope using an appropriate GFP-filter. The GFP signal localized to the beads indicates a positive SUMO-SIM interaction. The affinity of SIM_{MCAF1} was measured and calculated as previously described [16].

(**D**) The GFP signals on the beads-immobilized GST-SIM_{p150} (*dark gray*), GST-SIM_{MCAF1} (*light gray*), GST-SIM_{SETDB1} (*gray*) and GST-alone (*black*), were quantified using an image-analyzing system. *a.u.,* arbitrary relative signal intensity units.

Supplemental Figure 2. p150 may not be efficiently modified with SUMO2/3.

(**A**) Failure of detection of endogenously SUMOylated p150. Using NFH-p150 293S cell lysate, an immunoprecipitation assay was performed using control IgG (lanes 1) and anti-Flag antibody (lanes 2) under high salt (stringent) conditions. Immunoprecipitated fractions were subjected immunoblot assay

using anti-Flag antibody. The *arrowhead* indicates the position at FH-p150.

(**B**) A small amount of SUMO modification of p150 in the bacterial SUMOylation system. GST, fused either with MBD1 (*left panel*) or p150 (*right panel*) protein, was subjected to bacterial SUMOylation [17] and purified with glutathione Sepharose beads. Purified products were subjected to SDS-PAGE and stained with Coomassie blue (CB). The a*sterisks* represent the positions of SUMO modified GST-MBD1. Molecular size markers are indicated on the *right* side.

Supplemental Figure 3. Characterization of the NFH-p150 293S cell line

(**A**) FH-p150 was stably over-expressed in the NFH-p150 293S cell line. Immunoblot analysis of 293S cells and NFH-p150 293S cells using antibodies against p150 (*upper panel*) and Flag (*middle panel*) are shown. For the loading control, immunoblot analysis using anti-gamma tubulin antibody is shown (*bottom panel*). The *numbers* at the bottom of each lane represent the relative amounts of the band intensities at around 150 kDa detected by anti-p150 antibody.

(**B**) The cell cycle progression is not perturbed in NFH-p150 293S cells. Growth curves of 293S cells and NFH-p150 293S cells are compared. The cell numbers were counted at 24 hr intervals for 5 days and the relative ratio at each point was plotted.

Supplemental Figure 1

A

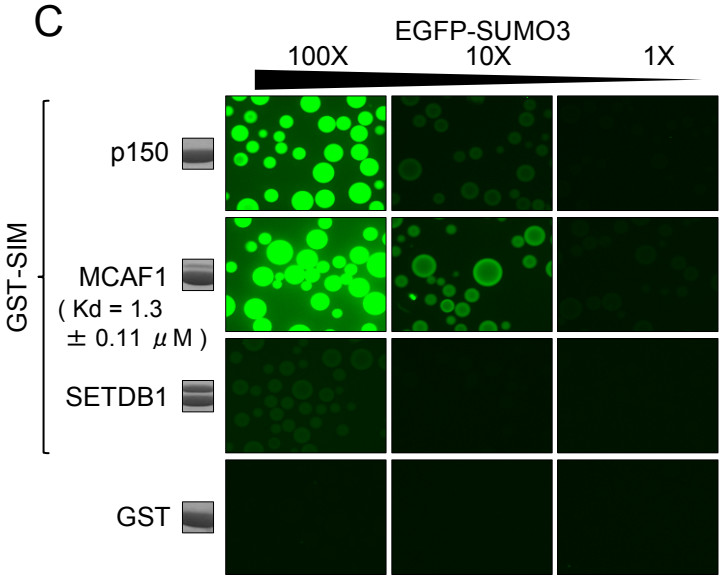
Human CAF-1 p150	88-	IETSIGQSTV	IIDLTEDS	NEQPDSLVDH -115
Mouse CAF-1 p150	99-	ATIKPVPSVV	IIDLTENC	SDIPDSPEGH -126
Zebrafish CAF-1 p150	101-	RSPLRSAPEA	TIDLTEDS	NDSAKQQPAP -128
Fruit Fly CAF-1 p180	123-	VKTTDSVVED	VIELDEDE	ADKEIEDQDQ -150
Yeast CAC1	31-	SNKFLTKEKD	VITLDDPK	EDVSGPMIET -58

B

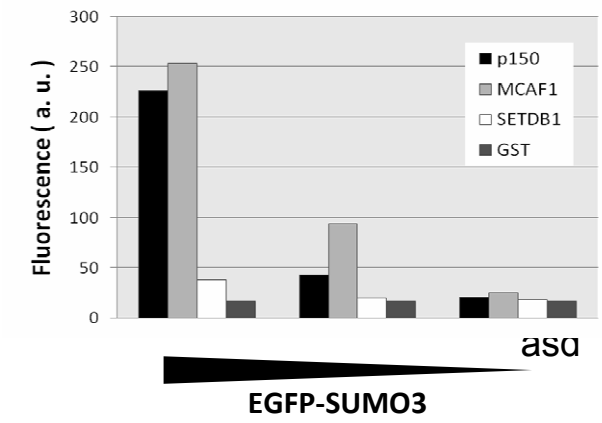
Human CAF-1 p150	93-	GQSTV	IIDL	TEDSNEQPDSLVD -114
MCAF1	961-	SDSSG	VIDL	TMDDEESGASQDP -982
SETDB1	117-	SRPTE	IIEI	PDEDDDVLSIDSG -138
PIAS1	454-	NKKVE	VIDL	TIDSSSDEEEEEP -475
DAXX	728-	CDPEE	IIVL	SDSD -740
PML	551-	EAEER	VVVI	SSSEDSDAENSSS -572
SP100	318-	NQASD	IIVI	SSEDSEGSTDVDE -339
TOPORS	902-	SRSPV	VITI	DSDSDKDSEVKED -923

E/D rich

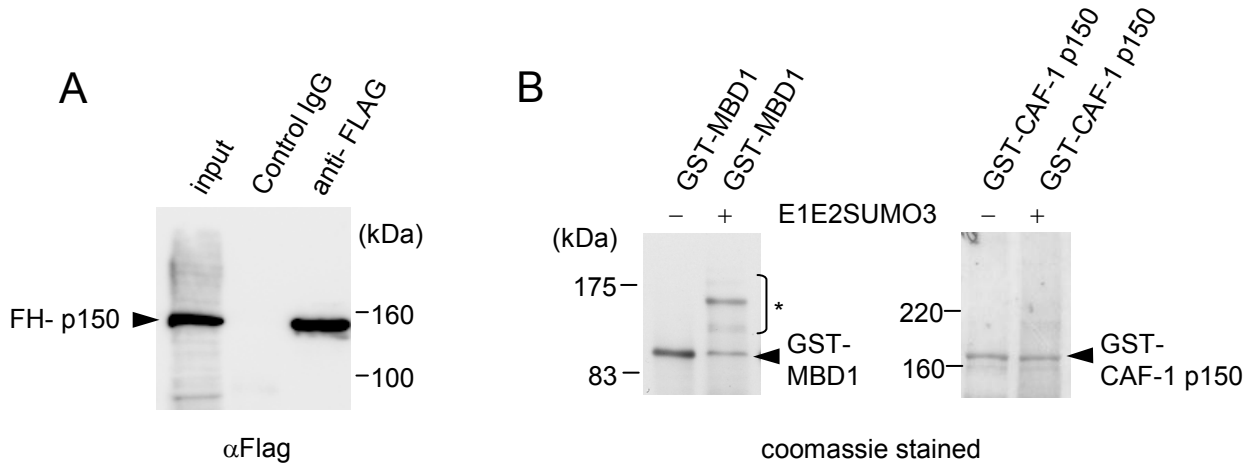
C



D



Supplemental Figure 2



Supplemental Figure 3