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SUMO-1 interacts with mutant ataxin-1 and colocalizes to its aggregates in Purkinje cells of SCA1 transgenic mice

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ABSTRACT

Spinocerebellar ataxia type 1 (SCA1) is one of several progressive neurodegenerative diseases caused by the expanded polyglutamine tract in ataxin-1, the SCA1 gene product. In SCA1 patients and transgenic mice, the affected neuronal cells contain a large ubiquitin-positive aggregate which is derived from the mutant ataxin-1. Small ubiquitin-like modifier-1 (SUMO-1) is one of the most intriguing ubiquitin-like modifiers being conjugated to target proteins and modulating a number of cellular pathways. Recent findings that the aggregates from several neurodegenerative diseases are SUMO-1-positive prompted us to examine the implication of SUMO-1 in SCA1 pathogenesis. In our yeast two-hybrid experiments using mutant ataxin-1 as bait, we identified a SUMO-1 protein that directly binds to ataxin-1 protein. Interestingly, we found that most of the mutant ataxin-1-derived aggregates were SUMO-1-positive both in Purkinje cells of SCA1 transgenic mice and in HeLa cells, but not wild-type ataxin-1 in HeLa cells. In addition, the aggregates in Purkinje cells of SCA1 transgenic mice were positive against both anti-SUMO-1 and anti-ubiquitin antibodies. These results show that the SUMO-1 protein interacts with mutant ataxin-1 and colocalizes with its aggregates which suggests the involvement of the SUMO-1 system in the pathogenesis of SCA1 disease.

Key words
Aggregates • Polyglutamine • Mutant ataxin-1 • SCA1 • SUMO-1

Introduction

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurological disorder characterized by ataxia, progressive motor deterioration, and loss of cerebellar Purkinje cells and brainstem neurons. SCA1 is caused by the expansion of a CAG trinucleotide repeat which results in an expanded polyglutamine tract in its gene product, ataxin-1 (Orr et al., 1993). The abnormal polyglutamine expansion in SCA1 leads to nuclear aggregates and degeneration of selective groups of neurons. Although the relationship between protein aggregates and polyglutamine diseases is still unknown, a number of neurodegenerative disorders such as Huntington disease (HD) (DiFiglia et al., 1997; Furukawa et al., 2009; Gutekunst et al., 1999; Nekooki-Machida et al., 2009), dentatorubral-pallidoluysian atrophy (DRPLA) (Igarashi et al., 1998; Terashima et al., 2002; Katsuno et al., 2008) and spinocerebellar ataxia 3 (SCA3) (Ikeda et al., 1996; Paulson et al., 1997; Yamada et al., 2006) correlate well with the appearance of cytoplasmic or nuclear aggregates. The aggregates have been shown to affect nuclear distribution and function of a variety of proteins which are components of the proteasome machinery or molecular chaperones and transcription factors or regulatory nuclear proteins (Cummings et al., 1998; Shimohata et al., 2000; Okazawa et al., 2002; Hong et al., 2008; Parfitt et al., 2009).

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Redistribution of subnuclear structure is involved in certain human diseases such as neurodegenerative diseases and leukemia. Mutant ataxin-1 (82Q) has been reported to be colocalized with promyelocytic leukemia (PML) nuclear bodies, thus altering the normal nuclear distribution of PML nuclear bodies (Skinner et al., 1997). Since it is known that small ubiquitin-like modifiers (SUMOs) critically regulate transport of PML between nucleoplasm and nuclear body (Yang et al., 2002; Weidtkamp-Peters et al., 2008), these previous data suggest the involvement of SUMOs in the pathology of polyglutamine diseases. Recently, it was reported that SUMO modification of ataxin-1 enhances the aggregation of ataxin-1, which suggests that SUMO-1 may have roles in SCA1 pathogenesis (Ryu et al., 2010). Although the function of SUMOs is not well understood, the perturbations within the modification system has been reported to contribute to the etiology of various diseases such as cancers, inflammatory diseases, and neurodegenerative processes (Kerscher et al., 2006). SUMO-1, which is 18% identical to ubiquitin, utilizes a similar conjugation pathway consisting of activation by a heterodimeric SAE1 and SAE2 activating enzyme (E1) and conjugation onto substrates by Ubc9 (Johnson and Blobel, 1997; Desterro et al., 1999), a protein with a strong sequence similarity to ubiquitin-conjugating enzymes (E2s). In addition, several SUMO E3-like factors were identified in yeast and mammalian cell (Johnson and Gupta, 2001; Kahyo et al., 2001; Sachdev et al., 2001; Pichler et al., 2002; Saitoh et al., 2006; Palvimo, 2007; Yang and Sharrocks, 2010). These findings suggest a possible similarity between SUMO- and ubiquitin-systems.

To understand the involvement of SUMO-1 system in SCA1 pathogenesis, we have studied the effects of normal (30Q) and/or mutant (82Q) ataxin-1 in both mammalian cells and SCA1 transgenic mice.

Materials and methods

Plasmid constructs

SUMO-1 was amplified from the human fetal brain cDNA library (Clontech) by polymerase chain reaction (PCR) with *Pfu* polymerase (Stratagene) using the following primers: SUMO-1: 5'-ATGTCTGACCAGGAGGCAAAACC-3', 5'-CTAAACTGTTGAATGACCCC-3'. The amplification product was cloned into pBluscript KS(+) (Stratagene). SUMO-1 was then subcloned into pcDNA3.1/HisC (Invitrogen) and pB42AD (Clontech). For pLexA-ataxin-$1_{1\text{-}400}$, pLexA-ataxin-$1_{539\text{-}816}$, and pLexA-ataxin-$1_{278\text{-}594}$ constructs, the truncated ataxin-$1_{1\text{-}400}$, ataxin-$1_{539\text{-}816}$, and ataxin-$1_{278\text{-}594}$ were amplified using PCR using following primers: truncated ataxin-$1_{1\text{-}400}$: 5'-ATGAAATCCAACCAAGAGCG-3', 5-GGCCTGTTGCACCTCCAGGTC-3, ataxin-$1_{539\text{-}816}$: 5'-GTCACCCAGGCCGCCTACCC-3', 5'-CTACTTGCCTACATTAGACCG-3', ataxin-$1_{278\text{-}594}$: 5'-CCCACCAGACGATGATCCC-3', 5'-TTTTTAAGTCTTCCACCTTC-3. The cDNAs were cloned into pLexA-BD and pcDNA3. The plasmid pcDNA/amp FlagSCA1[30] and [82] were kind gifts from H.T. Orr (Institute of Human Genetics, University of Minnesota, Minnesota).

Yeast two-hybrid assay

The LexA-Ataxin-1 constructs were transformed together with human fetal brain cDNA library into *Saccharomyces cerevisiae* strain *EGY48* as described (Hong et al., 2002; Hong et al., 2008). The transformants were plated on a selective medium devoid of uracil, histidine, tryptophan, and leucine, and β-galactosidase activities were determined from three separate liquid yeast cultures according to the instructions of the Matchmaker Two-Hybrid System (CLONTECH).

Antibodies

To generate polyclonal antibodies against ataxin-1, the GST-fusion protein, ataxin-1 C-terminus (leucine 539 to lysine 816), was expressed in *E. coli*, purified through gel electrophoresis, and used for the immunization of rats as described (Hong et al., 2002). Anti-A539, the resulting immune serum, could detect both the wild-type and the mutant ataxin-1 through Western blots and immunofluorescence of the transfected COS-7 cells. The identity of the protein as ataxin-1 was confirmed using FLAG- or Xpress-tagged antibodies. The anti-SUMO-1, anti-ubiquitin and anti-Ubc9 were purchased from Santa Cruz.

Immunohistochemistry

SCA1 transgenic mice (line B05) and wild-type nontransgenic mice were generously provided by Dr H.T. Orr. Female B05 SCA1 transgenic mice

(n = 10) and nontransgenic mice (n = 5) were sacrificed. Animals were anesthetized by an i.p. overdose of pentobarbital (150mg/kg) and perfused intracardially with heparinized saline (0.1% heparin) followed by 4% paraformaldehyde (PFA). PFA-fixed paraffin sections of mouse brain were immunohistochemically stained with antibodies to SUMO-1, ubiquitin or ataxin-1. The procedures have previously been described elsewhere (Hong et al., 2002; Vig et al., 2000). Briefly, 8-10 μm thick tissue sections were deparaffinized and were then incubated with 5% blocking goat, rabbit or mouse serum for 10 min with thorough intervening washes of PBS. The sections were incubated for 48 h at 4°C with antibodies. After washing in PBS, the sections were stained with hematoxylin, and were photomicrographed using a Zeiss microscope.

Immunofluorescence assay

Forty-eight hours after transfection, HeLa cells were prepared for immunofluorescence assay as described (Hong et al., 2002). Cells were washed in PBS, fixed in 3.7% formaldehyde for 15 min, rinsed three times in PBS. Subsequently, the cells were permeabilized for 10 min in PBS containing 0.1% Triton X-100. Coverslips with the cells were then incubated in a block buffer (2% bovine serum albumin in PBS) for 1 h at 4°C. The cells were then incubated for another 1 h at room temperature with the following primary antibodies diluted in a blocking buffer: anti-SUMO-1 (1:500), anti-Ubc9 (1:500) or anti-A539. The coverslips were rinsed three times with PBS and incubated for 1 h with goat anti-rat FITC (Jackson Laboratories, West Grove, PA), goat anti-mouse Texas-Red (Jackson Laboratories), and/or mouse anti-goat Texas-Red (Jackson Laboratories), each at 1:800 in a blocking buffer. They were again rinsed three times with PBS and mounted on glass slides using FluoroGuard™ Antifade Reagent (Bio-Rad Laboratories, Hercules, CA). Confocal images were obtained from a Leica TCS-NT laser confocal microscope (Heidelberg, Germany).

In vivo sumoylation assay

The *in vivo* sumoylation was performed as previously described (Buschmann et al., 2001). Briefly, HEK293 cells that maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum were co-transfected with FLAG-tagged ataxin-1(30Q) or ataxin-1(82Q) and HA-tagged SUMO-1 plasmids using Lipofectamine 2000 transfection reagent (Invitrogen). Forty-eight hours after transfection, cells were directly lysed in 500ul of lysis buffer (50 mM Tris-HCl pH 7.9, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% SDS, 20mM N-ethylmaleimide [NEM], 1 mM dithiothreitol [DTT], proteinase inhibitors) and incubated on ice for 40 min. The cell lysates were centrifuged at 13,000g for 15 min at 4°C and the supernatants were immunoprecipitated with a monoclonal anti-FLAG (Sigma). After the immunoprecipitation reactions were incubated overnight at 4°C, Protein G-Sepharose beads (GE Healthcare Life Sciences) were added, and the bead-bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Immunodetections were performed with a polyclonal anti-HA (Sigma).

Results

Yeast two-hybrid screening

To identify a specific protein that interacts with mutant ataxin-1 (82Q), we used mutant ataxin-1 (82Q) as bait in yeast two-hybrid screening (Fig. 1). Yeast cells expressing LexA-ataxin-1 (82Q) were transformed with a human brain cDNA library, and one of positive clones that were identified by screening approximately 2.5 x 10^6 Trp^+, Leu^+ auxotrophic transformants was matched to the sequence of SUMO-1 (Fig. 1). To compare the strength of interaction between SUMO-1 and ataxin-1(82Q) or ataxin-1(30Q), pB42AD-SUMO-1 was retransformed into yeast expressing LexA-ataxin-1 (82Q) or LexA-ataxin-1 (30Q) or LexA-BD, and the Trp^+, Leu^+ yeast transformants were then selected. LexA-ataxin-1 (30Q) or LexA-ataxin-1 (82Q) and pB42AD-SUMO-1-expressing yeast cells grown on a selective medium lacking Trp and Leu showed a strong β-galactosidase activity by the statistical analysis using *t*-test (Fig. 1), but there was no difference between them (data not shown). However, no significant β-galactosidase activity was detected in cells containing pB42AD-SUMO-1 and pLexA-BD, or pB42AD and LexA-ataxin-1 (82Q) or pLexA-BD and pB42AD, indicating that a specific interaction exists between SUMO-1 and ataxin-1. The positive control, LexA-p53 and pB42AD-T_{Ag}, showed a strong β-galactosidase activity.

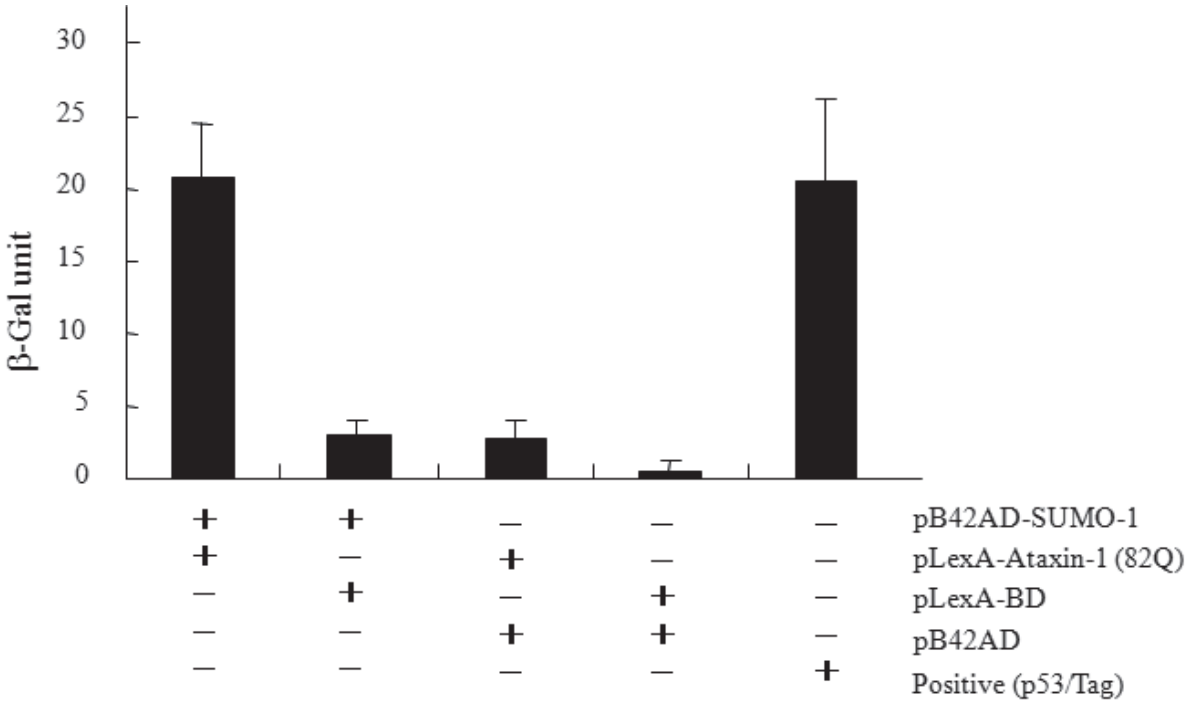

Fig. 1. - The interaction between ataxin-1 (82Q) and SUMO-1. The interaction shown between ataxin-1 and SUMO-1 by liquid β-galactosidase assay. Mutant ataxin-1 was cloned into the yeast expression vector pLexA-BD. β-galactosidase activity was quantified after the yeast was co-transformed with the indicated construct and pB42AD-SUMO-1. As a negative control, pLexA-BD and pB42AD alone were used. As the positive control, pLexA-p53 and pB42AD-Tag were used. Data were generated from three independent experiments, and the statistical analysis was performed using a t-test ($p < 0.05$).

Strong SUMO-1-positive aggregates in HeLa cells transfected with mutant ataxin-1

We subsequently did immunofluorescence analysis of HeLa cells that were transfected with flag-tagged-wild-type ataxin-1 (30Q)/pcDNA1 or mutant ataxin-1 (82Q) with anti-SUMO-1 antibody. Most (93%) of the cells that were transfected with mutant ataxin-1 (82Q) had large (72%) and small (21%) aggregates and the remainder of the cells showed a diffuse staining (6%), in the nucleus. In contrast, 83% of the cells that were transfected with wild-type ataxin-1 (30Q) had large (43%) and small (40%) aggregates, and 14% of the cells showed a diffuse staining pattern (Fig. 2A). SUMO-1 in nontransfected cells was diffusely stained in the nucleus (Fig. 3). All aggregates that were formed by mutant ataxin-1 (82Q) were strongly SUMO-1-positive (a-h in Fig. 2B) in all HeLa cells, but only a few of the aggregates by wild-type ataxin-1 were weakly (arrow head) or very weakly (arrow) SUMO-1-positive in HeLa cells (a-d in Fig. 2C). We observed no significant differences in the strength of immunoreactivity between SUMO-1-positive micropunctate and large aggregates in cells containing mutant (82Q) aggregates (Fig. 2A-C). However, our statistical analysis revealed that the percentage of cells containing SUMO-1 positive aggregates was dramatically different between the wild-type (30Q) and mutant ataxin-1 (82Q). The percentage of cells (86%) with SUMO-1-positive aggregates formed by mutant ataxin-1 (82Q) was found to be 10-fold higher than that observed in wild-type ataxin-1 (30Q) (7.3%) (Fig. 2A).

When the HeLa cells were transfected with flag-tagged-mutant ataxin-1 $(82Q)^{K16A}$/pcDNA1, the cells formed aggregates and all the aggregates were SUMO-1-positive with mutant ataxin-1 (82Q) (data not shown). These results suggest that the mutant ataxin-1 (82Q), but not wild-type ataxin-1, recruited most of the other sumoylated proteins that interact with only mutant ataxin-1 protein to its aggregates, and thus the aggregates formed by site-directed

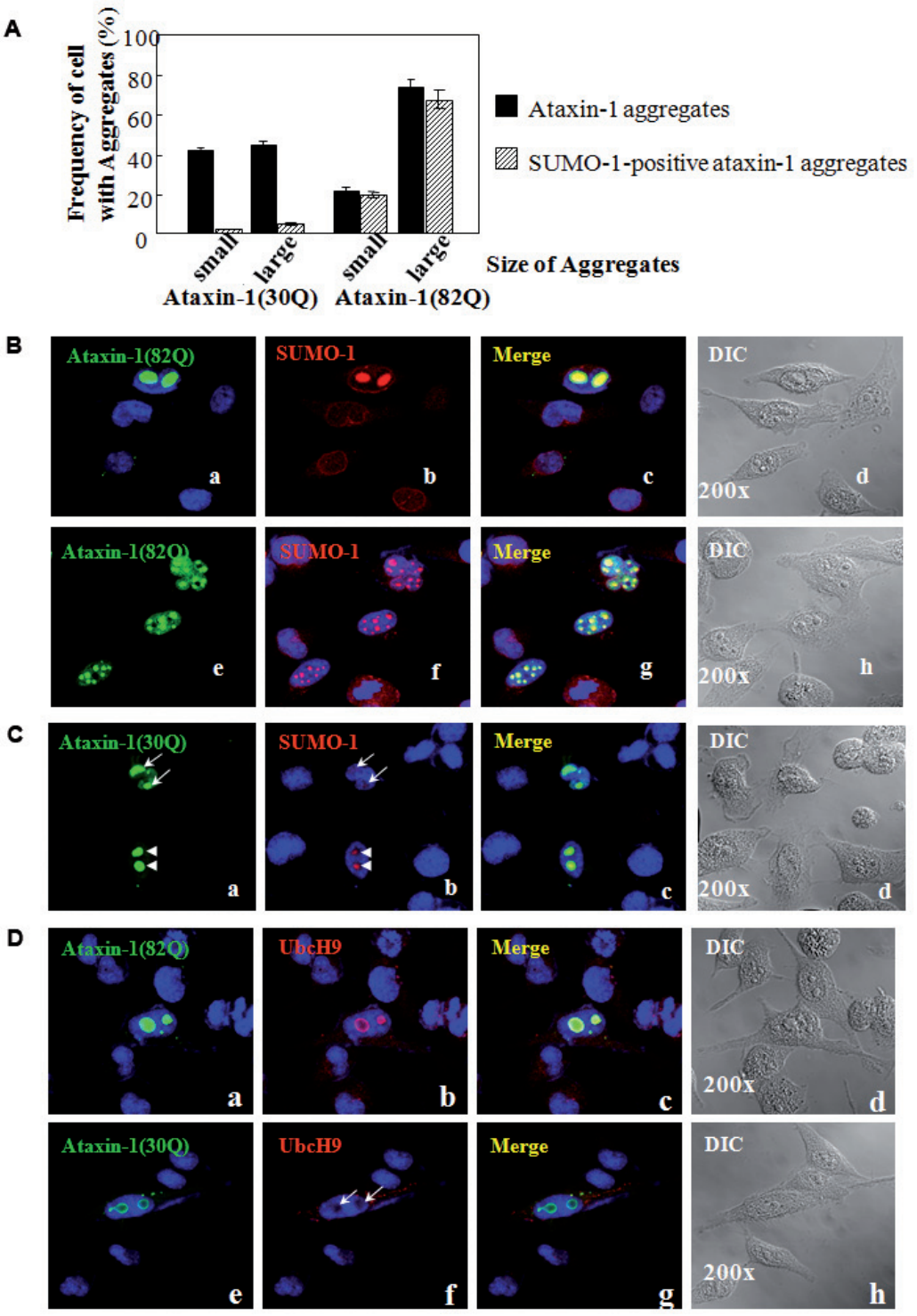

Fig. 2. - Immunoreactivity of SUMO-1 or UbcH9 in HeLa cells transfected with ataxin-1. (A) Frequency of cells with the SUMO-1-positive ataxin-1 nuclear aggregates in cells transfected with wild-type (30Q) or mutant (82Q) ataxin-1. Data were generated from three independent experiments, and the statistical analysis was performed using a t-test ($p < 0.05$). (B) Nuclear aggregates (including large and small aggregates) of mutant ataxin-1 were strongly immunoreactive to anti-SUMO-1 antibody (a-h). (C) Nuclear aggregates of wild-type ataxin-1 were weakly (arrow head) and/or very weakly (arrow) immunoreactive to SUMO-1 antibody (a-d). (D) Nuclear aggregates of mutant ataxin-1 were strongly immunoreactive to anti-UbcH9 antibody (a-d), but not wild-type ataxin-1 (e-h; arrow). Polyclonal A539 anti-ataxin-1 coupled to FITC-conjugated antibody (green), and with anti-SUMO-1 or anti-UbcH9 coupled to Texas Red-conjugated antibody (red). Blue color shows nuclei of cells, counter-stained with DAPI. Yellow signals show the expected overlap of the red and green signals.

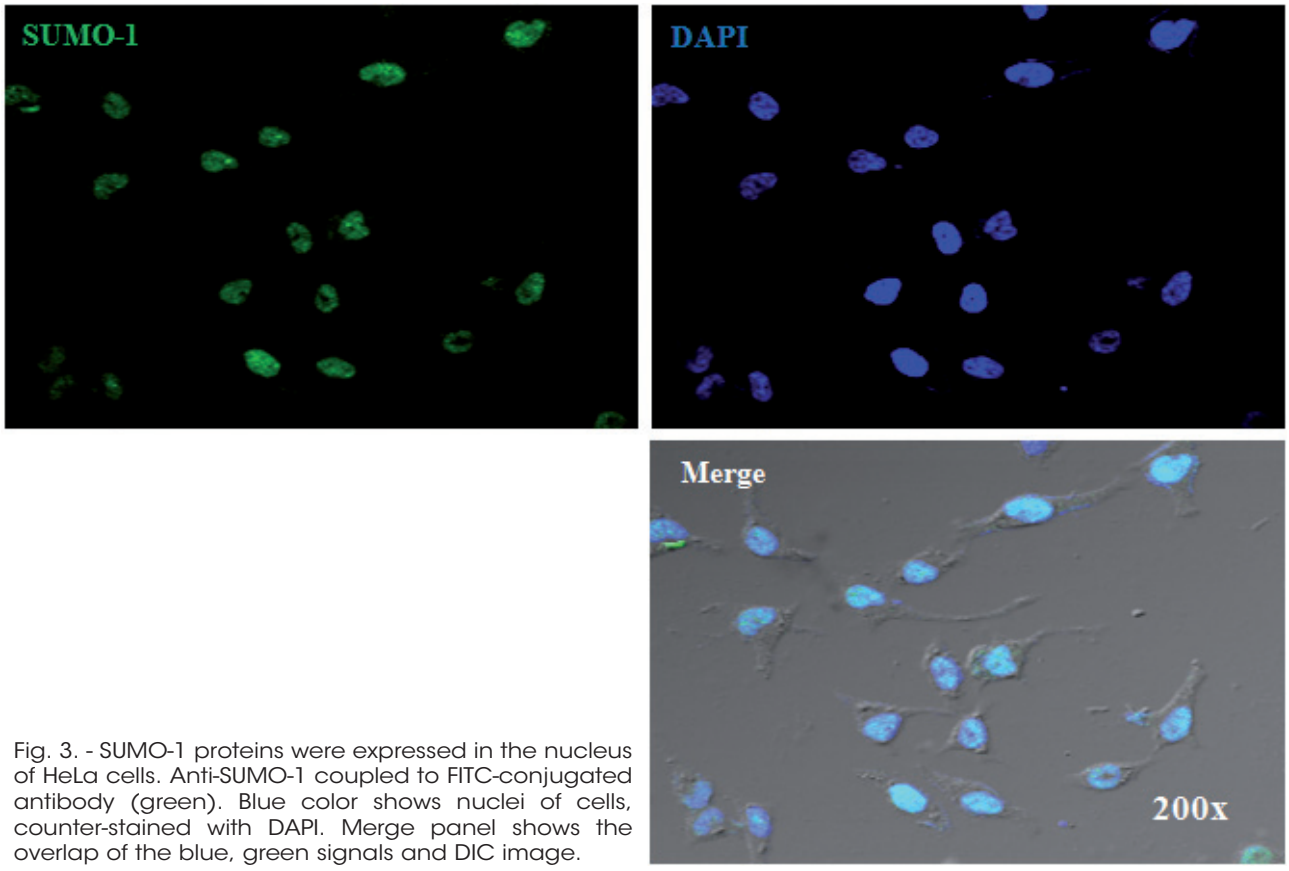

Fig. 3. - SUMO-1 proteins were expressed in the nucleus of HeLa cells. Anti-SUMO-1 coupled to FITC-conjugated antibody (green). Blue color shows nuclei of cells, counter-stained with DAPI. Merge panel shows the overlap of the blue, green signals and DIC image.

mutant ataxin-1 $(82Q)^{K16A}$ of mutant ataxin-1 (82Q) might also contain the sumoylated proteins.

Given the role of UbcH9 enzyme in SUMO-1 system which covalently links the ubiquitin-like protein SUMO-1 to several target proteins (Desterro et al., 1997; Anckar and Sistonen, 2007; Yunus and Lima, 2009), we next examined the localization of UbcH9 in HeLa cells that were transfected with wild-type (30Q) or mutant ataxin-1 (82Q). UbcH9 proteins were localized to both nuclear and cytoplasm of HeLa cells. In the HeLa cells that were transfected with mutant ataxin-1 (82Q), UbcH9 enzymes colocalized to the ataxin-1 aggregates (a-d in Fig. 2D) but not those of wild-type ataxin-1 (30Q) (e-h in Fig. 2D). These results suggest that the mutant ataxin-1 (82Q), but not wild-type ataxin-1, could also recruit the UbcH9 enzymes or other UbcH9-binding proteins to its aggregates.

Distinct SUMO-1-positive aggregates in Purkinje cells of SCA1 transgenic mice

To ascertain whether the SUMO-1 proteins are positive to ataxin-1 aggregates *in vivo*, we examined Purkinje cells of SCA1 transgenic mice expressing the mutant SCA1 allele (B05 line containing 82Q) and nontransgenic mice by immunohistochemistry experiments. In the Purkinje cells of all nontransgenic control mice (n = 5), the SUMO-1 protein was found diffusely in both nuclear and cytoplasm (Fig. 4B). In contrast, the SUMO-1 proteins stained intensely positive to a single large nuclear aggregate in the Purkinje cells of all B05 mice (n = 10) (Fig. 4A). The remainder of the nucleus showed diffuse staining as observed in nuclei of Purkinje cells from nontransgenic control mice.

We next examined the localization of UbcH9 enzyme in the Purkinje cells of SCA1 transgenic mice and nontransgenic mice. The UbcH9 proteins were distributed in the cytoplasm in areas of micropunctate staining with limited nuclear staining, but were not colocalized to ataxin-1 nuclear aggregates. Purkinje cells in nontransgenic controls did not show definitive positive immunoreactivity of the UbcH9 protein (Fig. 5). Taken together, these results suggest that the SUMO-1 or other sumoylated proteins that interact with only mutant ataxin-1 proteins can be

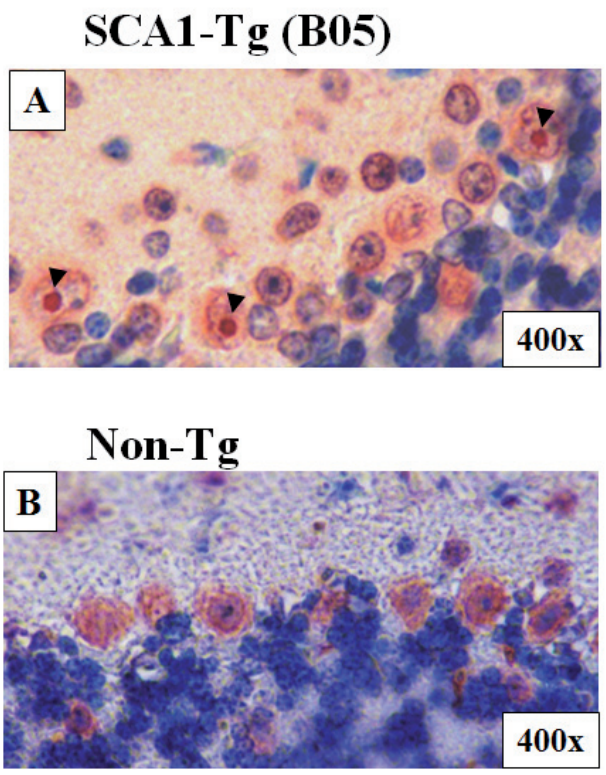

Fig. 4. - Immunoreactivity of SUMO-1 in Purkinje cells of SCA1 transgenic mice and nontransgenic littermate control. Note the redistribution of the SUMO-1 to ataxin-1 aggregates in the transgenic mice. The SUMO-1 colocalizes with ataxin-1 aggregates in mice expressing mutant ataxin-1 with 82 glutamines (A). In contrast, the staining for the SUMO-1 in cerebellar tissue from nontransgenic mice (B) is diffuse in the nuclei of Purkinje cells. Data were generated from each brain section of ten transgenic mice and five control mice.

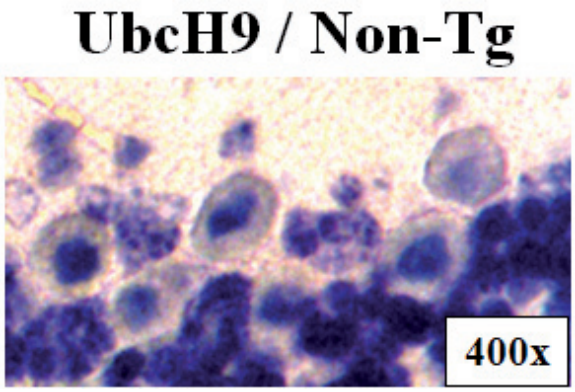

Fig. 5. - Immunoreactivity of UbcH9 in Purkinje cells of nontransgenic littermate control. The staining for the UbcH9 in cerebellar tissue from nontransgenic mice was not definitive positive immunoreactivity in the nuclei of Purkinje cells. Data were generated from each brain section of five control mice.

recruited to the aggregates in Purkinje cells of SCA1 transgenic mice.

SUMO-1 and ubiquitin proteins in same cellular compartment in SCA1 transgenic mice and HeLa cells transfected with mutant ataxin-1

To examine whether SOMO-1 and ubiquitin proteins are colocalized to a single aggregate, we performed immunofluorescence analysis with anti-SUMO-1 and anti-ubiquitin antibodies in Purkinje cells of SCA1 transgenic mice and HeLa cells that were transfected with mutant ataxin-1. We found that ataxin-1 aggregates were definitely both SUMO-1- and ubiquitin-positive in Purkinje cells (A in Fig. 6) and in the HeLa cells (B in Fig. 6). The SUMO-1 proteins overlapped ubiquitin proteins on the aggregates in Purkinje cells and in the HeLa cells suggesting that the two systems could cooperatively or independently function in the same cellular compartments. When we performed immunofluorescence analysis with anti-SUMO-1 and anti-ubiquitin antibodies in Purkinje cells of non-transgenic mice and HeLa cells, we could not find any aggregates in the cells and the SUMO-1 and ubiquitin proteins were diffusely expressed in the nucleus and both nucleus and cytoplasm of HeLa cells, respectively (Fig. 7A-B).

Discussion

One of the most intriguing UBLs (ubiquitin-like modifiers) is SUMO-1 which function as a modifier in a manner analogous to that of ubiquitin (Jentsch and Pyrowolakis, 2000; Melchior, 2000; Hochstrasser, 2001). The structural similarity between SUMO and ubiquitin (Hay, 2001, 2007) and remarkable the structural and functional similarity between Ubc9, SUMO-conjugating enzyme (Johnson and Blobel, 1997), and the large family of ubiquitin-conjugating enzymes (Tong et al., 1997; Giraud et al., 1998) suggest a similarity between sumoylation and ubiquitination modifications.

In this study, we have shown that SUMO-1 interacts with mutant ataxin-1 in yeast two-hybrid system, and colocalizes to the aggregates in HeLa cells containing mutant ataxin-1 and Purkinje cells of SCA1 transgenic mice. Although mutant ataxin-1,

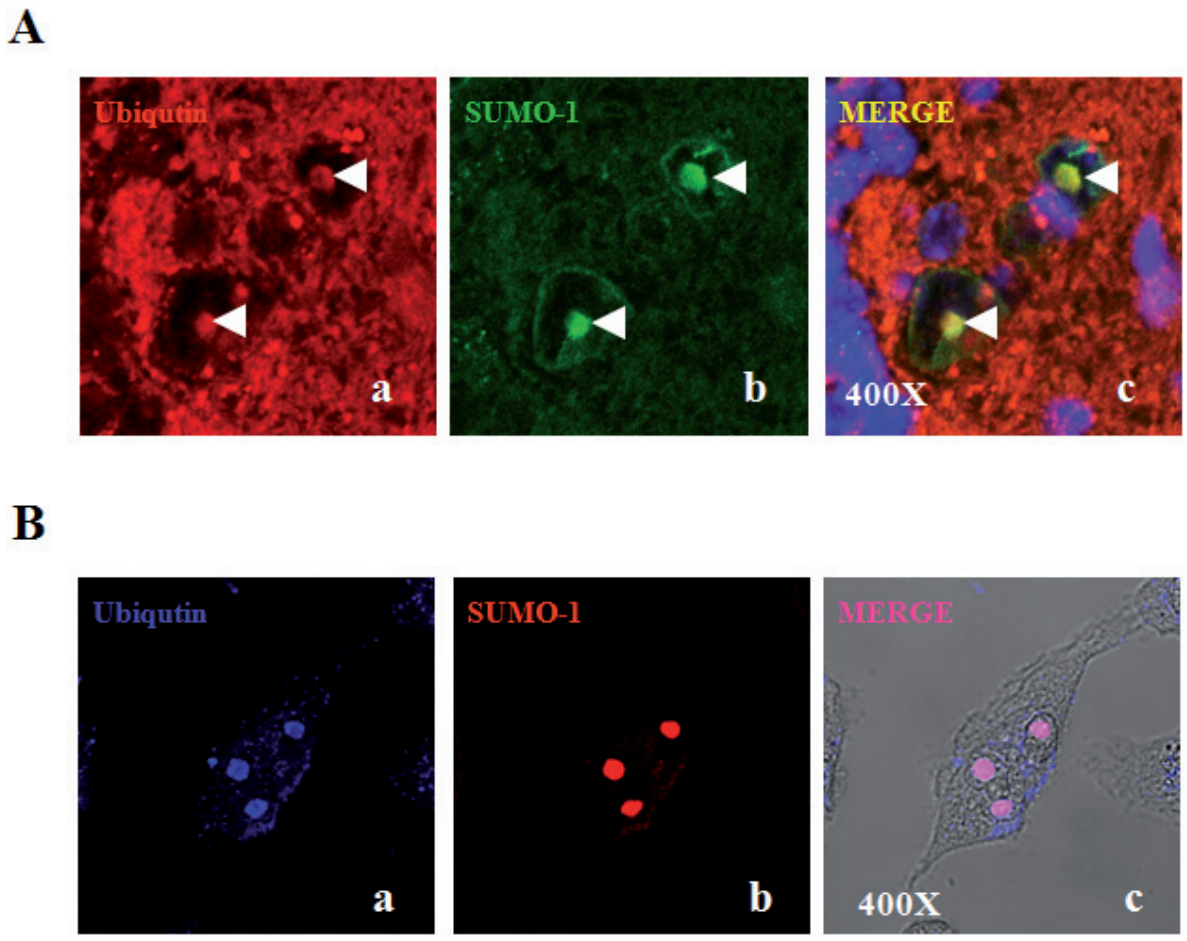

Fig. 6. - Nuclear inclusions of ataxin-1 were immunoreactive to both anti-SUMO-1 and anti-ubiquitin antibodies in Purkinje cells of SCA1 transgenic mice and HeLa cells transfected with mutant ataxin-1 (82Q). (A) Purkinje cells immunolabeled with anti-ubiquitin coupled to Texas Red-conjugated antibody (a) and anti-SUMO-1 coupled to FITC-conjugated antibody (b). SUMO-1- or ubiquitin-positive inclusions in the nucleus showed definite immunoreactivity. Colocalization of both proteins under a fluorescent microscope is indicated in yellow, and blue color shows nuclei of cells, counter-stained with DAPI (c). Data were generated from each brain section of ten transgenic mice and five control mice. (B) HeLa cells with nuclear aggregates of mutant ataxin-1 were strongly immunoreactive to SUMO-1 and ubiquitin antibody. Anti-ubiquitin coupled to Cy5-conjugated antibody (a), and with anti-SUMO-1 coupled to Texas Red-conjugated antibody (b). Pink signals show the expected overlap of the blue and red signals (c).

which is destabilized by the presence of the expanded polyglutamine tract in normal ataxin-1, are modified in the ubiquitin system (Cummings et al., 1998; Cummings et al., 1999; Hong et al., 2002; Al-Ramahi et al., 2006; Choi et al., 2007; Hong et al., 2008), our results in Fig. 6 suggest that the SUMO-1 system could be involved in the modification of mutant ataxin-1 proteins in the same cellular compartment. Unfortunately, our *in vitro* and *in vivo* biochemical sumoylation experiments failed to demonstrate SUMO-1 conjugation onto an ataxin-1 protein (Fig. 8, *in vitro* data not shown). This suggests that desumoylation activity of mammalian SUMO-specific proteases such as SENP1 (Gong et al., 2000; Kim et al., 2005; Cheng et al., 2007; Li et al., 2008), SUSP1(Kim et al., 2000) and SMT3IP1 containing a lot of unidentified enzymes may lead to desumoylation of many target proteins just like deubiquitination by ubiquitin-specific proteases such as USP7 in ubiquitin systems. A simple technical problem in our sumoylation experiments could also cause the failure. Nevertheless, it has been reported that the aggregates are distinctly SUMO-1-positive in HD, SCA1, SCA3, and DRPLA patients, animal

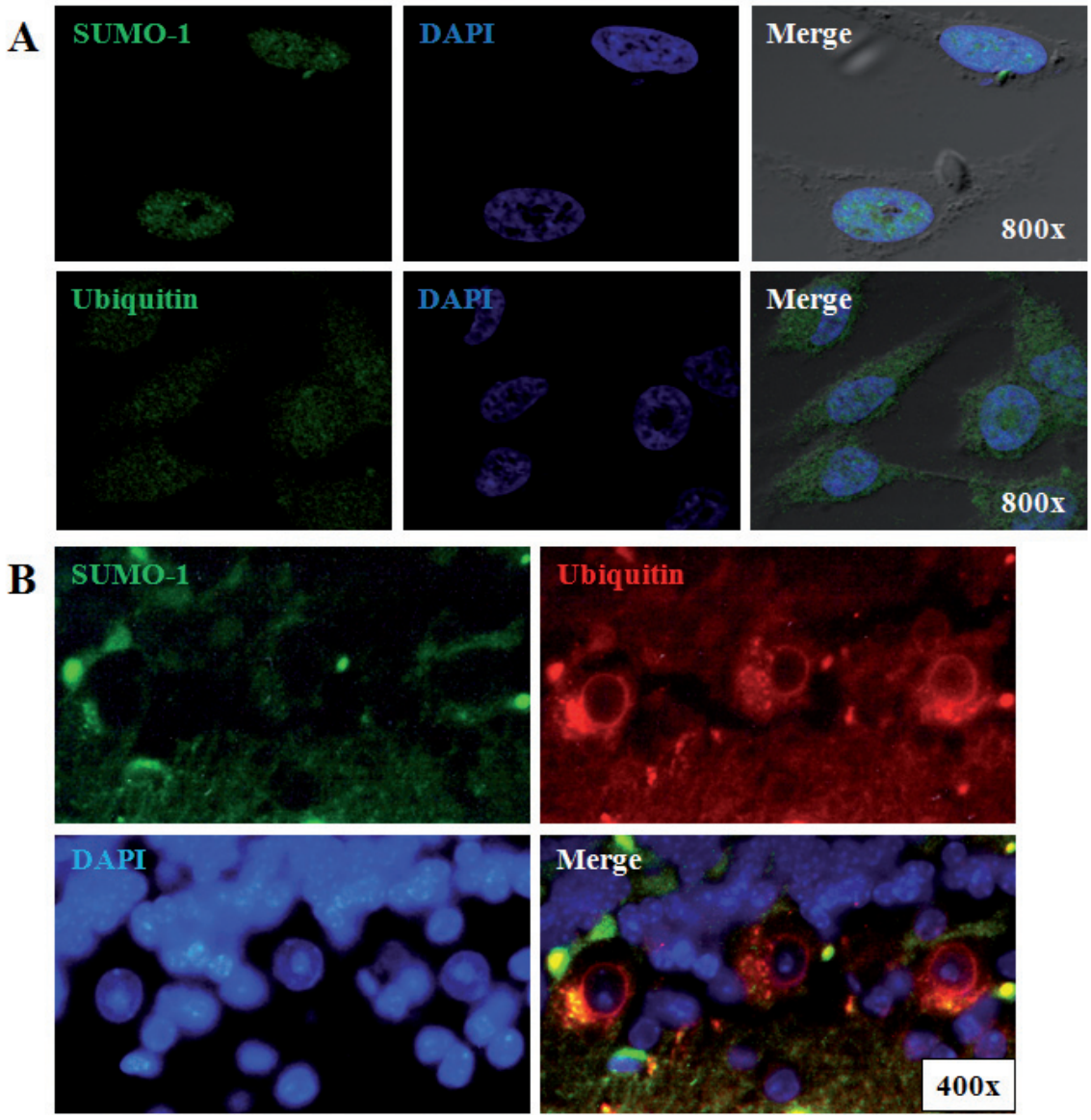

Fig. 7. - Expression of SUMO-1 and ubiquitin proteins in HeLa cells and Purkinje cells of nontransgenic mice. (A) SUMO-1 and ubiquitin proteins were expressed in nucleus and both nucleus and cytoplasm of HeLa cells, respectively. Anti-SUMO-1 or anti-ubiquitin coupled to FITC-conjugated antibody (green). Blue color shows nuclei of cells, counter-stained with DAPI. Merge panel shows the overlap of the blue, green signals and DIC image. (B) Immunoreactivity of both anti-SUMO-1 and anti-ubiquitin antibodies in Purkinje cells of nontransgenic mice. Nuclear inclusions of ataxin-1 were not found, and the SUMO-1 and ubiquitin proteins did not show definite immunoreactivity in Purkinje cells. Merge panel shows the overlap of the green, red and blue signals. Data were generated from each brain section of five control mice.

models and cellular model systems (Yamada et al., 2001; Terashima et al., 2002; Ueda et al., 2002; Pountney et al., 2003; Steffan et al., 2004; Dorval and Fraser, 2007; Ryu et al., 2010) strongly suggesting a direct link of the SUMO-1 system in the pathology of SCA1 disease.

In our liquid β-galactosidase assay in the yeast-two-hybrid system, we found that SUMO-1 specifically interacted with full-length mutant and wild-type ataxin-1, and that no different β-galactosidase activities were observed between full-length wild-type ataxin-1 and mutant ataxin-1. However, the N-terminus (a.a. 1-400) of ataxin-1 with 82 glutamines was found to be 1.5-fold higher than that observed in the truncated ataxin-1 (a.a. 1-400) with 30 glutamines (data not shown). In addition,

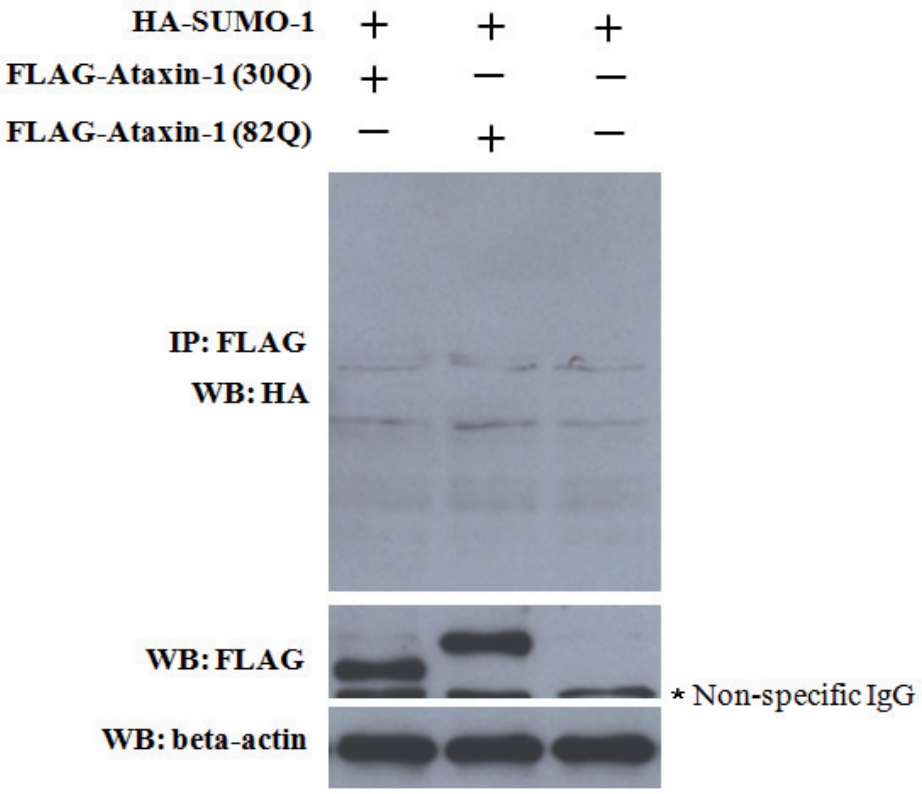

Fig. 8. - *In vivo* sumoylation of ataxin-1. HEK293 cells were co-transfected with FLAG-tagged ataxin-1 (30Q) or (82Q) and the HA-tagged SUMO-1constructs as indicated on the top. The cell extracts and immunoprecipitates were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies. Anti-beta-actin antibody was used as control. Asterisk (*) indicates non-specific IgG immunoglobulin proteins.

UbcH9 interacted with the N-terminus (a.a.1-400) of ataxin-1 with 82 glutamines, but not the N-terminus (a.a. 1-400) of ataxin-1 with 30 glutamines and truncated ataxin-1 without the polyglutamine tract, such as LexA-ataxin-$1_{539\text{-}816}$ and LexA-ataxin-$1_{278\text{-}594}$ (data not shown).

In our immunofluorescence assays, we observed that the percentage of SUMO-1-positive aggregates in cells containing mutant ataxin-1 (82Q) was 10-fold higher than that by wild-type ataxin-1. Also, we found that UbcH9 was also colocalized to aggregates in HeLa cells with mutant ataxin-1, but not wild-type ataxin-1. These results imply that the mutant ataxin-1 (82Q) could directly induce the activation of SUMO-1 system and would be able to conjugate SUMO-1 to other proteins that could interact with mutant ataxin-1 in the HeLa cells.

The results that were described in this report show that mutant ataxin-1 interacts with SUMO-1, and SUMO-1 proteins was positive stained in mutant ataxin-1 aggregates which may suggest the involvement of the SUMO-1 system in the pathogenesis of SCA1 disease. However, the function and regulation of ataxin-1 sumoylation in SCA1 disease should be investigated at the molecular and cellular levels.

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