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Research Article

PRMT1-mediated arginine methylation controls ATXN2L localization

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ABSTRACT

Arginine methylation is a posttranslational modification that is of importance in diverse cellular processes. Recent proteomic mass spectrometry studies reported arginine methylation of ataxin-2-like (ATXN2L), the paralog of ataxin-2, a protein that is implicated in the neurodegenerative disorder spinocerebellar ataxia type 2. Here, we investigated the methylation state of ATXN2L and its significance for ATXN2L localization. We first confirmed that ATXN2L is asymmetrically dimethylated *in vivo*, and observed that the nuclear localization of ATXN2L is altered under methylation inhibition. We further discovered that ATXN2L associates with the protein arginine-N-methyltransferase 1 (PRMT1). Finally, we showed that neither mutation of the arginine–glycine-rich motifs of ATXN2L nor methylation inhibition alters ATXN2L localization to stress granules, suggesting that methylation of ATXN2L is probably not mandatory.

Introduction

Posttranslational modifications (PTMs) provide a basis for the structural and functional diversity of the proteome and are important for the regulation of essential cellular processes by modulating protein localization, protein–protein interactions, and biochemical properties, hence, a protein's function. Arginine methylation is an abundant and evolutionary conserved PTM that plays a key role in signal transduction, chromatin structure and epigenetic regulation, DNA damage repair, transcriptional regulation, as well as in diverse steps of the cellular mRNA metabolism, such as splicing, translation, and nucleocytoplasmic transport [1–3]. In view of that, proteomic approaches revealed that RNA-binding proteins represent main targets for arginine methylation [4,5], which commonly takes place on arginines in the arginine–glycine-rich (RGG) domains of these proteins; the relevant arginines are found to be monomethylated or dimethylated in a symmetric or asymmetric manner [1]. These modifications are catalyzed by a family of protein arginine-N-methyltransferases (PRMTs) that comprises nine members in mammals [1,4]. Depending on the modification they catalyze, PRMTs have been classified into different types, of which the majority belongs to type I and type II. Type I PRMTs catalyze monomethylation and asymmetric dimethylation, whereas type II PRMTs catalyze monomethylation and symmetric dimethylation. Notably, the activity of a single type I PRMT, PRMT1, is accountable for 90% of all cellular methylation events in mammalian cells [1,6]. Of note, *Prmt1* null mice are embryonic lethal, indicating an important role of PRMT1 in early development [7].

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Over the last years a number of studies demonstrated that arginine methylation affects the cellular localization, protein–protein interactions as well as RNA-binding properties of RNA-binding proteins. In this context, methylation of the TATA-binding protein-associated factor 15 (TAF15) is substantial for proper nuclear localization and its gene regulatory function [8]. The methylation state of the alternative splicing factor (ASF/SF2) determines its nuclear localization and splicing activities [9]. Mutations within ASF/SF2 that block methylation are accountable for its cytoplasmic accumulation, which results in splicing defects, increased translation and effects on the nonsense-mediated mRNA decay pathway [9]. Additionally, the importance of arginine methylation for the subcellular localization of the serine–arginine-rich splicing factor 9 (SFRS9/SRp30c) has been demonstrated [10]. Moreover, the nuclear import of RNA helicase A is dependent on arginine methylation [11]. The interaction between the survival motor neuron (SMN) protein and the small nuclear ribonucleoproteins SmD1 and SmD3 is stronger if these proteins are methylated [12]. Arginine methylation of the nuclear poly(A)-binding protein 1 (PABPN1) weakens the interaction with transportin, thereby affecting the transportin-mediated nuclear import of PABPN1. Interestingly, methylation of PABPN1 on the other hand increases its binding to RNA [13]. In this regard, methylation of the cellular nucleic acid-binding protein (CNBP) interferes with its RNA-binding activity, while its subcellular localization is not affected [14]. RNA-binding of the RNA-export factor (REF/ALY) is reduced by arginine methylation, whereas the interaction between REF/ALY and the TIP-associated protein/ nuclear RNA-export factor 1 (TAP/NXF1) is not altered [15]. The ability of the SRC-associated in mitosis 68 protein (SAM68) to bind to poly(U) sequences is weakened if methylated, and protein–protein interactions are also modulated in a specific and selective manner [16]. The dimerization of the fragile X mental retardation protein (FMRP) and its paralog, the fragile X mental retardation-related protein 1 (FXR1), is affected by the methylation state of FXR1, and binding of FMRP to mRNA is also modulated by methylation [17].

Eukaryotic cells have evolved various response mechanisms against environmental stresses on different cellular levels. In regard to mRNA metabolism, cells respond to stress with the formation of stress granules (SGs). These are ribonucleoparticles that assemble in response to various stressful conditions, such as oxidative stress, heat or osmotic stress, to store and regulate untranslated mRNA molecules under such unfavorable conditions [18]. They therefore contain translationally stored 48 S pre-initiation complexes, a number of RNA-binding proteins and proteins implicated in cell signaling [18,19]. The process of SG formation is classified by different stages. Posttranslational modifications, *e.g.* phosphorylation and glycosylation, of several SG components that are involved in the primary nucleation step play a significant role in the regulation of SG assembly/disassembly and function [20]. Moreover, several RNA-binding proteins, representing SG components, have been found to be asymmetrically and symmetrically dimethylated, suggesting that arginine modification is important in the process of SG formation as well [21]. The recruitment of FMRP to SGs is reduced under methylation inhibition [17]. Furthermore, methylation of the cold-inducible RNA-binding protein (CIRP) is obligatory for its localization to SGs [22]. On the other hand, methylation does not seem to be mandatory for recruitment of the protein fused in sarcoma (FUS) into SGs [23].

In our earlier study we report that the protein ataxin-2-like (ATXN2L), the paralog of ataxin-2 (ATXN2), which is implicated in the neurodegenerative disorder spinocerebellar ataxia type 2, is a component of SGs and further plays a role in the regulation of SG formation [24]. In addition to its potential role in the cellular mRNA metabolism, ATXN2L might function in cytokine signaling, since ATXN2L associates with the erythropoietin receptor myeloproliferative leukemia protein (MPL) [25]. Since recent proteomic mass spectrometry approaches identified ATXN2L as being arginine methylated [26–28], we investigated in this study whether methylation of ATXN2L might be important for its localization. First, we showed that ATXN2L is asymmetrically dimethylated exploiting specific anti-dimethyl arginine antibodies. We observed that the nuclear localization of ATXN2L is altered under methylation inhibiting cellular conditions. Furthermore, we showed that ATXN2L associates with PRMT1. Finally, we investigated whether arginine methylation within the RGG motifs is essential for ATXN2L localization to SGs, and concluded from our findings that methylation of ATXN2L is likely not mandatory.

Material and methods

Plasmids

The plasmids pCMV-MYC-ATXN2L-WT, pCMV-MYC-ATXN2L-R361Q, pCMV-MYC-ATXN2L-R370Q and pCMV-MYC-ATXN2L-R361Q/R370Q were constructed in a two-step reaction. First, DNA fragments encoding the N-terminal region of ATXN2L were PCR amplified using plasmid RSV-ATXN2L-MYC [24,25] as template and primer pair ATXN2L-*Eco*RI-fw (5′-GC GAATTCAG TTG AAG CCT CAG CCG CTA CAA CAG-3′; *Eco*RI restriction site underlined) and ATXN2L-int-R361R-rev (5′-GCT GCA TCG AAC TCC TCC CCG GGG ACC-3′) or ATXN2L-*Eco*RI-fw and ATXN2L-int-R361Q-rev (5′-GCT GCA TCG AAC TCC TCC CTG GGG ACC-3′; modified triplets underlined). DNA fragments encoding the C-terminal region of ATXN2L were amplified using the primer pair ATXN2L-int-R370R-fw (5′-GTT CGA TGC AGC AGC TCT CGG GGC GGT-3′) and ATXN2L-*Sal*I-rev (5′-GTC GTCGAC TTA TTG GGG GTG TCC GAT GTA GGG GTA-3′; *Sal*I restriction site underlined) or ATXN2L-int-R370Q-fw (5′-GTT CGA TGC AGC AGC TCT CAG GGC GGT-3′; modified triplets underlined) and ATXN2L-*Sal*I-rev. In a second step the primers ATXN2L-*Eco*RI-fw and ATXN2L-*Sal*I-rev were used to amplify the complete ATXN2L sequence using the overlapping fragments as DNA templates. Afterward, the amplified DNA fragments were purified, treated with *Sal*I and *Eco*RI, subcloned into the *Xho*I/*Eco*RI sites of the expression vector pCMV-MYC (Clontech), and validated by sequencing.

For the generation of yeast-2-hybrid (Y2H) bait constructs encoding LexA-ATXN2L-LSm, LexA-ATXN2L-LSm+RR and LexA-ATXN2L-LSm+QQ, DNA fragments were amplified using pCMV-MYC-ATXN2L-WT or pCMV-MYC-ATXN2L-R361Q/R370Q as template and the primer pair ATXN2L-LSm-*Sal*I-fw (5′-GC GTCGACG TCC AGA ATG CTG CAT-3′; *Sal*I restriction site underlined) and ATXN2L-LSm-*Not*I-rev (5′-TTA GCGGCCGCA TTA CTG CCG CTG GAC T-3′; *Not*I restriction site underlined) or ATXN2L-LSm-*Sal*I-fw and ATXN2L-LSm+methyl-sites-*Not*I-rev (5′-TTA GCGGCCGCA TTA GCT GTT GTC CAG ATG-3′; *Not*I restriction site underlined).

The purified DNA fragments were subcloned into the *Sal*I/*Not*I sites of pBTM117c and validated by sequencing.

To generate plasmid pTL-FLAG-PRMT1-WT, the complete coding sequence of PRMT1 (ENSEMBLE-ID: ENSP00000375724) was amplified using cDNA from HeLa cells and primer pair PRMT1-*Sal*I-fw (5′-CTAGATTTA GTCGACT GCG GCA GCC GA-3′; *Sal*I restriction site underlined) and PRMT1-*Not*I-rev (5′-TTA GCGGCCGCA TCA GCG CAT CCG GTA-3′; *Not*I restriction site underlined). The amplified DNA fragment was purified, treated with *Sal*I and *Not*I and subcloned into the *Xho*I/*Not*I sites of the expression vector pTL-FLAGc. For the generation of the respective Y2H bait and prey constructs, the DNA fragment was subcloned into the *Sal*I/*Not*I sites of the vector pBTM117c or pACT4-1b, and validated by sequencing. To generate an enzymatically inactive PRMT1 (VLD to AAA at position 74–76) as described [29,30], plasmid pTL-FLAG-PRMT1-WT was used as template. A DNA fragment encoding the N-terminal region of PRMT1 was amplified using the primer pair PRMT1-*Sal*I-fw and PRMT1-AAA-rev (5′-GAC GGC CGC CGC CAC CTT GTC CTT G-3′; modified triplets underlined). The fragment coding for the C-terminal region of PRMT1 was generated using the primer pair PRMT1-AAA-fw (5′-C AAG GAC AAG GTG GCG GCG GCC GTC-3′; modified triplets underlined) and PRMT1-*Not*I-rev. The complete PRMT1 sequence was amplified using the overlapping DNA fragments as templates and the primer pair PRMT1-*Sal*I-fw and PRMT1-*Not*I-rev. The amplified DNA fragment was purified, treated with *Sal*I and *Not*I, subcloned into the *Xho*I/*Not*I sites of the expression vector pTL-FLAGc, and validated by sequencing.

Cell culture, transfection, RNA interference and stress induction

HeLa cells were cultivated at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Biochrom) and 1% penicillin/streptomycin (Biochrom). Cells were grown in 24-well-plates for 24 h. For inhibition of methyltransferase activity, cells were then treated with adenosine-2,3-dialdehyde (AdOx, Sigma) or DMSO as control for 24 h.

Plasmid transfections were carried out in 0.5 ml DMEM supplemented with 10% FBS and 1% penicillin/streptomycin using 0.5 μg of plasmids pCMV-MYC-ATXN2L-WT, pCMV-MYC-ATXN2L-R361Q, pCMV-MYC-ATXN2L-R370Q, pCMV-MYC-ATXN2L-R361Q/R370Q, pTL-FLAG-PRMT1-WT or pTL-FLAG-PRMT1-inactive and 1.67 μl polyethylenimine (PEI, linear, MW 25 kDa, 1 mg/ml, pH 7, Polysciences Inc.) per well. Transfected cells were grown for 48 h to allow transient expression of proteins. For inhibiting the general methyltransferase activity, cells were treated with AdOx for 24 h and then transfected with the respective plasmids. To allow expression of proteins, cells were incubated for additional 24 h.

RNA interference experiments were performed in 0.5 ml DMEM supplemented with 10% FBS using 0.6 μl 20 μM siRNA molecules [PRMT1 On Target Plus Smart Pool, On Target Plus Non-targeting Pool (Dharmacon)] and 1.5 μl Lipofectamine RNAiMax reagent (Life Technologies) per well. Cells were then incubated for 72 h.

For induction of oxidative stress, cells were treated with 0.5 mM sodium arsenite (Merck) at 37 °C for 1 h. To apply heat stress, cells were incubated at 44 °C in a water bath for 30 min.

Immunofluorescence

For immunofluorescence experiments HeLa cells were plated on glass coverslips in 24-well plates and treated as described above. Then, cells were washed with phosphate buffered saline (PBS), fixed with 2% formaldehyde (Polysciences, Inc.) for 10 min and ice-cold methanol for 30 min. Next, cells were washed with PBS and blocked with 3% bovine serum albumin (BSA, Sigma) in PBS for 30 min. Cells were then incubated with the respective primary antibodies in 3% BSA/PBS for 1 h at room temperature [rabbit anti-ATXN2L (Bethyl A301-370A, 1:300), mouse anti-ATXN2 (BD Biosciences 611378, 1:200), mouse anti-dimethyl arginine (dmArg, Santa Cruz 3C110, 1:100), rabbit anti-dimethyl arginine (ASYM24, Millipore 07-414, 1:200), mouse anti-FLAG (Sigma M2, 1:500), rabbit anti-FLAG (Sigma F7425; 1:500), rabbit anti-FUS (Sigma, HPA008784, 1:300), mouse anti-MYC (Millipore 4A6, 1:500), rabbit anti-MYC (Sigma C3956, 1:500), mouse anti-PRMT1 (Sigma P1620, 1:200), mouse anti-SR proteins (Life Technologies 16H3, 1:200) and mouse anti-TIAR (BD Biosciences 610352, 1:200)]. Afterward, cells were incubated with secondary antibodies for 1 h at room temperature [goat anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (Molecular Probes, 1:500)]. Finally, nuclei were stained using Hoechst 33258 (bisbenzimide, Sigma), samples were mounted with Fluoromount-G (Southern Biotech) and analyzed using a confocal microscope (LSM 700, Zeiss) on an inverted stand (Axiovert 200 M, Zeiss) using objective Plan-NEOFLUAR 40 × 1.3 oil DIC. Images were acquired using ZEN version 5.5 software and processed using AxioVision software (Zeiss). All experiments were performed at least three times with reproducible results.

Proximity ligation assay

For proximity ligation assay (PLA) experiments HeLa cells were plated on glass coverslips in 24-well-plates, treated as described above, then washed with PBS and fixed with 2% formaldehyde for 10 min and ice-cold methanol for 30 min. Subsequently, cells were incubated with 3% BSA/PBS, the indicated combinations of primary antibodies were added, and cells were further incubated for 1 h at room temperature. Coverslips were then processed according to the manufacturer's instructions (Duolink *In Situ* Fluorescence, Olink Bioscience) using open droplet reactions at 37 °C and high humidity. Briefly, cells were washed with wash buffer and incubated with anti-rabbit (plus) and anti-mouse (minus) PLA probes for 1 h. Afterward, ligase solution was added for 30 min followed by an incubation with amplification-polymerase solution for 100 min. After washing steps with wash buffer and PBS, cells were incubated with a secondary antibody (goat anti-mouse Alexa Fluor 488) for 1 h at room temperature to immunolabel PLA target proteins. Nuclei were stained using Hoechst 33258 (bisbenzimide, Sigma), and samples were mounted with Fluoromount-G (Southern Biotech). PLA samples were analyzed by confocal microscopy as described above. Red PLA signals were detected using excitation at 594 nm and quantified using Duolink Image Tool (Olink). Cells were segmented and nuclear as well as cytoplasmic signals were quantified using the following parameters: nuclei size 40–60, cytoplasm size 60–100, signal size 5, signal threshold 150. *P*-value was calculated using Student's *t*-test. All experiments were performed at least three times with reproducible results.

Co-immunoprecipitation

For co-immunoprecipitation (co-IP) experiments, HeLa cells were harvested by washing with PBS and lysis was carried out in lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5% protease inhibitor ("complete" tablets, Roche), and 25 U/ml benzonase (Merck)] for at least 30 min at 4 °C. Then, 500 µg cell lysate was incubated with 1.5 µl primary antibody [rabbit anti-ATXN2L (Bethyl A301-370A), mouse anti-PRMT1 (Sigma P1620)] or without primary antibody as control overnight at 4 °C. Next, samples were incubated with 15 µl IgG conjugated M-280 Dynabeads (Dynal) for 2–3 h. Subsequently, Dynabeads were pulled down magnetically and washed three times with 3% BSA/PBS and with PBS. After adding Laemmli's sample buffer containing 100 mM DTT, samples including 50 µg input lysate were incubated at 95 °C and loaded onto a 10% SDS gel. After separation, proteins were transferred to a PVDF membrane (Millipore) using a BIO-RAD blotting system. Membranes were incubated overnight with primary antibodies as indicated [rabbit anti-ATXN2L (Bethyl A301-370A, 1:1000), mouse anti-dimethyl arginine (Abcam 21C7, 1:500), rabbit anti-dimethyl arginine (ASYM24, Millipore 07-414, 1:1000), mouse anti-PRMT1 (Sigma P1620, 1:1000)] for the detection of precipitated proteins. Finally, membranes were incubated with respective secondary antibodies [POD-conjugated goat anti-mouse or anti-rabbit (1:10000, Sigma)] for 1 h and proteins were visualized using Western Lightning ECL solutions (Perkin-Elmer). If necessary, equal protein loading was confirmed by Coomassie Brilliant Blue (Sigma) staining of a separate SDS gel.

Yeast two-hybrid analysis

Yeast strain L40ccua was co-transformed using the bait constructs encoding LexA-ATXN2L-LSm, LexA-ATXN2L-LSm+RR, LexA-ATXN2L-LSm+QQ, or LexA-PRMT1 and the prey construct encoding AD-PRMT1. As control, cells were co-transformed with the respective bait constructs and empty prey vector. Transformants were grown on synthetic defined (SD) medium lacking tryptophan and leucine, isolated and spotted onto solid SD medium lacking tryptophan and leucine. For analysis of reporter gene activity, yeast cells were spotted onto medium lacking tryptophan, leucine, uracil and histidine (containing 2.5 mM 3-amino-1,2,4-triazole (5-AT, Sigma) to suppress slight autoactivity) and growth was analyzed after 3 days. For analysis of the *LacZ* reporter gene activity, yeast cells were spotted onto a nylon membrane (Micron Separations Inc.) on SD medium lacking tryptophan and leucine. After 3 days, membranes were incubated in liquid nitrogen and then incubated with X-Gal buffer (0.15% X-Gal [Carl Roth] and 10 mM DTT in phosphate buffer) for at least 4 h at 37 °C.

Results

ATXN2L is asymmetrically dimethylated *in vivo*

Protein arginine methylation commonly occurs at arginines in RGG motifs of RNA-binding proteins and these are found to be monomethylated or dimethylated in a symmetric or asymmetric manner [1]. Previously, mass spectrometry studies revealed that ATXN2L is asymmetrically dimethylated at arginine 361 within one RGG motif [26] (Fig. 1A), but also monomethylated at this and other positions [27,28]. We decided first to validate the identified dimethylation of ATXN2L *in vivo* by performing immunoprecipitation experiments taking advantage of a specific antibody against asymmetric dimethyl arginine (dmArg). For this, endogenous ATXN2L was precipitated with a specific antibody (Fig. 1B, lower panel), and the methylation status of precipitated ATXN2L was analyzed by immunoblotting. As shown in Fig. 1B (upper panel), a signal was obtained with the dmArg antibody in the precipitate, which was absent in the control, indicating that ATXN2L is asymmetrically dimethylated *in vivo*. To further corroborate this finding, we set out to perform PLA experiments. This assay is based on the recognition of two target proteins or posttranslational modifications and brings DNA strands attached to the antibodies into close proximity, which are then ligated and amplified for a highly sensitive detection of protein-protein interactions or posttranslational modifications *via* incorporated fluorescent dyes [31,32] (Fig. 1C). HeLa cells were prepared, incubated with antibodies against ATXN2L and asymmetric dimethyl arginine (dmArg), and further processed for the microscopic analysis. PLA signals were detected in the nucleus and in particular in the cytoplasm (Fig. 1D, left panel). We also observed unspecific signals in the control, in which no primary antibodies were added (Fig. 1D, right panel); however, these signals were significantly less (Fig. 1E). To end with, we set out to validate the dimethylation of ATXN2L with another combination of specific antibodies. To transiently overexpress ATXN2L, HeLa cells were transfected with the expression plasmid pCMV-MYC-ATXN2L-WT or with the empty vector, fixed 48 h posttransfection and prepared for the PLA approach using antibodies against the MYC-tag and asymmetric dimethyl arginine (ASYM24). Transfected cells were afterward immunostained for the MYC-tag. Strong cytoplasmic PLA signals were detected in cells expressing MYC-ATXN2L, whereas almost no signals were detectable in non-transfected cells or in the control assay performed without primary antibodies (Fig. 1F and G). Thus, our findings validate that ATXN2L is asymmetrically dimethylated *in vivo* as initially identified in the mass spectrometry studies [26].

Methylation inhibition alters ATXN2L localization to nuclear splicing speckles

ATXN2L is a predominantly cytoplasmic protein, but also localizes to nuclear splicing speckles [24]. Since protein methylation is known to modulate the localization of proteins [1,2], we examined next if ATXN2L localization is affected by its methylation state. HeLa cells were treated with the methyltransferase inhibitor AdOx and cells were processed for microscopic analysis. We verified that AdOx treatment reduced cellular protein methylation on a general level (Fig. S1A and C). PLA analyses furthermore indicate that dimethylation of ATXN2L was significantly reduced to about 65% (Fig. S1D and E). We observed that the cytoplasmic localization of ATXN2L in AdOx-treated cells appears unaffected at both AdOx concentrations used (Fig. 2). On the contrary, the nuclear localization of ATXN2L to splicing speckles was altered in AdOx-treated cells, as these ATXN2L-positive structures appear brighter, enlarged and more regularly shaped (Fig. 2; enlarged images). This observation was more evident in cells that was treated with 100 µM AdOx. Notably, the localization of serine/arginine-rich (SR) marker proteins to splicing speckles seems unaffected, indicating that AdOx treatment influences ATXN2L

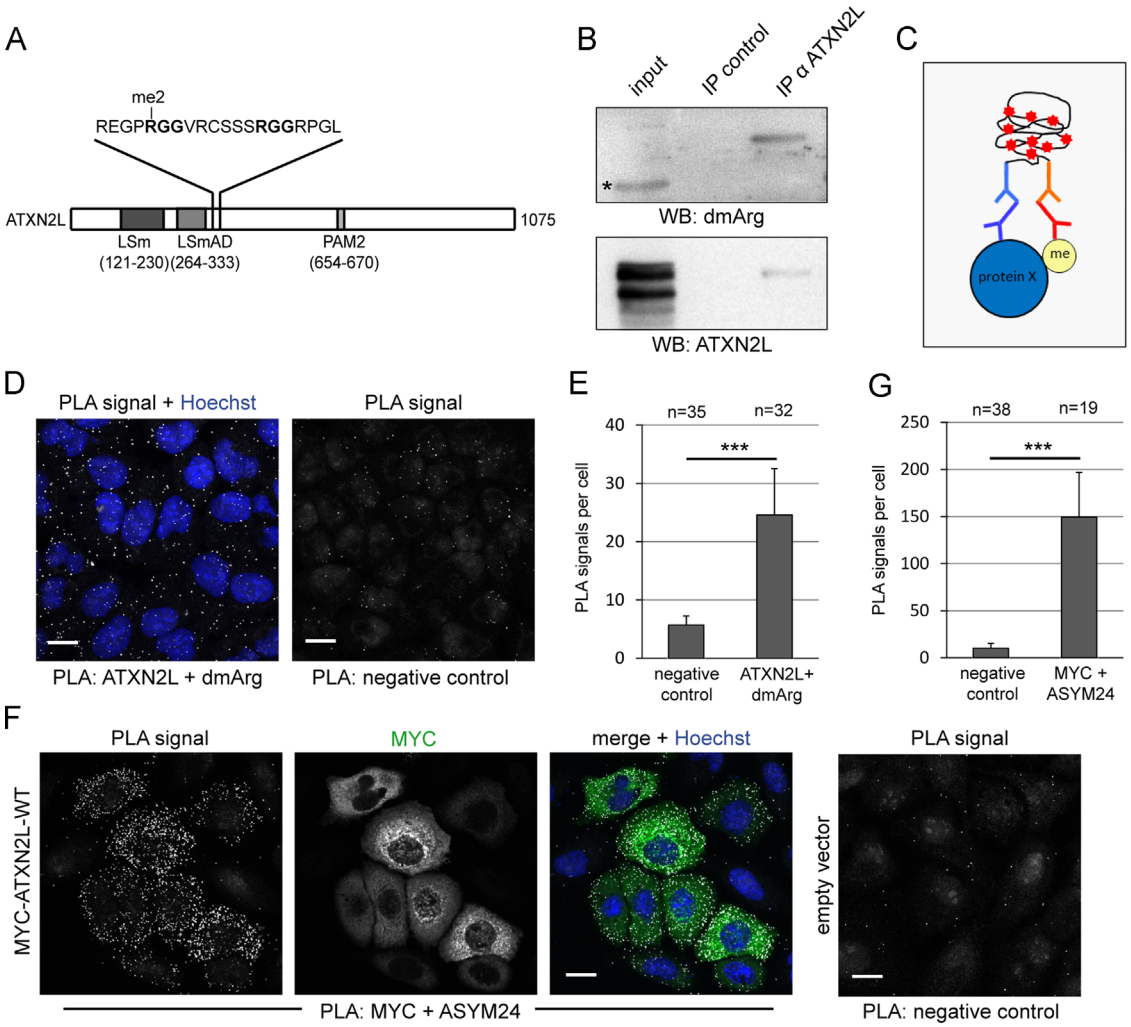

Fig. 1 – ATXN2L undergoes asymmetric arginine dimethylation *in vivo*. (A) Schematic representation of the domain architecture of ATXN2L. RGG motifs and the identified dimethylated arginine residue are highlighted. (B) *In vivo* analysis of ATXN2L dimethylation. Cell lysates were prepared from HeLa cells and ATXN2L was immunoprecipitated (lower panel). Asymmetric arginine dimethylation of precipitated ATXN2L was examined with the respective antibody (dmArg, Abcam) (upper panel). Asterisk marks most prominent dimethylated proteins in the input lysate. (C–G) Proximity ligation assay (PLA). (C) Two primary antibodies bind to the target protein and the methylated arginine residues. Oligonucleotides bound to the respective secondary antibodies hybridize. After ligation, the circular DNA is amplified and can be detected *via* incorporated red fluorescent dye molecules. (D) PLA was performed using specific antibodies against ATXN2L and dimethyl arginine (dmArg, Santa Cruz) (left panel) or without primary antibodies as control (right panel). Nuclei were stained with Hoechst. Scale bars represent 20 μm. (E) PLA signals per cell were quantified by image software-assisted analysis as described in *Material and methods*. Number of analyzed cells and the mean+SD is shown. *P*-value was determined by Student's *t*-test (*: $P<0.001$). (F) HeLa cells were transfected with expression plasmid pCMV-MYC-ATXN2L-WT or the respective empty vector and incubated for 48 h. PLA was performed using specific antibodies against the MYC-tag and dimethyl arginine (ASYM24) or without primary antibodies as control (right panel). Co-localization of PLA signals with subsequent immunostaining of the MYC-tag is shown. Nuclei were stained with Hoechst. Scale bars represent 20 μm. (G) Quantification of PLA signals per cell. Number of analyzed cells and the mean+SD is shown. *P*-value was determined by Student's *t*-test (***: $P<0.001$).**

nuclear localization to splicing speckles rather than the integrity of these structures *per se*.

PRMT1 activity regulates ATXN2L localization to splicing speckles

The major PRMT responsible for 90% of all cellular methylation events in mammalian cells is PRMT1 [1,6]. For this reason, we decided to investigate whether PRMT1 activity might be substantial for ATXN2L localization to splicing speckles. To examine this, we carried out RNAi experiments to reduce the intracellular PRMT1 activity. HeLa cells were transfected with siRNA against PRMT1 as indicated. We analyzed first whether a lowered PRMT1 level would affect the global asymmetric dimethylation state of the cell. As shown in Fig. S1B and C, a reduced asymmetric dimethylation was observed in siPRMT1-treated cells. In the next

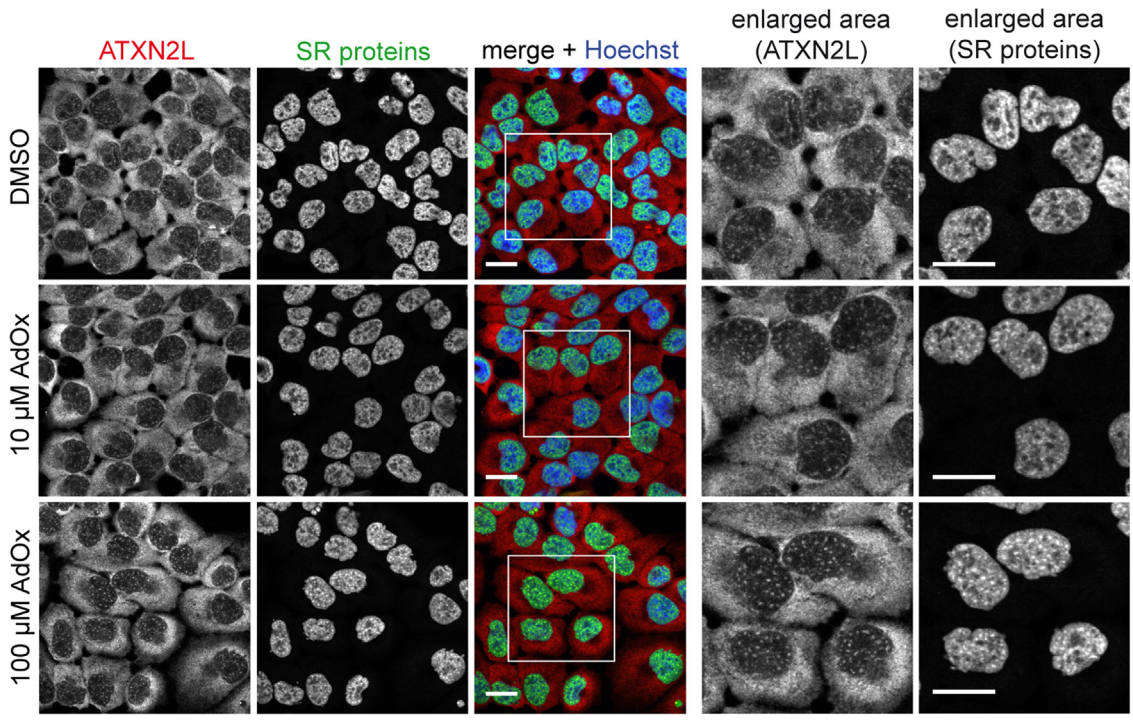

Fig. 2 – AdOx treatment alters the nuclear localization of ATXN2L, but not the integrity of nuclear splicing speckles *per se*. HeLa cells were treated with the indicated concentrations of AdOx for 24 h and processed for confocal microscopy to analyze the localization of ATXN2L (red) and SR proteins as markers for splicing speckles (green). Enlarged areas of ATXN2L and SR staining are shown on the right. Nuclei were stained with Hoechst. Scale bars represent 20 µm.

step, we observed that ATXN2L-positive splicing speckles are altered in siPRMT1-treated cells in comparison to cells that were treated with non-targeting control siRNA (Fig. 3A). Similar to the effects of AdOx treatment (Fig. 2), ATXN2L-positive splicing speckles appear larger and rounder, whereas SR protein-positive splicing speckles seem unaffected. To further corroborate the effect of PRMT1 activity on ATXN2L localization, we conducted overexpression experiments using a catalytically active or inactive variant of PRMT1. This inactive PRMT1 was constructed by exchange of amino acids 74–76 (VDL to AAA) as described before [29,30]. We first verified regular or compromised catalytic activity of both active and inactive PRMT1 on asymmetric arginine dimethylation (Fig. S2). To investigate the effect on ATXN2L localization, HeLa cells were transfected with expression plasmids pTL-FLAG-PRMT1-WT or pTL-FLAG-PRMT1-inactive. We observed that in cells overexpressing the active PRMT1 variant ATXN2L-positive nuclear structures were almost completely absent, whereas ATXN2L-positive nuclear structures were detectable in the presence of inactive PRMT1 (Fig. 3B). Of note, nuclear splicing speckles *per se* visualized by SR proteins appeared to be unaffected (Fig. 3C). In summary, ATXN2L nuclear localization and association with nuclear splicing speckles seems to be regulated by PRMT1 activity.

ATXN2L associates with PRMT1

Given that PRMT1 activity influences the nuclear ATXN2L localization, we examined therefore if ATXN2L is a target protein of PRMT1 performing co-IP experiments. Cell lysates were prepared and endogenous ATXN2L or PRMT1 was precipitated with specific antibodies as indicated. As shown in Fig. 4A, endogenous ATXN2L was precipitated with an antibody directed against PRMT1 (upper panel), and *vice versa*, PRMT1 was precipitated with an antibody directed against ATXN2L (lower panel). To further strengthen this finding, we once more performed the PLA approach described using antibodies against ATXN2L and PRMT1. As a control we further used antibodies against PRMT1 and FUS in this assay, since FUS represents a known PRMT1 target protein [30,33]. Predominantly cytoplasmic PLA signals were detected in cells if ATXN2L and PRMT1 antibodies were used (Fig. 4B, left panel and C), whereas only few weak signals were detectable in a control assay performed without primary antibodies (Fig. 4B, right panel). A comparable number of PLA signals with a strong nuclear localization were detected if FUS and PRMT1 antibodies were used (Fig. 4B, middle panel and C). Thus, this result demonstrates an association between ATXN2L and PRMT1 that mainly takes place in the cytoplasm.

RGG sequences within RNA-binding proteins serve as substrate recognition sites for PRMTs and are also known to mediate protein-protein interactions [2]. Since ATXN2L comprises two RGG motifs, we exploited the Y2H system to further investigate whether these sites are important for the association of ATXN2L and PRMT1. For this analysis we generated an expression plasmid encoding an ATXN2L fusion protein encompassing the LSm and LSmAD domain with both RGG motifs (ATXN2LSm+RR) as well as an expression plasmid encoding an ATXN2L fusion protein, in which the arginines have been exchanged to glutamines (ATXN2L-LSm+QQ). We decided to use glutamine instead of lysine, which often serves as substitute in methylation studies, since lysine methylation of non-histone proteins can impact

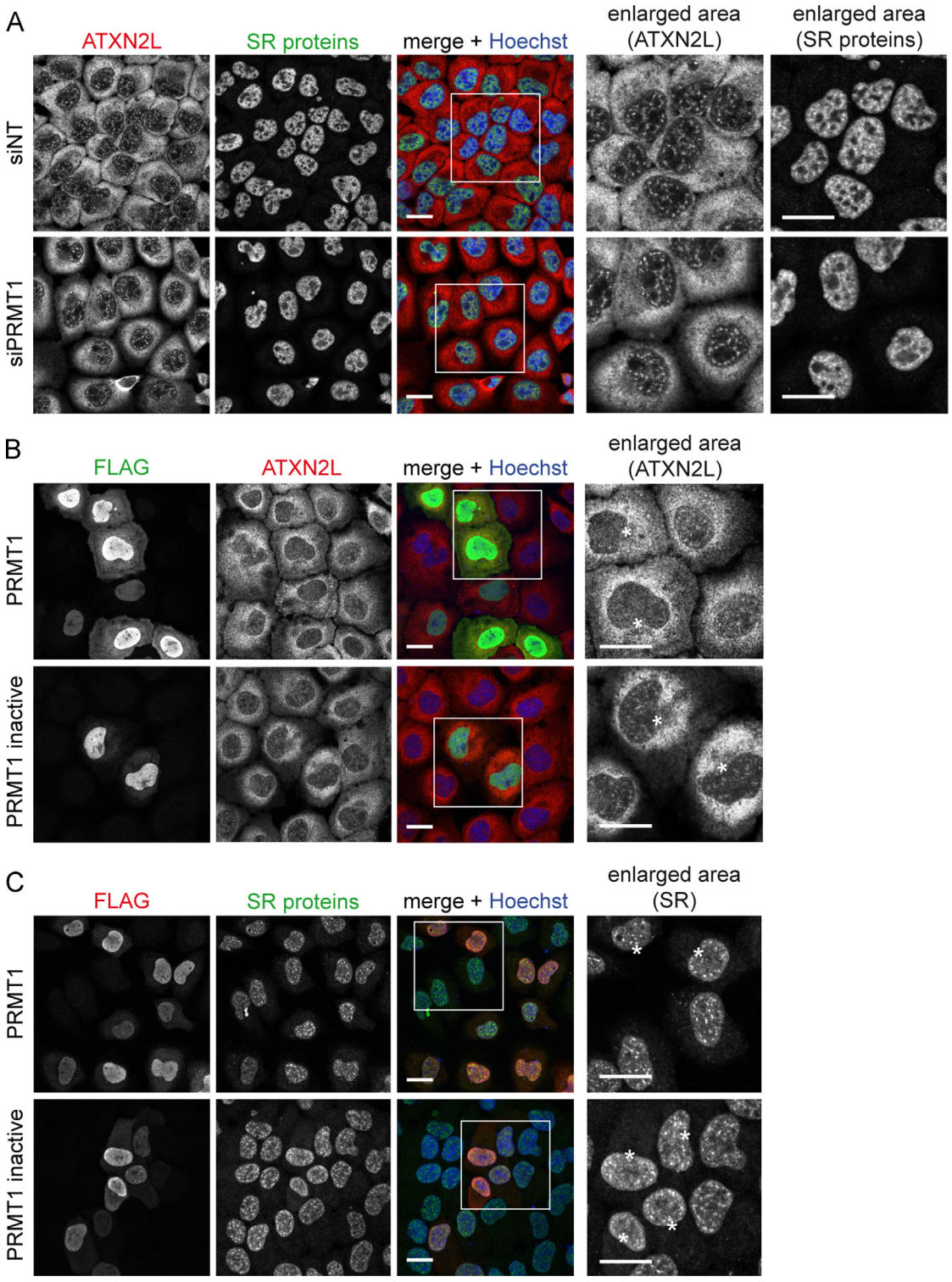

Fig. 3 – PRMT1 activity modulates nuclear localization of ATXN2L. (A) HeLa cells were transfected with siRNA against PRMT1 transcripts, or non-targeting control siRNA (siNT). 72 h posttransfection cells were fixed and processed for immunostaining of ATXN2L (red) and SR proteins (green). Nuclei were stained with Hoechst. Scale bars represent 20 μm. (B, C) HeLa cells were transfected with the expression plasmids pTL-FLAG-PRMT1-WT or pTL-FLAG-PRMT1-inactive to overexpress an active or inactive PRMT1 enzyme, respectively. 48 h posttransfection, cells were fixed and stained with antibodies directed against (B) the FLAG-tag (green) and ATXN2L (red), or (C) the FLAG-tag (red) and SR proteins as markers for splicing speckles (green). Nuclei were stained with Hoechst. Enlarged areas of ATXN2L or SR staining are shown on the right. Overexpressing cells are marked by asterisks. Scale bars represent 20 μm.

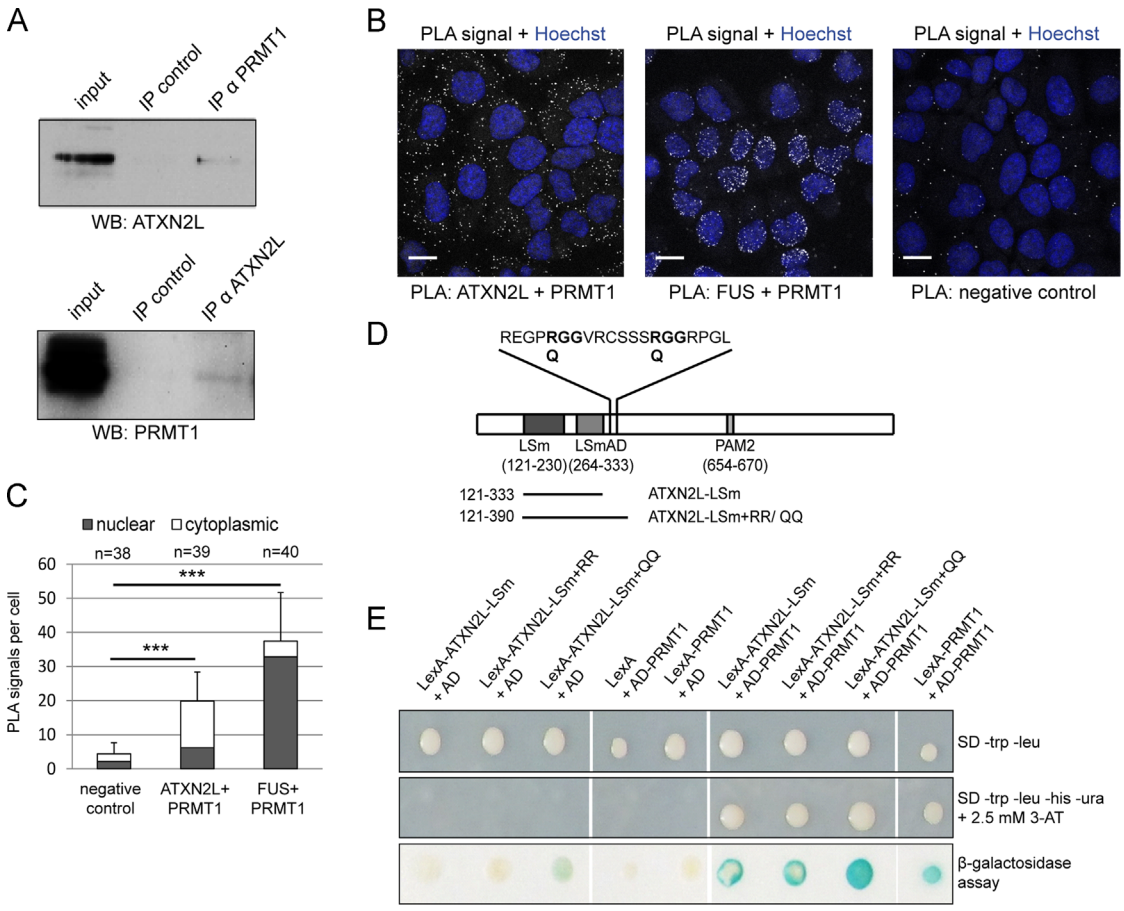

Fig. 4 – ATXN2L interacts with the methyltransferase PRMT1. (A) Co-immunoprecipitation experiments. Cell lysates were prepared from HeLa cells, and incubated with an antibody directed against PRMT1. Precipitated ATXN2L was detected with a specific antibody as described in *Material and methods* (upper panel). Additionally, ATXN2L was precipitated from HeLa cell lysates and co-precipitated PRMT1 protein was detected using a specific antibody (lower panel). (B) PLA approach. HeLa cells were incubated with antibodies against ATXN2L and PRMT1 (left panel). A control assay was performed without primary antibodies (right panel). Additionally, a positive control was included in this assay using specific antibodies against FUS and PRMT1 (middle panel). Nuclei were stained with Hoechst. Scale bars represent 20 μm. (C) Quantification of nuclear and cytoplasmic PLA signals per cell. Number of analyzed cells and the mean+SD is shown. *P*-value was determined by Student's *t*-test (*: $P<0.001$). (D) Schematic representation of ATXN2L and regions used for Y2H analysis. (E) Y2H analysis. Yeast strain L40ccua was co-transformed with the indicated bait and prey plasmids, and transformants were spotted onto selective media. Reporter gene activity was determined by growth on selective media (containing 2.5 mM 3-AT) as well as by β-galactosidase assay.**

protein stability, protein-protein interactions, and cellular function [34,35]. Therefore, we wanted to avoid the possibility that ATXN2L could be methylated at an introduced lysine in our experiments. As control we included in our analysis an ATXN2L fusion protein comprising only the LSm and LSmAD domain (ATXN2L-LSm) as illustrated in Fig. 4D. Yeast strain L40ccua was co-transformed with respective bait and prey plasmids encoding PRMT1 or the respective ATXN2L domains as described. As a positive control, we co-transformed bait and prey plasmids encoding PRMT1, since dimerization of PRMT1 itself was reported [36,37]. Since in initial experiments slight autoactivation of reporter genes through LexA-ATXN2L protein expression alone was observed (data not shown), we performed this analysis in the presence of 2.5 mM 3-AT in the media to suppress this observed reporter gene activity. As shown in Fig. 4E, growth of yeast expressing LexA-ATXN2L-LSm+RR and AD-PRMT1 as well as LexA-ATXN2L-LSm+QQ and AD-PRMT1 was detectable. None of the used constructs *per se* led to autoactivation of the analyzed reporter genes when co-expressed with the activation domain (AD) or LexA DNA-binding domain alone in the presence of 2.5 mM 3-AT. Therefore, this result indicates a direct interaction between ATXN2L and PRMT1, which seems to be independent of the presence of arginines within the RGG motifs. Surprisingly, growth of yeast expressing LexA-ATXN2L-LSm and AD-PRMT1 was also detected, indicating that the LSm domain rather than the RGG motifs, is responsible for the interaction between ATXN2L and PRMT1.

Since ATXN2L localizes to cytoplasmic SGs under various stress conditions [24] and PRMT1 localizes to SGs induced by RAP55 overexpression [38], we finally investigated whether ATXN2L and PRMT1 associate in these cytoplasmic structures as well. For this analysis, HeLa cells were treated with arsenite or heat, and localization of endogenous ATXN2L and PRMT1 was investigated by confocal microscopy. We observed a robust co-

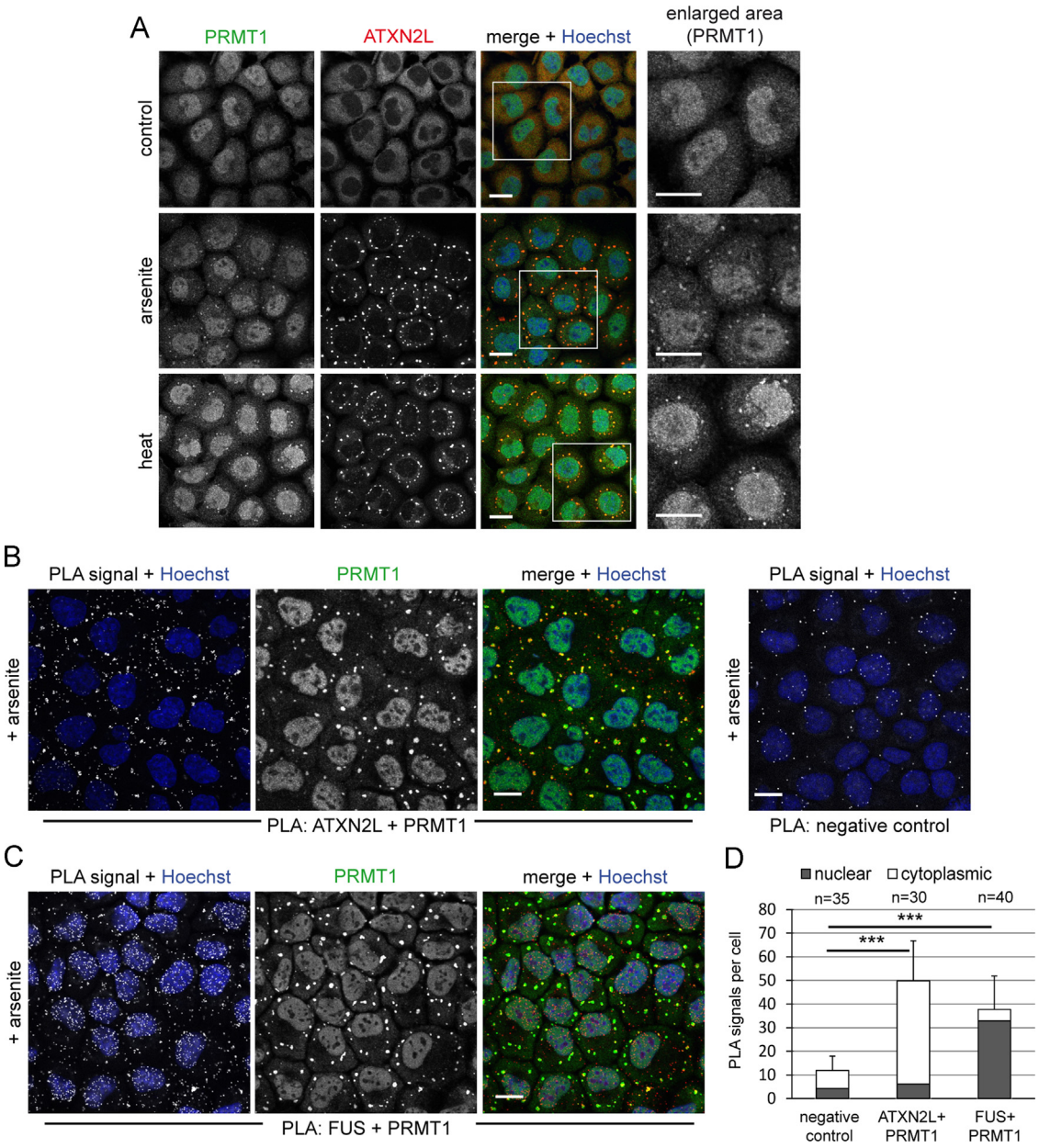

Fig. 5 – ATXN2L associates with PRMT1 in stress granules. (A) To induce oxidative or heat stress, HeLa cells were exposed to 0.5 mM arsenite for 1 h or to 44 °C for 30 min, while control cells were left untreated. Cells were then fixed and processed for confocal microscopy to analyze the localization of PRMT1 (green) and ATXN2L (red). (B) PLA approach. HeLa cells exposed to 0.5 mM arsenite for 1 h were fixed and incubated with specific antibodies against ATXN2L and PRMT1, whereas PLA without primary antibodies served as negative control (right panel). (C) PLA performed with specific antibodies against FUS and PRMT1 served as positive control. Co-localization of PLA signals with subsequent PRMT1 immunostaining is shown. Nuclei were stained with Hoechst. Scale bars represent 20 µm. (D) Quantification of nuclear and cytoplasmic PLA signals per cell. Number of analyzed cells and the mean+SD is shown. *P*-value was determined by Student's *t*-test (*: $P<0.001$).**

localization of PRMT1- and ATXN2L-positive cytoplasmic foci in arsenite or heat-treated cells (Fig. 5A). In addition, we again carried out the PLA approach to investigate an interaction between ATXN2L and PRMT1 under oxidative stress conditions using antibodies against ATXN2L and PRMT1. Strong PLA signals were observed in arsenite-treated cells that accumulated in cytoplasmic foci (Fig. 5B and D). Additional immunostaining of PRMT1 demonstrated that these PLA signals co-localize with PRMT1-positive SGs; only few weak diffuse signals were detectable in the respective control. As control we further used antibodies against PRMT1 and FUS to spot this known interaction as well. We observed PLA signals to a similar extent, which are primarily in the nucleus, although both proteins are components of SGs (Fig. 5C and D). Thus, an interaction between ATXN2L and PRMT1 occurs in SGs under oxidative stress conditions as well.

Methylation of ATXN2L is not crucial for its localization to SGs

Since the methylation state of some SG components might be important for their localization to SGs [20,21], we investigated whether arginine methylation in the RGG motifs of ATXN2L might play a role for SG localization as well. For this, we introduced a mutation at the arginine residue at position 361, which is described to be methylated [26–28]. As described earlier, we also introduced a mutation at arginine 370, as it is part of the second RGG motif. To examine the location of the RGG methylation-deficient proteins, HeLa cells were transfected with the respective plasmids pCMV-MYC-ATXN2L-WT, pCMV-MYC-ATXN2L-R361Q, pCMV-MYC-ATXN2L-R370Q or pCMV-MYC-ATXN2L-R361Q/R370Q. To begin with we examined whether localization of these transiently expressed proteins under normal conditions is altered, and observed no differences between ATXN2L wild type and the mutant proteins (Fig. S3). We then studied the localization of RGG methylation-deficient mutants under stress conditions. As shown in Fig. 6A, wild type MYC-ATXN2L co-localized with the SG marker protein T-cell-restricted intracellular antigen 1-related (TIAR) in arsenite-treated cells as described [24]. Nevertheless, the RGG methylation-deficient ATXN2L proteins co-localized with TIAR as well, indicating that methylation of arginines within the RGG motifs of ATXN2L might be dispensable for its recruitment to SGs. Since ATXN2L comprises additional arginines that might be methylated, we finally investigated the localization of overexpressed wild type ATXN2L under conditions of general methylation inhibition. For this, cells were treated with AdOx and then transfected with expression plasmid pCMV-MYC-ATXN2L-WT to avoid methylation of the overexpressed protein. We observed that overexpressed ATXN2L localizes to SGs under general methylation inhibition (Fig. 6B), indicating that methylation might not be obligatory for its localization to SGs. In addition to this set of experiments, we again treated cells with two concentrations of AdOx and analyzed the endogenous ATXN2L localization after arsenite treatment. ATXN2L-positive cytoplasmic foci were evident that co-localized with the SG component ATXN2 under methylation inhibition (Fig. 7A). Finally, we treated cells with siRNA against PRMT1 or with control siRNA, and observed ATXN2L-positive foci representing SGs under a reduced PRMT1 level (Fig. 7B). In sum, methylation of ATXN2L is likely dispensable for its localization to SGs.

Discussion

The physiological function of RNA-binding proteins is regulated by posttranslational modifications, of which methylation is fairly predominant [4]. Here we focused on investigating whether ATXN2L localization, and accordingly its function, is regulated by its arginine methylation state. We first validated results from recent mass spectrometry approaches [26], and showed that ATXN2L is asymmetrically dimethylated *in vivo*. Methylation inhibition or reduction of PRMT1 activity alters ATXN2L localization to splicing speckles. Moreover, we demonstrated that in the presence of overexpressed active PRMT1 nuclear ATXN2L-positive foci were absent, whereas overexpression of an inactive PRMT1 protein has no significant effect on these foci. Therefore it is tempting to speculate that nucleocytoplasmic shuttling of ATXN2L occurs and might be regulated through arginine methylation presumably by PRMT1. Since overexpression of PRMT1 depletes ATXN2L from nuclear splicing speckles, dimethylated ATXN2L

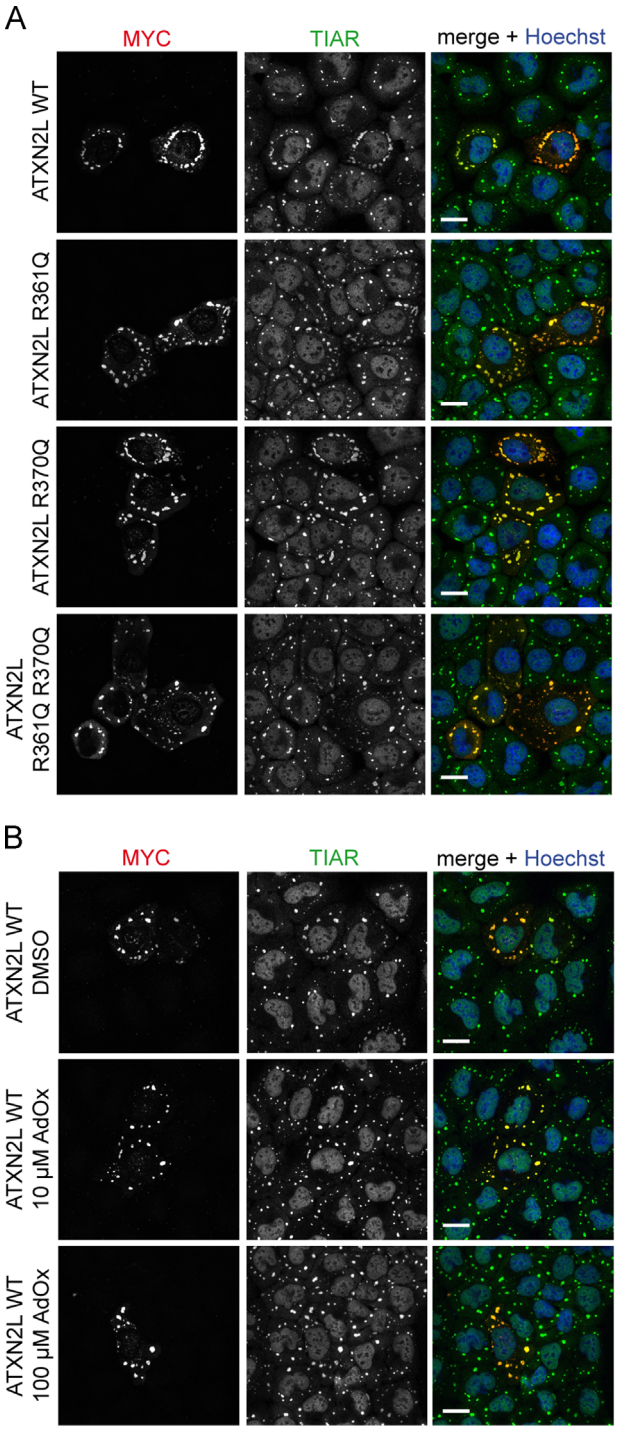

Fig. 6 – Arginine methylation-deficient ATXN2L mutants do not fail to localize to stress granules. (A) HeLa cells were transfected with the expression constructs pCMV-MYC-ATXN2L-WT, pCMV-MYC-ATXN2L-R361Q, pCMV-MYC-ATXN2L-R370Q or pCMV-MYC-ATXN2L-R361Q/R370Q to overexpress wild type ATXN2L or arginine methylation-deficient mutants, respectively. (B) Cells were treated with the indicated concentrations of AdOx and subsequently transfected with pCMV-MYC-ATXN2L-WT. 48 h posttransfection, cells were exposed to 0.5 mM arsenite for 1 h and fixed. Cells were then stained with antibodies directed against the MYC-tag (red) and TIAR as stress granule marker (green). Nuclei were stained with Hoechst. Scale bars represent 20 µm.

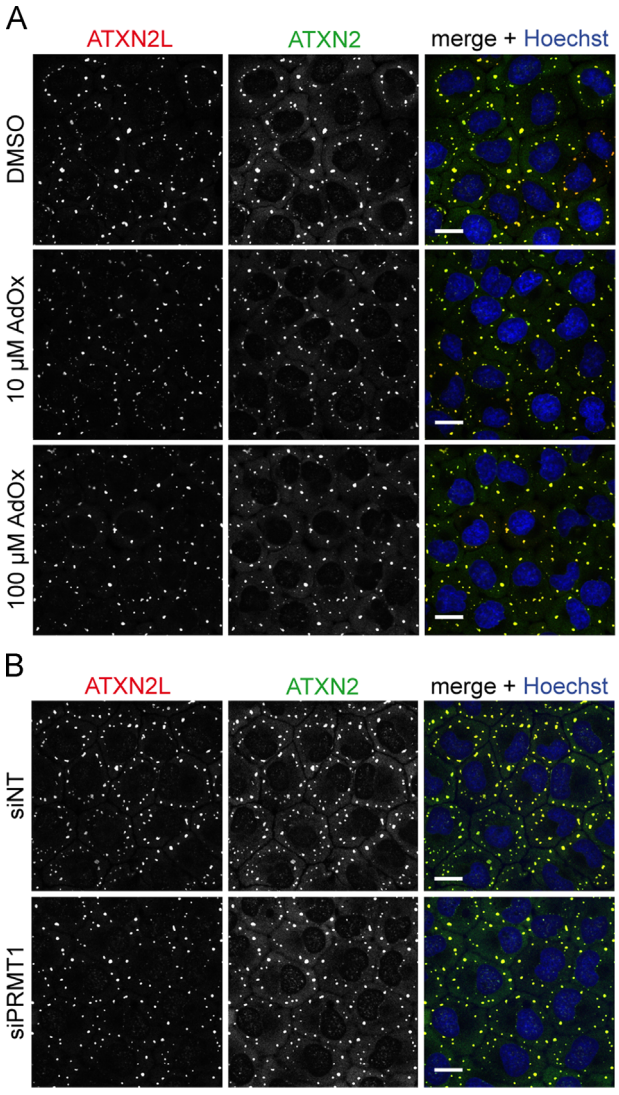

Fig. 7 – Arginine methylation of ATXN2L is dispensable for its localization to stress granules. (A) HeLa cells were treated with the indicated concentrations of AdOx for 24 h, exposed to 0.5 mM arsenite for 1 h and processed for confocal microscopy to analyze the localization of ATXN2L (red) and ATXN2 as SG marker protein (green). (B) HeLa cells were transfected with siRNA against PRMT1 transcripts or non-targeting control siRNA (NT). 72 h posttransfection cells were exposed to 0.5 mM arsenite for 1 h, fixed and processed for immunostaining of ATXN2L (red) and ATXN2 (green). Nuclei were stained with Hoechst. Scale bars represent 20 µm.

likely acts in the cytoplasm, whereas unmethylated ATXN2L might be involved in splicing-associated nuclear processes. In this light, PRMT1 overexpression slightly increases the cytoplasmic localization of FUS, whereas nuclear localization of FUS was increased if inactive PRMT1 was overexpressed [39]. We additionally discovered that ATXN2L associates with PRMT1, and that the arginines within the RGG sites of ATXN2L are not mandatory for this association. Instead we discovered that the LSm domain of ATXN2L alone mediates the interaction with PRMT1 in the Y2H system.

It has been suggested that the localization of proteins to SGs is probably regulated through methylation, as symmetrically and asymmetrically dimethylated proteins have been detected in these structures [21]. Since we observed an interaction of ATXN2L and PRMT1 in SGs, dimethylated ATXN2L is likely present in these structures under stress conditions. Nevertheless, our studies suggest that ATXN2L methylation is likely dispensable for its localization to SGs. However, one should keep in mind that methylation is a rather stable modification. Accordingly, general inhibition of methylation *via* AdOx affects newly translated proteins, whereas already methylated proteins are likely to persist during the course of investigations [21,40]. In this regard, we performed ATXN2L overexpression experiments, in which cells have been treated with AdOx before transfection. Furthermore, we investigated ATXN2L localization under low PRMT1 activity; under both experimental settings ATXN2L was localized to SGs. In addition, mutation of arginines within both RGG sites of ATXN2L appears irrelevant for SG localization. Notably, the proline-/RG-rich domain within SAM68 plays a role in its recruitment to SGs [41]. Moreover, the RGG domains within the FUS protein are important for SG localization, however, methylation does not seem to be mandatory for incorporation of FUS into SGs [23]. The tudor domain of the tudor domain-containing protein 3 (TDRD3) is both required and sufficient for its recruitment to SGs [42]. Interestingly, this domain recognizes methylated arginines in proteins and, therefore, the recruitment of TDRD3 is regulated by methyl-dependent protein-protein interactions. Moreover, recruitment of TDRD3 to SGs was slightly delayed under methylation inhibition. Of note, SG disassembly was also delayed under methylation inhibition, indicating that methylation indeed plays a role in SG dynamics and compositions [17,42]. We furthermore observed that PRMT1 localized to SGs in arsenite-treated cells as well. This is in agreement with other studies that reported the presence of PRMT1 in SGs [38,39], suggesting that methylation could perhaps occur for some SG components within these structures. Since additional posttranslational modifications of SG components have been reported, further studies are required to understand the functional consequence of posttranslational modifications for SG biology.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.02.022.

REFERENCES

[1] M.T. Bedford, S.G. Clarke, Protein arginine methylation in mammals: who, what, and why, Mol. Cell 33 (2009) 1–13.

[2] P. Thandapani, T.R. O'Connor, T.L. Bailey, S. Richard, Defining the RGG/RG motif, Mol. Cell 50 (2013) 613–623.
[3] K.K. Biggar, S.S. Li, Non-histone protein methylation as a regulator of cellular signalling and function, Nat. Rev. Mol. Cell Biol. 16 (2015) 5–17.
[4] T.B. Nicholson, T. Chen, S. Richard, The physiological and pathophysiological role of PRMT1-mediated protein arginine methylation, Pharmacol. Res. 60 (2009) 466–474.
[5] S.M. Carlson, O. Gozani, Emerging technologies to map the protein methylome, J. Mol. Biol. 426 (2014) 3350–3362.
[6] Z. Yu, T. Chen, J. Hebert, E. Li, S. Richard, A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation, Mol. Cell Biol. 29 (2009) 2982–2996.
[7] M.R. Pawlak, C.A. Scherer, J. Chen, M.J. Roshon, H.E. Ruley, Arginine N-methyltransferase 1 is required for early postimplantation mouse development, but cells deficient in the enzyme are viable, Mol. Cell Biol. 20 (2000) 4859–4869.
[8] L. Jobert, M. Argentini, L. Tora, PRMT1 mediated methylation of TAF15 is required for its positive gene regulatory function, Exp. Cell Res. 315 (2009) 1273–1286.
[9] R. Sinha, E. Allemand, Z. Zhang, R. Karni, M.P. Myers, et al., Arginine methylation controls the subcellular localization and functions of the oncoprotein splicing factor SF2/ASF, Mol. Cell Biol. 30 (2010) 2762–2774.
[10] G.C. Bressan, E.C. Moraes, A.O. Manfiolli, T.M. Kuniyoshi, D.O. Passos, et al., Arginine methylation analysis of the splicing-associated SR protein SFRS9/SRP30C, Cell. Mol. Biol. Lett. 14 (2009) 657–669.
[11] W.A. Smith, B.T. Schurter, F. Wong-Staal, M. David, Arginine methylation of RNA helicase a determines its subcellular localization, J. Biol. Chem. 279 (2004) 22795–22798.
[12] W.J. Friesen, S. Massenet, S. Paushkin, A. Wyce, G. Dreyfuss, SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets, Mol. Cell 7 (2001) 1111–1117.
[13] K. Fronz, S. Guttinger, K. Burkert, U. Kuhn, N. Stohr, et al., Arginine methylation of the nuclear poly(a) binding protein weakens the interaction with its nuclear import receptor, transportin, J. Biol. Chem. 286 (2011) 32986–32994.
[14] H.M. Wei, H.H. Hu, G.Y. Chang, Y.J. Lee, Y.C. Li, et al., Arginine methylation of the cellular nucleic acid binding protein does not affect its subcellular localization but impedes RNA binding, FEBS Lett. 588 (2014) 1542–1548.
[15] M.L. Hung, G.M. Hautbergue, A.P. Snijders, M.J. Dickman, S.A. Wilson, Arginine methylation of REF/ALY promotes efficient handover of mRNA to TAP/NXF1, Nucleic Acids Res. 38 (2010) 3351–3361.
[16] J. Rho, S. Choi, C.R. Jung, D.S. Im, Arginine methylation of Sam68 and SLM proteins negatively regulates their poly(U) RNA binding activity, Arch. Biochem. Biophys. 466 (2007) 49–57.
[17] N. Dolzhanskaya, G. Merz, J.M. Aletta, R.B. Denman, Methylation regulates the intracellular protein-protein and protein-RNA interactions of FMRP, J. Cell. Sci. 119 (2006) 1933–1946.
[18] P. Anderson, N. Kedersha, Stress granules: the Tao of RNA triage, Trends Biochem. Sci. 33 (2008) 141–150.
[19] N. Kedersha, P. Ivanov, P. Anderson, Stress granules and cell signaling: more than just a passing phase?, Trends Biochem. Sci. 38 (2013) 494–506.
[20] T. Ohn, P. Anderson, The role of posttranslational modifications in the assembly of stress granules, Wiley Interdiscip. Rev. RNA 1 (2010) 486–493.
[21] W. Xie, R.B. Denman, Protein methylation and stress granules: posttranslational remodeler or innocent bystander?, Mol. Biol. Int. 2011 (2011) 137459.
[22] F. De Leeuw, T. Zhang, C. Wauquier, G. Huez, V. Kruys, et al., The cold-inducible RNA-binding protein migrates from the nucleus to cytoplasmic stress granules by a methylation-dependent mechanism and acts as a translational repressor, Exp. Cell Res. 313 (2007) 4130–4144.
[23] D.M. Baron, L.J. Kaushansky, C.L. Ward, R.R. Sama, R.J. Chian et al., Amyotrophic lateral sclerosis-linked FUS/TLS alters stress granule assembly and dynamics, Mol. Neurodegener. 8 (2013) 30.
[24] C. Kaehler, J. Isensee, U. Nonhoff, M. Terrey, T. Hucho, et al., Ataxin-2-like is a regulator of stress granules and processing bodies, PLoS One 7 (2012) e50134.
[25] C. Meunier, D. Bordereaux, F. Porteu, S. Gisselbrecht, S. Chretien, et al., Cloning and characterization of a family of proteins associated with Mpl, J. Biol. Chem. 277 (2002) 9139–9147.
[26] S.E. Ong, G. Mittler, M. Mann, Identifying and quantifying *in vivo* methylation sites by heavy methyl SILAC, Nat. Methods 1 (2004) 119–126.
[27] K.B. Sylvestersen, H. Horn, S. Jungmichel, L.J. Jensen, M.L. Nielsen, Proteomic analysis of arginine methylation sites in human cells reveals dynamic regulation during transcriptional arrest, Mol. Cell. Proteomics 13 (2014) 2072–2088.
[28] A. Guo, H. Gu, J. Zhou, D. Mulhern, Y. Wang, et al., Immunoaffinity enrichment and mass spectrometry analysis of protein methylation, Mol. Cell. Proteomics 13 (2014) 372–387.
[29] F. Herrmann, F.O. Fackelmayer, Nucleo-cytoplasmic shuttling of protein arginine methyltransferase 1 (PRMT1) requires enzymatic activity, Genes Cells 14 (2009) 309–317.
[30] K. Wada, K. Inoue, M. Hagiwara, Identification of methylated proteins by protein arginine N-methyltransferase 1, PRMT1, with a new expression cloning strategy, Biochim. Biophys. Acta 1591 (2002) 1–10.
[31] A. Blokzijl, M. Friedman, F. Ponten, U. Landegren, Profiling protein expression and interactions: proximity ligation as a tool for personalized medicine, J. Intern. Med. 268 (2010) 232–245.
[32] O. Soderberg, M. Gullberg, M. Jarvius, K. Ridderstrale, K.J. Leuchowius, et al., Direct observation of individual endogenous protein complexes *in situ* by proximity ligation, Nat. Methods 3 (2006) 995–1000.
[33] J. Lee, M.T. Bedford, PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays, EMBO Rep. 3 (2002) 268–273.
[34] K.S. Egorova, O.M. Olenkina, L.V. Olenina, Lysine methylation of nonhistone proteins is a way to regulate their stability and function, Biochemistry (Moscow) 75 (2010) 535–548.
[35] X. Zhang, H. Wen, X. Shi, Lysine methylation: beyond histones, Acta Biochim. Biophys. Sin. (Shanghai) 44 (2012) 14–27.
[36] D.O. Passos, G.C. Bressan, F.C. Nery, J. Kobarg, Ki-1/57 interacts with PRMT1 and is a substrate for arginine methylation, FEBS J. 273 (2006) 3946–3961.
[37] X. Zhang, X. Cheng, Structure of the predominant protein arginine methyltransferase PRMT1 and analysis of its binding to substrate peptides, Structure 11 (2003) 509–520.
[38] K. Matsumoto, H. Nakayama, M. Yoshimura, A. Masuda, N. Dohmae, et al., PRMT1 is required for RAP55 to localize to processing bodies, RNA Biol. 9 (2012) 610–623.
[39] A. Yamaguchi, K. Kitajo, The effect of PRMT1-mediated arginine methylation on the subcellular localization, stress granules, and detergent-insoluble aggregates of FUS/TLS, PLoS One 7 (2012) e49267.
[40] D.H. Chen, K.T. Wu, C.J. Hung, M. Hsieh, C. Li, Effects of adenosine dialdehyde treatment on *in vitro* and *in vivo* stable protein methylation in HeLa cells, J. Biochem. 136 (2004) 371–376.
[41] J. Henao-Mejia, J.J. He, Sam68 relocalization into stress granules in response to oxidative stress through complexing with TIA-1, Exp. Cell Res. 315 (2009) 3381–3395.
[42] I. Goulet, S. Boisvenue, S. Mokas, R. Mazroui, J. Cote, TDRD3, a novel Tudor domain-containing protein, localizes to cytoplasmic stress granules, Hum. Mol. Genet. 17 (2008) 3055–3074.