

Effects of Adenosine Dialdehyde Treatment on *In Vitro* and *In Vivo* Stable Protein Methylation in HeLa Cells

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Adenosine dialdehyde (AdOx) is an indirect methyltransferase inhibitor broadly used in cell culture to accumulate methyl-accepting proteins in hypomethylated states for *in vitro* protein methylation analyses. In this study we included a translation inhibitor, cycloheximide, in the AdOx treatment of HeLa cells. The methyl-accepting proteins disappeared in the double treatment, indicating that they were most likely newly synthesized in the AdOx incubation period. AdOx treatment could also be used in combination with *in vivo* methylation, another technique frequently used to study protein methylation. AdOx treatment prior to *in vivo* methylation accumulated methyl-accepting proteins for the labeling reaction. The continued presence of AdOx in the *in vivo* labeling period decreased the methylation of the majority of *in vivo* methyl-accepting polypeptides. The level and pattern of the *in vivo* methylated polypeptides did not change after a 12-h chase, supporting the notion that the methylated polypeptide as well as the methyl groups on the modified polypeptides are stable. On the other hand, methylarginine-specific antibodies detected limited but consistent reduction of the methylarginine-containing proteins in AdOx-treated samples compared to the untreated ones. Thus, AdOx treatment probably only blocked a small fraction of stable protein methylation. Overall, it is likely that base-stable methylation are formed soon after the synthesis of the polypeptide and remain stable after the modification.

Key words: adenosine dialdehyde, cycloheximide, *in vivo* methylation, protein methylation.

Abbreviations: AdOx, adenosine dialdehyde; PRMT, protein arginine methyltransferase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosyl-L-homocystein; CHX, cycloheximide; ALLN, N-acetyl-leu-leu-norleucinal; sDMA, symmetric dimethylarginine; aDMA, asymmetric dimethylarginin; MMA, monomethylarginine.

Posttranslational modification of proteins can greatly enrich the diversity of the protein world beyond the combination of twenty amino acids encoded by the direction of the genetic information. Protein methylation can occur on various amino acid side chains such as glutamyl and isoaspartyl residues to form base labile methylesters or arginine or lysine residues to form chemically base-stable methylamines (1). It has been demonstrated that the majority of methyl-accepting sites are occupied in cells (2). To obtain methyl-accepting proteins with empty methyl-accepting sites for further studies, general inhibition of protein methylation can be accomplished by treating cells with methyltransferase inhibitors. For example, adenosine dialdehyde (AdOx), an indirect inhibitor that can be incorporated by cells, has been routinely used. AdOx inhibits S-adenosyl-L-homocystein hydrolase, resulting in the accumulation of S-adenosyl-L-homocystein (AdoHcy), a product inhibitor of methyltransferases that utilize S-adenosyl-L-methionine (AdoMet) as the methyl group donor (3, 4). In this way, the methyl-accepting polypeptides can be retained in hypomethyl-

ated state upon the treatment and can later be probed by *in vitro* methylation with radioactive [methyl-³H]-AdoMet.

Protein arginine methylation accounts for the majority of AdOx-accumulated stable protein methylation in rat pheochromocytoma PC12 cells (5) and human lymphoblastoid cells (6, 7). Protein arginine methylation has been implicated in signal transduction, subcellular localization, transcription as well as protein–protein interactions (8, 9). Unlike reversible modifications, such as phosphorylation of proteins by kinase and reversal of the modification by phosphatase, arginine methylation is chemically stable and irreversible. The reaction is catalyzed by a group of protein arginine methyltransferases (PRMT) that can catalyze the transfer of methyl groups from AdoMet to the side-chain ω-guanido nitrogens of arginine residues in protein substrates to form of monomethylarginines (MMA) and asymmetric N^G,N^G-dimethylarginines (aDMA; type I activity) or symmetric N^G,N^G-dimethylarginines (sDMA; type II activity) (10–16). As no demethylase has been identified, the methyl group stays on the arginyl residues of the proteins once it is added.

In vivo methylation has frequently been employed to metabolically methylate proteins in cells. In this approach, the direct methyl group donor AdoMet cannot enter cells, so its precursor [methyl-³H]-methionine is

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generally used as the source of radioactive methyl groups. To prevent the incorporation of methyl groups into proteins by translation but not posttranslational modification, *in vivo* methylation has to be conducted in the presence of protein synthesis inhibitors (17). This technique has been widely used in different cell types to evaluate the protein methylation under different treatments or conditions (18). Specific *in vivo* methylation of certain proteins such as Sam 68 can also be demonstrated directly by their labeling during the *in vivo* methylation period (19, 20).

HeLa cells have been used for hnRNP protein methylation studies (17) and other investigations related to protein methylation (20). In this study we evaluated the effect of methylation inhibitor AdOx on both the *in vitro* and *in vivo* protein methylation in HeLa cells.

MATERIALS AND METHODS

Cell Culture and Treatment—HeLa cells (ATCC CCL-2) were grown in MEM medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C in a 5% CO₂ incubator. Treatment of cells with AdOx for various time periods was performed as indicated. The cells were harvested and washed with phosphate-buffered saline, resuspended in buffer A (phosphate-buffered saline with 5% glycerol, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.5% Triton X-100 and Complete protease inhibitor cocktail [Roche]). Adenosine dialdehyde (AdOx, Sigma) was added to the medium at the indicated final concentration. Cycloheximide (CHX, Sigma) and *N*-acetyl-leu-leu-norleucinal (ALLN, Sigma) were added to the culture medium at the final concentration of 100 µg/ml and 15 µM respectively.

Extract Preparation and Methyltransferase Assay—HeLa cell extracts (20 µg of proteins) were incubated with 4 µCi of [methyl-³H]-AdoMet (60 Ci/mmol, Amersham) in a total volume of 20 µl in buffer B (50 mM sodium phosphate, pH 7.5). After incubation at 37°C for 60 min, the reaction was terminated by the addition of one third of the volume of 3× SDS sample buffer, and the samples were subjected to SDS-PAGE (12.5% acrylamide) as described by Laemmli (21). The polyacrylamide gels were stained with Coomassie Brilliant blue, destained, and treated with EN³HANCE (Du Pont NEN). Subsequently, the gels were dried and exposed to X-ray film (Kodak, MS) at -75°C for 3 d. Recombinant rat GST-PRMT1 and mouse fibrillar protein were prepared as described (7, 22).

Western Blotting—Equal amounts of protein (30 µg) were separated by 12.5% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Gelman Science). The membranes were blocked in 5% skimmed dry milk in TTBS (10 mM Tris-HCl, pH = 7.5; 100 mM NaCl; 0.1% tween 20) for 30 min, incubated with primary antibodies (1:200 dilution for 7E6 antibody from Abcam; 1:550 dilution for SYM10, 1:900 dilution for SYM11 and 1:900 dilution for ASYM24 from Upstate) for 1 h, washed three times in TTBS, then incubated with secondary antibody (anti-mouse or rabbit IgG horse radish peroxidase conjugate from Sigma) for 1 h. Chemiluminescent detection was performed using the Supersignal kit according to the manufacturer's instructions (Pierce).

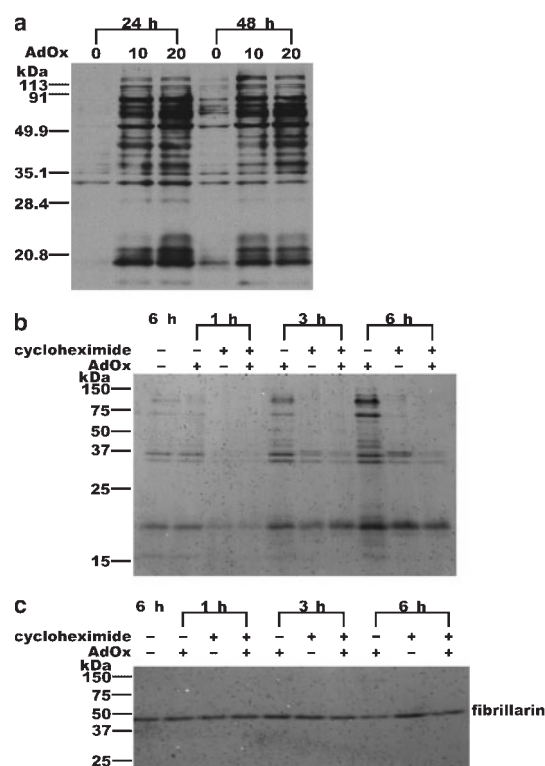


Fig. 1. Treatment of HeLa cells with AdOx and cycloheximide. (a) HeLa cell extracts were prepared from cells grown in the presence of 0, 10, 20 or 40 µM AdOx for 24 and 48 h. Accumulation of methyl-accepting proteins in HeLa cells was evaluated by *in vitro* methylation reaction as described in "MATERIALS AND METHODS." (b) HeLa cell extracts were prepared from cells incubated in the presence of both 20 µM AdOx and 0.08 µg/ml of cycloheximide or only either one for 1, 3 or 6 h. *In vitro* methylation of the extracts without the addition of exogenous substrate was performed as described. (c) Methylation of the extracts was performed with recombinant mouse fibrillar protein (1 µg) as the exogenous methyl-accepting substrate. The molecular mass standards are indicated on the left in kilodaltons.

***In Vivo* Methylation**—HeLa cells grown to near confluency were treated with AdOx for the indicated times. Cycloheximide (100 µg/ml) and chloramphenicol (40 µg/ml) were added 30 min prior to the labeling. For *in vivo* methylation, the medium was replaced with DMEM medium without methionine (GIBCO), 10% FBS (GIBCO, dialyzed), [methyl-³H] methionine (10 µCi/ml) and translation inhibitors for the indicated labeling time. The cells were then collected, lysed by SDS-PAGE sample buffer and analyzed by SDS-PAGE and fluorography.

RESULTS

The Translation Inhibitor Cycloheximide Block AdOx-Accumulated Methyl-Accepting Proteins—We first incubated HeLa cells with various concentrations of AdOx for different time periods to determine the best treatment time and dose of AdOx. Total cell extract was prepared, and *in vitro* methylation was performed to examine the level of methyl-accepting proteins accumulated upon the treatment. As shown in Fig. 1a, when no AdOx was present in the cell culture, a few methyl-accepting

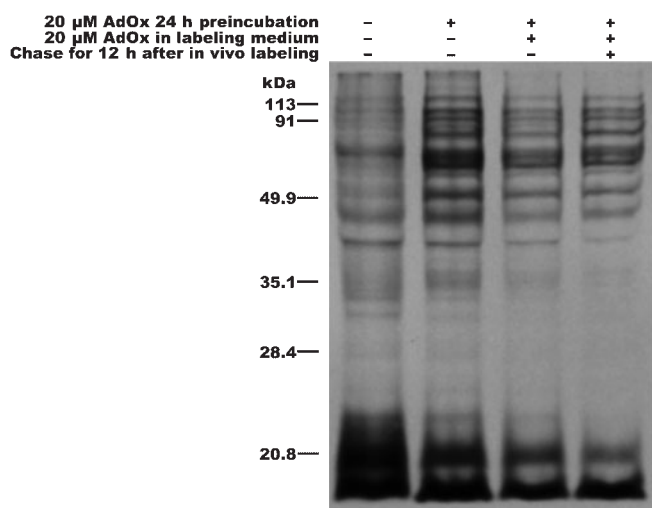


Fig. 2. *In vivo* methylation of HeLa cells treated with AdOx. *In vivo* methylation was performed as described in "MATERIALS AND METHODS." HeLa cells were *in vivo* labeled either by pretreatment with AdOx for 24 h, or by incubation in the labeling medium containing AdOx. Cells were also chased for 12 h in non-radioactive medium after *in vivo* labeling. Cell lysates were collected, separated by SDS-PAGE, and the *in vivo* methylated proteins were detected by fluorography.

polypeptides were modified only to a limited level, demonstrating that normally the methyl-accepting sites were mostly occupied. Addition of AdOx clearly enhanced the methylation signals of the methyl-accepting polypeptides that can be detected without AdOx treatment. AdOx concentration of 20 μ M for 24 h was enough to reach the maximum accumulation of methyl-accepting proteins in cells.

The origin of the hypomethylated proteins accumulated upon AdOx treatment was further studied. HeLa cells were incubated with a translation inhibitor, cycloheximide (CHX), at the same time as the AdOx treatment. Since cells appeared to die upon double treatment for 12 h, we performed the experiments for periods of 1, 3 and 6 h. As shown in Fig. 1b, the AdOx treatment can accumulate methyl-accepting proteins in short intervals of 3 and 6 h. However, the presence of CHX completely blocked the accumulation of methyl-accepting proteins by AdOx treatment at 3 and 6 h. Cycloheximide treatment did not effect the type I arginine methyltransferase activity in HeLa cells as assayed by the addition of an exogenous methyl-accepting protein, fibrillarlin (Fig. 1c). Thus the disappearance of methyl-accepting proteins by CHX treatment is probably due not to reduced protein methyltransferase activity in the extract but to the blockage of new protein synthesis. The results indicated that the methyl-accepting proteins accumulated for *in vitro* methylation by AdOx treatment are most likely to be newly synthesized during the incubation period.

Effects of AdOx Treatment on *In Vivo* Methylation—*In vivo* protein methylation in mammalian cells with [methyl- 3 H] methionine has been used to evaluate the protein methylation under native conditions (17, 18). To prevent the incorporation of radioactive methyl groups into proteins by translation but not posttranslational modification, protein synthesis inhibitors have to be

included throughout the *in vivo* methylation period. Various methyl-accepting proteins were detected in earlier studies, which somehow seems not to agree with our results in Fig. 1b suggesting that methyl-accepting sites are on newly synthesized proteins. We performed *in vivo* methylation in HeLa cells following the protocol of Liu *et al.* (17) and as reported previously, detected many labeled polypeptides (Fig. 2). We then treated the cells with AdOx prior to the *in vivo* methylation. Except for few polypeptides, the pattern of the signals of AdOx-pretreated cells was basically the same as that of the untreated ones. However, addition of AdOx for 24 h before *in vivo* methylation clearly increased the labeling of polypeptides of molecular mass between 40 to 100 kDa. No new protein synthesis was detected under the *in vivo* methylation conditions as monitored by incubation with 35 S-Met (data not shown). The methyl groups were probably present on the unmodified proteins newly synthesized before the *in vivo* methylation period in the presence of CHX.

In addition, when AdOx was present in the labeling medium after the 24-h pretreatment, the elevated *in vivo* methylation pattern appeared to be suppressed back to a low level. When the *in vivo* labeling was followed by a 12-h chase in the non-radioactive medium, the majority of the *in vivo* labeled polypeptides were of similar level, indicating that the modified proteins were rather stable. Similar results were obtained from the 12-h chase experiments of no AdOx treatment or AdOx-pretreatment/labeling samples (data not shown).

The Level of Methylarginine-Containing Proteins in AdOx-Treated or Untreated Cell Extracts Detected by Methylarginine-Specific Antibodies—AdOx can accumulate proteins in hypomethylated states for *in vivo* and *in vitro* methylation. Nevertheless, the fraction of the AdOx-affected polypeptides in the whole methyl-modified protein is unknown. Protein arginine methylation accounts for the majority of AdOx-stable protein methylation in rat pheochromocytoma PC12 cells (5) and human lymphoblastoid cells (6, 7). We also found that the major methyl-accepting substrates accumulated by AdOx treatment can be recognized by the predominant protein arginine methyltransferase PRMT1 and are likely to be modified to form methylarginines (data not shown). We thus determined the level of total methylarginine-containing proteins in the extracts by Western blot analysis. Methylarginine-specific antibodies SYM 10, SYM11 and ASYM24 are rabbit polyclonal antibodies against alternative RG sequences containing symmetric dimethylarginine (sDMA), SmD3 RG sequences containing sDMA, and alternative RG sequences containing asymmetric dimethylarginine (aDMA) in all the arginyl positions, respectively (23). Another antibody, 7E6, is specific for monomethylarginine (MMA) and aDMA (24). As shown in Fig. 3, reduced recognition of certain specific polypeptides by SYM10, SYM11, ASYM24 was observed for the AdOx-treated cell extracts. For SYM10 recognition, the intensity of the strongest 30-kDa signal was the same in both samples, while most of the signals above 36 kDa were reduced in the AdOx-treated sample (Fig. 3a). For SYM11, except for the highest signal above 113 kDa, most of the signals were slightly stronger in the untreated sample (Fig. 3b). The polypeptides with molec-

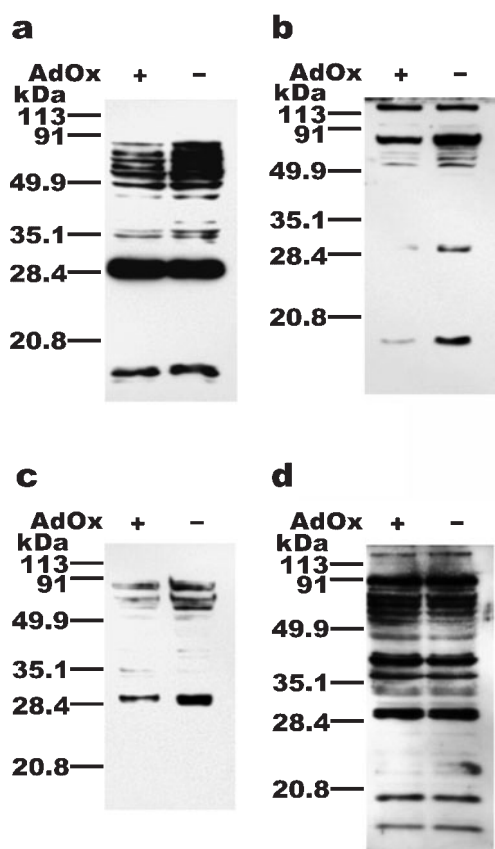


Fig. 3. **Detection of methylarginine-containing polypeptides by methylarginine-specific antibodies.** HeLa cell extracts of AdOx-treated or untreated cells were immunoblotted with commercially available methylarginine-specific antibodies SYM10 (a), SYM11 (b), AYM 24 (c) and 7E6 (d).

ular mass of 80, 28 and 16 kDa are probably the previously identified p80 coilin, SmB and SmD3, respectively (25). As for AYM24, generally the signals appeared to be slightly reduced in the AdOx-treated samples, probably including Sam68 at the molecular mass of 68 kDa (20). However, no difference was detected between the AdOx-treated and untreated cell extracts using the 7E6 methylarginine-specific antibodies (Fig. 3d). Whether this antibody might cross-react with certain other epitopes, thus masking minor differences in the level of methylarginines, needs more investigation.

Treatment of HeLa Cells with a Proteasome Inhibitor ALLN Does Not Affect AdOx-Mediated Accumulation of Hypomethylated Proteins—The *in vivo* labeled proteins appeared to be rather stable in our 12-h chase period. We then investigated whether the normally methylated proteins might be degraded rapidly when rendered unmethylated by AdOx-treatment. *N*-acetyl-leu-leu-norleucinal (ALLN) is an inhibitor that blocks proteolytic activity of both type I calpain and the 26 S proteasome. We treated the cells with ALLN to evaluate the effect of protein degradation on the accumulation of methyl-accepting sites by AdOx treatment. No significant changes to the AdOx induced hypomethylation pattern upon the addition of ALLN for 24 h were observed (Fig. 4). Thus, under our experimental conditions, ALLN-blocked proteasome deg-

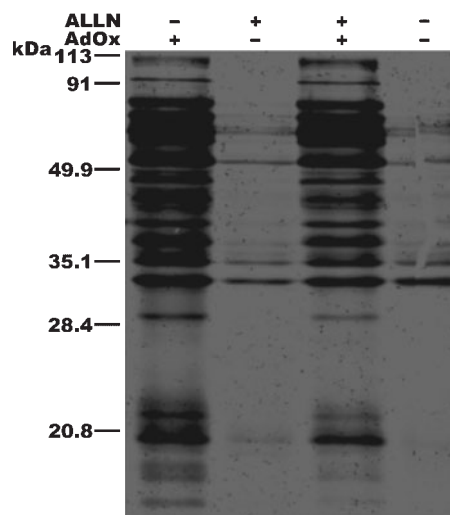


Fig. 4. **Treatment of HeLa cells with AdOx and ALLN.** HeLa cell extracts were prepared from cells incubated in the presence of 20 μ M AdOx or 15 μ M ALLN, or both for 24 h. *In vitro* methylation reaction of the cell extracts was performed as described in "MATERIALS AND METHODS."

radation has no effect on the level of hypomethylated polypeptides.

DISCUSSION

In this study we demonstrated the best treatment time and dose of AdOx, a frequently utilized general protein methylation inhibitor, to accumulate methyl-accepting proteins in HeLa cells for further *in vitro* methylation. We showed that AdOx-accumulated hypomethylated polypeptides for *in vitro* methylation disappeared in the presence of a protein synthesis inhibitor, CHX, indicating that they were probably newly translated in the AdOx-incubation period. Since methyl-accepting sites on polypeptides are generally occupied in cells, it is likely that protein methylation occurs soon after the synthesis of the polypeptide and these sites are occupied thereafter.

In vivo protein methylation to label methyl-accepting proteins metabolically has been performed with protein translation inhibitors in various cell lines (17–20). The proteins that can be *in vivo* labeled thus are likely to be restricted to those that are newly synthesized but have not been modified before the labeling period. Interestingly, we demonstrated that *in vivo* methylation could also be affected by AdOx treatment. Firstly, pre-treatment (24 h) with AdOx increased the level of *in vivo* methylation. The pattern of the signals was similar to that of the untreated control, indicating that AdOx can accumulate methyl-accepting proteins in more hypomethylated states not only for *in vitro* methylation, but also for *in vivo* methylation. Secondly, when AdOx was present throughout the *in vivo* methylation period, the level of methylated proteins was reduced. Furthermore, when the *in vivo* labeled proteins were chased in the non-radioactive medium for 12 h, the radioactive proteins were of the same level as the non-chase samples. These results indicated that the methylated polypeptides were stable and no demethylation occurred in the chase period.

In comparison, the proteasome inhibitor ALLN did not change the appearance of methyl-accepting proteins upon AdOx treatment. This result indicated that the accumulated hypomethylated proteins probably are not subject to rapid proteasome degradation, otherwise inhibition of protein degradation by ALLN should accumulate more of these sites.

Our results represent the base-stable methylation of the AdOx-accumulated methyl-accepting proteins catalyzed by all endogenous methyltransferases in the extracts. Base-labile methylation that cannot survive the mild alkaline conditions of SDS-PAGE, such as carboxyl methylation, can be excluded; but C-terminal carboxyl methylation and N-methylation such as lysyl or arginyl methylation could not be distinguished (1). The irreversible modification on the guanidino nitrogen of the arginyl residues is known to account for the majority of the AdOx-accumulated stable protein methylation events in PC12 and lymphoblastoid cells (5, 6). We detected limited but consistent reduction of the methylarginine-containing proteins in AdOx-treated samples compared to untreated ones by use of sDMA- and aDMA-specific antibodies. Specifically, decreased signals that probably correspond to methylation of coilin, SmB and Sm D3 were detected in AdOx-treated extracts. It is possible that since the cells treated with AdOx are of high confluency, most of the methyl-accepting proteins are already synthesized and methylated. If they are stable and turn over slowly, as indicated by the chase *in vivo* methylation experiments, the newly synthesized proteins will be responsible for only a small fraction of the total pool of the methyl-accepting proteins in the cell, and the difference will be barely detectable. This is consistent with our inability to detect differences between the overall protein patterns from AdOx treated cells and untreated cells by two-dimensional gel electrophoresis (26). Thus, AdOx probably only affects a small portion of the total methyl-containing proteins, at least for the methylarginine-containing proteins.

However, application of AdOx to inhibit specific protein methylation has already been shown in different cells. For example, we demonstrated that certain methylarginine-containing trypsin-digested peptides of hnRNPA2/B1 could be detected from AdOx-untreated lymphoblastoid cells but not from AdOx-treated cells (26). Nichols *et al.* also showed that of AdOx could affect the subcellular localization of hnRNPA2/B1 in HEK293 cells, presumably by affecting the methylation in the RGG domain (27). These investigations support the appropriateness of applying of AdOx treatment in specific protein methylation studies.

In summary, although AdOx treatment had been frequently used to accumulate methyl-accepting proteins for further *in vitro* methylation, the protein methylation inhibition is restricted to newly translated proteins during the treatment, which blocks only a small fraction of protein methylation in cells. In this study, we also show that AdOx can be applied to *in vivo* methylation as well as *in vitro* methylation. The timing of the AdOx treatment can be adjusted to enhance or to inhibit the *in vivo* methylation. In combination with chase experiments, *in vivo* methylation can be applied to follow the methylation of methyl-accepting proteins.

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