BMB
reports

Identification of SUMOylated proteins in neuroblastoma cells after treatment with hydrogen peroxide or ascorbate

*Melissa M Grant**
School of Dentistry, University of Birmingham, Birmingham, B4 6NN, UK

The small ubiquitin-like modifier (SUMO) proteins have been implicated in the pathology of a number of diseases, including neurodegenerative diseases. The conjugation machinery for SUMOylation consists of a number of proteins which are redox sensitive. Here, under oxidative stress (100 μM hydrogen peroxide), antioxidant (100 μM ascorbate) or control conditions 169 proteins were identified by electospray ionisation fourier transform ion cyclotron resonance mass spectrometry. The majority of these proteins (70%) were found to contain SUMOylation consensus sequences. From the remaining proteins a small number (12%) were found to contain possible SUMO interacting motifs. The proteins identified included DNA and RNA binding proteins, structural proteins and proteasomal proteins. Several of the proteins identified under oxidative stress conditions had previously been identified as SUMOylated proteins, thus validating the method presented. [BMB reports 2010; 43(11): 720-725]

INTRODUCTION

Dynamic post-translational regulation of protein function can occur through a combination of a variety of reversible modifications. SUMOylation is a versatile regulatory mechanism of protein function that can confer an alteration in function, location and/or half-life. Small ubiquitin-like modifier (SUMO) is a member of the ubiquitin-like protein superfamily. There are, to date 4 isoforms, found in human cells: SUMO-1, -2, -3 and -4. SUMO-1 is an 11kDa polypeptide that shares only 18% sequence homology with ubiquitin. However, NMR studies revealed the characteristic ββαββαβ ubiquitin fold and a nearly overlapping tertiary structure (1). The most prominent difference between the SUMO family and ubiqutin is a flexible N terminal extension with unknown function, though it has been suggested that it may confer specific protein/protein interactions. SUMO-1 shares 48% homology with SUMO-2 and 46% homology with SUMO-3, whereas SUMO-4 has 86% sequence homology with SUMO-2, but phylogenic analysis revealed that SUMO-4 has highest functional property homology with SUMO-1 (2). However, very little is known about the functional differences between SUMO-1, -2, -3 and -4.

The SUMO family proteins are attached to target proteins by a series of conjugating enzymes similar to those found in the ubiquitin conjugation system. Like ubiquitin they are synthesized as inactive precursors, and first, Ulp-1, a protease cleaves the C terminal of the SUMO polypeptide to reveal a di-glycine motif that is essential for the conjugation of SUMO to the target protein (1). Then the C-terminal glycine binds to the E1 activating enzyme complex (Aos1/Uba2) via thioester linkage. This is then passed onto the E2 conjugating enzyme (Ubc9) which catalyses the transfer of the SUMO polypeptide on to a lysine on the target protein. The E3 ligase facilitates this transfer. There are number of E3 ligases and, if the similarities with the ubiqutin system persist, many of these will also have a structural role in presentation of the target and Ubc9. The reversibility of the conjugation is given by the isopeptidases (SenP family) that can cleave SUMO from the target protein. The specificity for the lysine that becomes SUMOylated is often promoted by a consensus binding motif that determines the interaction of the target with Ubc 9 (3). The consensus sequence (ΨKXE) consists of a hydrophobic amino acid (Ψ preferably leucine, isoleucine or valine), a lysine (K), any amino acid (X) and a glutamic acid (E). SUMO-2, -3 and -4 contain an internal consensus binding motif and so can be SUMOylated themselves making it possible for SUMO chains. However, SUMO-1 does not contain the consensus binding sequence and thus conjugation of SUMO-1 to a target protein will result in mono-SUMOylation or the termination of a chain.

Protein function can be modulated by the addition of a SUMO protein, but also by interaction with a SUMOylated protein. These downstream interacting effector proteins may have a SUMO interacting motif (SIM). These motifs have a consensus sequence that consists of a hydrophobic core sequence V/I-V/I-X-V/I, where X equates often to an acidic residue that is often associated with a stretch of acidic residues flanking either its N or C terminus (4). For example, SIM containing protein Srs2 interacts with SUMOylated PCNA to pre-

*Corresponding author. Tel: 0121-237-2884; Fax: 0121-237-2882; E-mail: melissa_grant@hotmail.com, m.m.grant@bham.ac.uk
DOI 10.5483/BMBRep.2010.43.11.720

Received 15 April 2010, Accepted 1 September 2010

Keywords: Oxidative stress, Proteomics, SIM, SUMOylation

vent unwanted recombination events in replicating chromosomes (5).

Neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, have common features of aggregation or accumulation of proteins and these are thought to play a key role in disease pathogenesis (6). SUMO proteins have been co-localised to neurones containing protein aggregates (7). It has been hypothesized that: the SIMs may help in the aggregation; that SUMOylation may compete for proteasomal degradation by using the same lysine residues as those for ubiquitin mediated degradation; or that SUMOylation of molecular chaperones may play a role in neurodegenerative disease pathologies.

Redox effects on the conjugation of a SUMO group could tip the balance of signalling in favour of a particular outcome. Particularly, high concentrations of hydrogen peroxide (100 mM) increase SUMOylation (8), probably via inhibition of the isopeptidases that reverse SUMOylation. While at concentrations lower than 1mM hydrogen peroxide cause formation of a disulphide bridge between Uba2 and Ubc9, contain active site cysteines, and which are sensitive to the redox environment of the cell (9). Furthermore very low concentrations of hydrogen peroxide (10 μM) have minimal effects of the conjugation machinery and changes to the SUMO proteome are mostly likely to alter signalling cascades (9), for instance through redox sensitive phosphatases. In particular, both oxidants, such as hydrogen peroxide (H_2O_2), and antioxidants have been shown to have cellular roles as second messengers, passing signals from the outside of the cell to the nucleus and via transcription activation elicit downstream changes in the proteome (10). Previously, Grant *et al*. (2005) (11) showed that both ascorbate (100 μM) and hydrogen peroxide (100 μM) can modulate the total and oxidised proteomes of the neuroblastoma SH-SY5Y cell line, a cell line previously used to study oxidative stress neurodegeneration models. Numerous transcription factors can be SUMOylated and due to the redox sensitive nature of the SUMOylation machinery (9) it is hypothesized that these chemicals could mediate cellular changes by altering SUMOyaltion patterns. Thus, this paper identifies proteins that are SUMOylated in response to the oxidant hydrogen peroxide or the antioxidant ascorbate in neuroblastoma cells by electospray ionisation fourier transform ion cyclotron resonance mass spectrometry (ESI-FT ICR MS).

RESULTS

To assess SUMOyaltion in SH-SY5Y cells, cells were treated with PBS, ascorbate (100 μM) or hydrogen peroxide (100 μM). Proteins were vizualised by Coomassie Blue staining and proteins between 25-55 kDa were excised from each lane (Fig. 1). Mass spectral analysis identified 97 proteins from the PBS

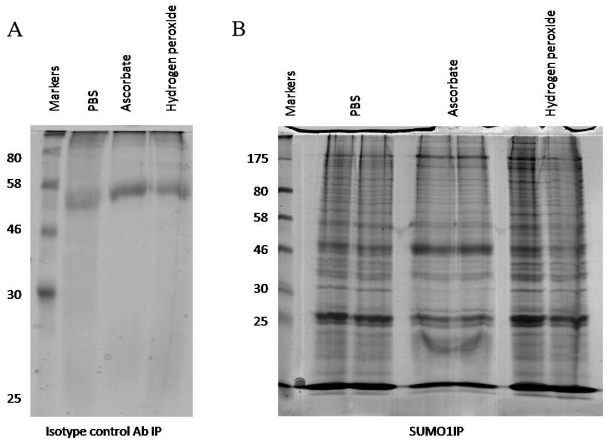

Fig. 1. Coomassie blue stained SDS-PAGE gels. (A) Immunoprecipitation of SY5Y neuroblastoma cell extracts by isotype control IgG antibody. (B) Immunoprecipitation of SY5Y neuroblastoma cell extracts by SUMO1 antibody. Cells were treated with PBS, ascorbate (100 μM) or hydrogen peroxide (100 μM) for 30 min before extraction.

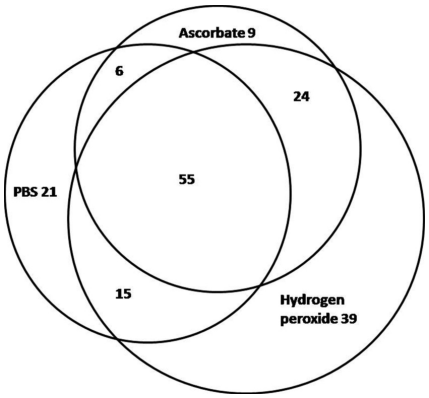

Fig. 2. Venn diagram showing the overlap of proteins found from cells treated with either PBS, ascorbate (100 μM) or hydrogen peroxide (100 μM).

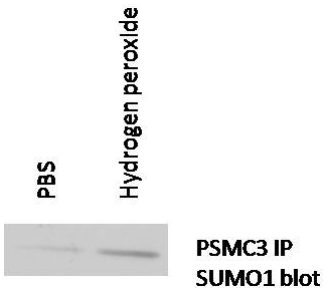

Fig. 3. Western blot of SY5Y neuroblastoma cell extracts immunoprecipitated by PSMC3 antibody and detected by SUMO1 antibody. Cells were treated with PBS or hydrogen peroxide (100 μM) for 30 min before extraction.

Table 1. Proteins identified by mass spectrometry without predicted SUMOylation site, showing accession number, number of peptides identified, sequence coverage, presence of predicted SIM sites and sequence of predicted SIM site

Protein name	Accession number	Mass	Number of peptides identified	Sequence coverage (%)	Potential SIM	SIM sequence
Proteins identified in PBS control, ascorabte and hydrogen peroxide treatments						
Actin, cytoplasmic 1	P60709	42052	9	44	Yes	^{248}VITI
Beta-actin-like protein 2	Q562R1	42318	5	15	Yes	^{248}VITI
Actin, alpha cardiac muscle 1	P68032	42334	6	29	Yes	^{249}VITI
Guanine nucleotide-binding protein subunit alpha-12	Q03113	44422	1	2	No	
Histone H2A type 1-B/E	P04908	14127	2	21	No	
Nascent polypeptide-associated complex subunit alpha-2	Q9H009	23209	1	6	No	
Poly(rC)-binding protein 1	Q15365	37987	2	6	No	
Poly(rC)-binding protein 2	Q15366	38955	3	10	No	
Protein disulfide-isomerase A6	Q15084	48490	2	6	No	
60S ribosomal protein L4	P36578	47953	8	31	No	
Tubulin alpha-1A chain	Q71U36	50788	8	28	No	
Tubulin alpha-1B chain	P68363	50804	8	31	No	
Putative tubulin-like protein alpha-4B	Q9H853	27819	2	10	No	
Tubulin beta-2B chain	Q9BVA1	50377	8	65	No	
Tubulin beta-2C chain	P68371	50255	8	68	No	
Tubulin beta-3 chain	Q13509	50856	10	47	No	
Tubulin beta chain	P07437	50095	8	68	No	
Tubulin beta-6 chain	Q9BUF5	50281	5	19	No	
Tubulin beta-8 chain	Q3ZCM7	50257	5	24	No	
Ubiquitin	P62988	8560	3	61	No	
Proteins identified in PBS control and ascorabte treatments						
Urocanate hydratase	Q96N76	75581	1	1	No	
Adenosylhomocysteinase	P23526	48255	6	14	No	
Proteins identified in PBS control and hydrogen peroxide treatments						
Histone H3-like	Q6NXT2	15318	2	11	No	
Regulator of chromosome condensation	P18754	45397	1	6	No	
Heterogeneous nuclear ribonucleoprotein A0	Q13151	30993	1	6	No	
Aspartyl-tRNA synthetase, cytoplasmic	P14868	57499	3	7	Yes	^{113}IVDV, ^{184}VIDL
T-complex protein 1 subunit beta	P78371	57794	2	6	No	
Proteins identified in ascorabte and hydrogen peroxide treatments						
Mitotic checkpoint protein BUB3	O43684	37587	3	10	No	
DNA polymerase subunit gamma-2, mitochondrial	Q9UHN1	55503	4	10	No	
RNA-binding motif protein, X-linked-like-2	O75526	42929	3	7	No	
Heterogeneous nuclear ribonucleoprotein H3	P31942	36960	2	19	No	
Tubulin alpha-3E chain	Q6PEY2	50626	7	27	No	
Tubulin beta-1 chain	Q9H4B7	50865	4	13	No	
Tubulin beta-4 chain	P04350	50010	10	59	No	
Proteins identified in PBS control						
Histone H4	P62805	11360	2	19	No	
Delta-1-pyrroline-5-carboxylate synthetase	P54886	87989	2	2	Yes	^{99}VEQV, ^{262}LIVL, ^{639}VEQV
26S protease regulatory subunit S10B	P62333	44430	1	3	No	
Tubulin beta-2A chain	Q13885	50274	7	66	No	
Trem-like transcript 2 protein	Q5T2D2	35503	1	3	No	
Exportin-2	P55060	111145	2	1	No	
Proteins identified in ascorabte treatment						
Probable D-tyrosyl-tRNA(Tyr) deacylase 2	Q96FN9	18877	1	5	No	

Table 1. Continued

Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase B	Q3V5L5	90732	1	1	No	
Proteins identified in hydrogen peroxide treatment						
Actin-related protein 3B	Q9P1U1	48090	1	2	No	
Fascin	Q16658	55123	4	12	No	
Hyaluronan synthase 1	Q92839	65645	1	1	No	
Septin-2	Q15019	41689	3	11	No	
Sortilin	Q99523	92979	2	2	Yes	^{123}VILV, ^{775}VLIV
Tubulin alpha-1C chain	Q9BQE3	50548	8	31	No	
Transformer-2 protein homolog beta	P62995	33760	2	10	No	

treated sample, 94 proteins from the ascorbate treated sample and 133 from the hydrogen peroxide treated sample. Supplemental Table 1 and Fig. 2 shows there was a great overlap between all treatments (55 proteins). Predicted SUMOylation sites were determined and this revealed that approximately 70% (120 out of 169) of all the proteins identified contained a consensus sequence for SUMOylation. The 26S protease regulatory subunit 6A (PSMC3) that was identified in hydrogen peroxide treatment alone was verified using immunoprecipitation of PSMC3 and detection with SUMO1 antibody by Western blotting (Fig. 3). It was not possible to identify the protein in whole cell lysates.

Proteins identified that did not contain a SUMOylation consensus sequence (49 out of 169, Table 1) may have co-immunoprecipitated with SUMOylated proteins or could contain a SIM. Proteins not containing a SUMOylation consensus sequence were searched for known SIM sequences. This identified six proteins with one or more potential SIM sequences (Table 1).

DISCUSSION

In this study 169 proteins were identified, and of these 120 were predicted to have SUMO consensus sequences. Although an approach using large numbers of cells was used instead of a transfected tagging system these numbers are comparable to previous proteomic studies of mammalian cell lines (HEK293: 122 protein identified (12); HeLa cells: 53 proteins identified (13)) and in yeast cells (141 proteins identified (14); 271 proteins identified (15)). However, here only a fraction of the total cell lysate was analysed (25-55 kDa) and thus many more proteins have potentially not been identified. The use of highly sensitive and accurate FT-ICR mass spectrometry may have aided in the detection of so many proteins found here. The use of a quantitative technique, such as iTRAQ or SILAC, will also aid in future comparative studies.

Some proteins detected by mass spectrometry and that were subsequently examined for SUMOylation consensus sequence did not contain one. These proteins could either have been co-immunopreciptated with other proteins that were SUMOylated or perhaps contain SUMO interacting motifs (SIMs). Co-immunoprecipitating proteins are a common problem with immunoprecipitation protocols (16), and as seen here homologous proteins of the same family (eg heterogeneous nuclear ribonucleoproteins (hnRNPs), with and without SUMOyaltion consensus sequences could be precipitated from complexes that are not disrupted by the isolation techniques employed. Although the hnRNPs are some of the most abundant RNA-binding proteins (17), as found previously (18) many of the proteins found here are involved in RNA metabolism and thus it may not be helpful to simply discard any proteins without SUMO consensus sequences.

The functions of SUMOylation are often mediated by downstream effector proteins (19) containing SIMs. These motifs are composed of a hydrophobic core and a stretch of acidic residues at either the N or C terminus (20) and confer a region that can bind SUMO (21). Rudimentary analysis of sequences from proteins without SUMO consensus sequences for SIM consensus sequences identified three actin species, aspartyl-tRNA synthetase, delta-1-pyrroline-5-carboxylate synthetase and sortilin as proteins with potential SIMs. However, this would have to be confirmed by further analysis.

The proteins found can broadly be assigned to: DNA associated proteins, such as histones, and transcription factors; RNA associated proteins, such as the heterogeneous nuclear ribonucleoproteins and RNA helicases; structural proteins, such as actin and tubulin; and proteasomal proteins. As would be expected the majority of the proteins are nuclear proteins and for the greater part these proteins have already been identified or proteins with similar functions have already been identified in screens for SUMOylated proteins (12, 13, 22). Cells were treated with hydrogen peroxide, inducing oxidative stress, as evidenced by the presence of topoisomerase II in extracts of these cells. This protein has previously been identified as a SUMOylation target that might be involved in DNA repair (23). The larger number of proteins seen in the hydrogen peroxide treatment (133) compared with the PBS control (97) or the ascorbate treatment (94) indicate that at the concentration used, Uba2 and Ubc9 are not inactivated, in contrast to results of Bossis & Melchior, 2006 (9). This may be due to differences in cell density or volumes of media between the current study and that of Bossis & Melchior (9).

Upon examining the proteins that were identified by the different treatments employed in this study, the PBS and ascorbate treatments revealed very similar groups. SUMOylated proteins that were present in hydrogen peroxide or ascorbate

treated cells that stood out were the proteasomal proteins. Of particular interest is the protein 26S proteasome regulatory subunit 6A, which is also known as TBP-1 or PSMC3 which was found in hydrogen peroxide treated cells only. Western blotting verified PSMC3 as SUMOylated in hydrogen peroxide treated cells, but also a small amount was found in untreated cells. Jakobs *et al*. (2007) (22, 24) have already demonstrated that this protein can be SUMOylated in a plasmid directed Ubc9 fusion dependent SUMOylation system. However, this may be the first time that it has been isolated as SUMOylated by cellular machinery. It is of particular interest because, although TBP-1/PSMC3 has been identified as part of the proteasomal equipment, it is also thought to have other functions such as control of transcription. Interaction of TBP-1/PSMC3 with oxidative stress induced transcription factor $p19^{ARF}$ causes stabilisation of $p19^{ARF}$ which may assist with check point responses to oxidative stress (25). Other proteasomal proteins were associated with ascorbate or hydrogen peroxide treatments, whether these too have further functions than protein degradation in regards to SUMOylation remains to be determined.

In conclusion, many proteins have been identified here from the SH-SY5Y neuroblastoma cell line in response to antioxidant or oxidative challenges. These have included proteins containing SUMOylation consensus sequences and potential interacting proteins either through complexes or SIMs. The sensitivity of the instrumentation used represents a way forward to finding novel SUMO targeted proteins in untransfected cells.

MATERIALS AND METHODS

Cell culture

Neuroblastoma cells (SH-SY5Y) were cultured using RPMI 1640 with Glutamax I (Gibco BRL, Grand Island, NY, USA) supplemented with fetal bovine sera (15%; Gibco) and non-essential amino acid solution (1%; Sigma, St. Louis, MO, USA).

Protein isolation and mass spectral analysis

For protein isolation, SH-SY5Y cells (10×10^6) were treated with PBS, ascorbate (100 μM) or hydrogen peroxide (100 μM) for 30 min and then extracted into ice cold RIPA buffer containing protease inhibitor cocktail (Complete mini, Roche). Cell experiments were performed three times and extracts were pooled prior to immunoprecipitation using a mouse mAb directed against human SUMO-1 (Abgent, Cambridge, UK; 4°C, 1 h) or mouse isotype IgG control antibody (Sigma) coupled to Protein G Dynabeads (Invitrogen, Paisley UK). After extensive washing immunoprecipitated proteins were separated by SDS-PAGE and proteins were stained with Imperial Coomassie Blue stain (Thermofisher, UK). Gel (between 25-55 kDa) was excised, tryptically digested and peptides extracted (26). On- line liquid chromatography was performed by use of a C_8 column and separated over a 60-min water: acetonitrile gradient. Peptides were eluted directly into a Thermo Finnegan LTQ FT mass spectrometer. Collision induced dissociation was performed with helium gas, as previously described (27). Data were analysed using Mascot software (version 2.2.04) and the SwissProt (version 56.3) human database, with a mass tolerance of 0.5 kDa and cysteine carbamidomethylation; glutamine or asparagine deamidation; methionine oxidation; serine, threonine or tryrosine phosphorylation as possible modifications.

Immunoprecipitation and Western blotting of PSMC3

Proteins were isolated as described above using PSMC3 antibody (Abcam, UK) and separated by SDS-PAGE. Gels were electroblotted to PVDF and proteins were detected with mouse mAb directed against human SUMO-1 (Abgent, Cambridge, UK) and horse radish peroxidise secondary antibody. Protein bands were visualised using chemiluminescence (Supersignal West Pico, Pierce, UK).

Data analysis

Venn diagram was drawn using EulerVennApplet http://www.cs.kent.ac.uk/people/staff/pjr/EulerVennCircles/EulerVennApplet.html, (28). SUMOylation consensus sequence site prediction was accomplished using SUMOsp 2.0 (29). Potential SIMs were determined by searching FASTA sequences of proteins without SUMOylation consensus sequences for known SIM sequences (5).

Acknowledgements

This study was made possible by funding from the Biochemical Society's Guildford Bench Methodology Fund.

REFERENCES

1. Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R. and Becker, J. (1998) Structure determination of the small ubiquitin-related modifier SUMO-1. *J. Mol. Biol.* **280**, 275-286.
2. Li, M., Guo, D., Isales, C. M., Eizirik, D. L., Atkinson, M., She, J. X. and Wang, C. Y. (2005) SUMO wrestling with type 1 diabetes. *J. Mol. Med.* **83**, 504-513.
3. Johnson, E. S. (2004) Protein modification by SUMO. *Annu. Rev. Biochem.* **73**, 355-382.
4. Sekiyama, N., Ikegami, T., Yamane, T., Ikeguchi, M., Uchimura, Y., Baba, D., Ariyoshi, M., Tochio, H., Saitoh, H. and Shirakawa, M. (2008) Structure of the small ubiquitin-like modifier (SUMO)-interacting motif of MBD1-containing chromatin-associated factor 1 bound to SUMO-3. *J. Biol. Chem.* **283**, 35966-35975.
5. Pfander, B., Moldovan, G. L., Sacher, M., Hoege, C. and Jentsch, S. (2005) SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature* **436**, 428-433.
6. Dorval, V. and Fraser, P. E. (2007) SUMO on the road to neurodegeneration. *Biochim. Biophys. Acta.* **1773**, 694-706.

7. Terashima, T., Kawai, H., Fujitani, M., Maeda, K. and Yasuda, H. (2002) SUMO-1 colocalized with mutant atrophin-1 with expanded polyglutamines accelerates intranuclear aggregation and cell death. *Neuroreport*. **13**, 2359- 2364.
8. Saitoh, H. and Hinchey, J. (2000) Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *J. Biol. Chem.* **275**, 6252-6258.
9. Bossis, G. and Melchior, F. (2006) Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol. Cell* **21**, 349-357.
10. Paulsen, C. E. and Carroll, K. S. (2010) Orchestrating redox signaling networks through regulatory cysteine switches. *ACS. Chem. Biol.* **5**, 47-62.
11. Grant, M. M., Barber, V. S. and Griffiths, H. R. (2005) The presence of ascorbate induces expression of brain derived neurotrophic factor in SH-SY5Y neuroblastoma cells after peroxide insult, which is associated with increased survival. *Proteomics*. **5**, 534-540.
12. Rosas-Acosta, G., Russell, W. K., Deyrieux, A., Russell, D. H. and Wilson, V. G. (2005) A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers. *Mol. Cell Proteomics*. **4**, 56-72.
13. Vertegaal, A. C., Andersen, J. S., Ogg, S. C., Hay, R. T., Mann, M. and Lamond, A. I. (2006) Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. *Mol. Cell Proteomics*. **5**, 2298-2310.
14. Hannich, J. T., Lewis, A., Kroetz, M. B., Li, S. J., Heide, H., Emili, A. and Hochstrasser, M. (2005) Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. *J. Biol. Chem.* **280**, 4102-4110.
15. Wohlschlegel, J. A., Johnson, E. S., Reed, S. I. and Yates, J. R. 3rd. (2004) Global analysis of protein sumoylation in Saccharomyces cerevisiae. *J. Biol. Chem.* **279**, 45662-45668.
16. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Séraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**, 1030-1032.
17. He, Y. and Smith, R. (2009) Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B. *Cell Mol. Life Sci.* **66**, 1239-1256.
18. Hay, R. T. (2005) SUMO: a history of modification. *Mol. Cell* **18**, 1-12.
19. Perry, J. J., Tainer, J. A. and Boddy, M. N. (2008) A SIM-ultaneous role for SUMO and ubiquitin. *Trends Biochem. Sci.* **33**, 201-208.
20. Hecker, C., Rabiller, M., Haglund, K., Bayer, P. and Dikic, I. (2006) Specification of SUMO1- and SUMO2-interacting motifs. *J. Biol. Chem.* **281**, 16117-16127.
21. Song, J., Zhang, Z., Hu, W. and Chen, Y. (2005) Small ubiquitin-like modifier (SUMO) recognition of a SUMO binding motif: a reversal of the bound orientation. *J. Biol. Chem.* **280**, 40122-40129.
22. Jakobs, A., Koehnke, J., Himstedt, F., Funk, M., Korn, B., Gaestel, M. and Niedenthal, R. (2007) Ubc9 fusion- directed SUMOylation (UFDS): a method to analyze function of protein SUMOylation. *Nat. Methods*. **4**, 245-250.
23. Gutierrez, G. J. and Ronai, Z. (2006) Ubiquitin and SUMO systems in the regulation of mitotic checkpoints. *Trends Biochem. Sci.* **31**, 324-332.
24. Jakobs, A., Himstedt, F., Funk, M., Korn, B., Gaestel, M. and Niedenthal, R. (2007) Ubc9 fusion-directed SUMOylation identifies constitutive and inducible SUMOylation. *Nucleic. Acids. Res.* **35**, e109.
25. Pollice, A., Vivo, M. and La Mantia, G. (2008) The promiscuity of ARF interactions with the proteasome. *FEBS Lett.* **582**, 3257-3262.
26. Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S. and Mische, S. M. (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20**, 601-605.
27. Creese, A. and Cooper, H. J. (2007) Liquid chromatography electron capture dissociation tandem mass spectrometry (LC-ECD-MS/MS) versus liquid chromatography collision-induced dissociation tandem mass spectrometry (LC-CID-MS/MS) for the identification of proteins. *J. Am. Soc. Mass. Spectrom.* **18**, 891-897.
28. Chow, S. and Ruskey, F. (2004) Drawing area-proportional Venn and Euler diagrams. *Proc. of Graph. Drawing* **2912**, 466-477.
29. Ren, J., Gao, X., Jin, C., Zhu, M., Wang, X., Shaw, A., Wen, L., Yao, X. and Xue, Y. (2009) Systematic study of protein sumoylation: development of a site-specific predictor of SUMOsp 2.0. *Proteomics*. **9**, 3409-3412.