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Developmental regulation of N-terminal H2B methylation in *Drosophila melanogaster*

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ABSTRACT

Histone post-translational modifications play an important role in regulating chromatin structure and gene expression *in vivo*. Extensive studies investigated the post-translational modifications of the core histones H3 and H4 or the linker histone H1. Much less is known on the regulation of H2A and H2B modifications. Here, we show that a major modification of H2B in *Drosophila melanogaster* is the methylation of the N-terminal proline, which increases during fly development. Experiments performed in cultured cells revealed higher levels of H2B methylation when cells are dense, regardless of their cell cycle distribution. We identified dNTMT (CG1675) as the enzyme responsible for H2B methylation. We also found that the level of N-terminal methylation is regulated by dART8, an arginine methyltransferase that physically interacts with dNTMT and asymmetrically methylates H3R2. Our results demonstrate the existence of a complex containing two methyltransferases enzymes, which negatively influence each other's activity.

INTRODUCTION

In the eukaryotic nucleus DNA is packaged into chromatin by its association with the basic core histones. The folding of chromatin fibers has a major impact on many aspects of nuclear function and is regulated by the post-translational modification of the histone tail domains. In multicellular organisms malfunction of the enzymatic machinery that establishes these modifications leads to abnormalities such as failures in embryonic development, cancer and other diseases (1–4). Histone modifications have been shown to mark specific chromosomal domains and serve as an indexing system of the genome to distinguish transcriptionally active from inactive regions (5–9). In addition, they also play a role during histone deposition (10–14) where an ordered appearance and removal of distinct modifications is required for proper chromatin assembly. The modifications on the histone N-terminal tails are recognized by specialized proteins that selectively bind modified histones (15,16). The specific binding to particular modifications can then either lead to structural changes of chromatin or recruit enzymatic activities to specific loci, which in turn can either stimulate or inhibit a subsequent modification. Examples of this phenomenon are the stimulation of the acetylation of H3K14 by a phosphorylation of H3S10 (17,18), the inhibition of H3K4 methylation by an adjacent dimethylation of H3R2 (19,20) or the inhibition of H3K4 demethylation by the phosphorylation of H3T6 (21). The two modifications that influence each other do not have to reside on the same molecule as it has been demonstrated that the ubiquitination of H2B by Rad6 facilitates the methylation of H3K4, suggesting a crosstalk of the two histone tails (22,23). Another example for such a crosstalk is the phosphorylation of H3S10 by the Pim1 kinase, which stimulates the acetylation of H4K16 (24).

Most of the global histone modification analyses done so far were performed on the two core histones H3 and H4 whereas the post-translational modifications of canonical H2A and H2B have been less well studied in metazoa. Only the ubiquitination of H2A and H2B has been suggested to have a specific function such as the silencing of genes (25) or the stimulation of H3K4 and H3K79 methylation, respectively (22,23,26). Human H2B is phosphorylated at S14 by the caspase cleaved mammalian Mst-1 kinase (27). This phosphorylation has been proposed to mediate chromatin condensation during apoptosis (27), which is counteracted by the acetylation of the adjacent K15 (28). H2A is phosphorylated at S1 (29) but so far this

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has not been shown to be regulated (29,30). The analysis of H2A and H2B methylation and acetylation in higher eukaryotes by mass spectrometry (MS) has been severely hampered by the multitude of different isoforms with similar molecular masses making it difficult to distinguish post-translational modifications from sequence variants (31). *Drosophila melanogaster*, in contrast to most higher organisms, has just a single H2B variant, which greatly facilitates the analysis of H2B PTMs.

Drosophila melanogaster H2B is ubiquitinated at K120 (32,33), phosphorylated at S33 (34) and methylated at the N-terminal proline (35). Although the methylation of the terminal α-amino group has been found in H2B of a variety of organisms and a number of proteins other than histones (36), its biological significance is largely unknown. *Drosophila* tissue culture cells show an increased proline methylation in response to heat shock and arsenite treatment (35,37). More recently, the N-terminal methylation of mammalian RCC1 has been shown to be crucial for its binding to chromatin and for proper mitotic chromosomal segregation (38). A pair of ortholog enzymes that perform N-terminal methylation of proteins in humans and yeast, NRMT (METTL11A) and YBR261C/Tae1, respectively, has been also isolated (36,39).

Here, we show that N-terminal methylation of H2B in *D. melanogaster* is not only regulated by cellular stress such as heat shock but also changes during development. In tissue culture cells the proportion of methylated histone depends on cell density, but not on the cell cycle distribution. Knockdown experiments and *in vitro* assays demonstrate that *Drosophila*'s ortholog of NRMT, dNTMT (CG1675) mono- and di-methylates the N-terminus of H2B. Interestingly, dNTMT forms a complex with an arginine-specific H3 methyltransferase dART8, which targets H3R2. Although the interaction has no effects on the activity of the enzymes *in vitro*, modulation of dART8 levels in cells has a substantial effect on H2B N-terminal methylation *in vivo*. A knockdown of dART8 results in an increase of H2B methylation whereas overexpression leads to a reduction, suggesting a repressive effect of dART8 levels on the modification of the H2B N-terminus.

MATERIALS AND METHODS

Cell culture

SL2 and Kc cells were maintained at 26°C in Schneider's *Drosophila* medium with glutamine (GIBCO) supplemented with 10% heat-inactivated FBS (Sigma) and 50 U/ml penicillin and 50 μg/ml streptomycin (CC Pro, Oberdorla, Germany). Except otherwise stated, cells were kept exponentially growing by diluting the cultures every 2–3 days with fresh medium to a density of 0.5×10^6 cells/ml. For each experiment, cells were harvested by centrifugation of the suspensions at 500*g* during 5 min (r.t.) and decanted. Then, the same number of cells per sample (typically, $4–8 \times 10^6$ cells) was seeded with a volume-to-surface ratio of 0.24 ml/cm^2.

Cell density and heat shock experiments

Exponentially growing cells were collected as indicated above, resuspended in fresh medium at the indicated densities and cultured for further 24 h (except otherwise stated) before harvesting. For heat shock experiments, exponentially growing cells were incubated at 37°C for 1 h. Histones were isolated immediately after heat shock.

Cell cytometry

One to two million cells per sample were harvested, washed once with PBS, resuspended in 0.17 ml ice-cold PBS and fixed by addition of 0.33 ml ice-cold absolute ethanol for 30 min. After fixation, each sample was washed twice with PBS, resuspended in 0.5 ml PBS and treated with 50 μg RNAse A for 15 min. After cooling on ice, DNA was stained with 2 μg propidium iodide (Sigma). Measurements were performed in a FACS Canto cytometer (BD Biosciences) equipped with a 488 nm laser. Typically, 3×10^4 events per sample were acquired.

Drug treatments

Sodium butyrate (Merck) was dissolved in milliQ water (2.0 mM final concentration) and rapamycin (Sigma) was reconstituted in ethanol (10 μM final concentration). Both solutions were sterile filtered (0.22 μm), aliquoted and stored at −20°C. For the experiments, cells were harvested, seeded in fresh medium (Kc cells: 0.5×10^6 cells/ml; SL2 cells 1×10^6 cells/ml) and treated with the indicated concentration of the chemical or the solvent only. Histones were isolated at the indicated time points after drug addition.

Histone extraction from cultured cells

Histone acid extraction was performed as previously described (40) with minor modifications. Typically, $4–8 \times 10^6$ cells were harvested as indicated above and subsequently washed with ice-cold RSB (10 mM Tris pH = 7.5, 10 mM NaCl, 3 mM $MgCl_2$). If necessary, at this point samples were stored at −20°C. Washed pellets were resuspended in 0.5 ml, 0.5M HCl and rotated at 4°C for 1 h. Solutions were cleared by centrifugation (13000*g* × 5 min × 4°C) and dialysed against 0.1 M acetic acid at 4°C (MWCO 6000–8000). Dialysates were freeze-dried and reconstituted in 50 mM Tris pH = 6.8 containing 5 mM tris(2-carboxyethyl)phosphine hydrochloride (Pierce) by using 6.2 μl buffer per million cells. For the isolation of histones using MNase, nuclei were isolated from SL2 cells using a nuclear extraction buffer (1 × PBS, 0.3% Triton X−100) and digested using 500mU of MNase (Roche) per 10^7 cells for 10 min at 26°C. To isolate chromatin, the MNase reaction was quenched using 0.5 vol of 0.5 M EGTA and nuclear debris was centrifuged for 20 min at 800 g. The released chromatin fibers were harvested and stored at 4°C.

Histone acid extraction from animals

All the samples were snap-frozen in liquid nitrogen immediately after collection. Embryos 0–24 h after egg laying were washed, dechorionated according to (40). For later

stages 50–100 unsorted larvae and 30–40 adult flies were pooled. Histones from frozen animals and frozen dechorionated embryos were grinding the frozen samples in either hydrochloric acid (0.5 M HCl, 5 mM DTT) or by sulfuric acid (0.2 M H_2SO_4, 1 mM DTT). After extraction histones were either dialysed against 0.1M acetic acid (HCL extraction) or precipitated by addition of 10 vol acetone and incubation at 4°C overnight (sulfuric acid extraction). Lyophilized samples were reconstituted in 300 µL 1 × Laemmli buffer whereas the acetone precipitated histones were reconstituted in 50 µl (sulfuric acid extraction) 50 mM Tris pH = 6.8 containing 5 mM tris(2-carboxyethyl)phosphine hydrochloride

Histones from staged embryos

Nuclei from staged embryos (2–3 h, 3–6 h, 6–9 h and 9–12 after egg laying) were prepared as described before (41) and histones were extracted with 3 vol nuclear pellet of 0.4 MH_2SO_4. Extracts were dialysed as indicated above, freeze-dried and reconstituted in 20 mM Tris pH = 6.8 containing 1 mM DTT.

H2B isolation and MS

Proteins in the extracts were separated by SDS–PAGE 18% acrylamide. After electrophoresis, gels were stained with Coomassie Brilliant Blue G250 (Merck) and bands corresponding to H2B were excised. Gel bands were destained, acylated and digested with trypsin as previously described (42). Alternatively, once the bands were destained, samples were digested with 40 ng Asp-N (Roche) at 25°C over night using 0.1 M Tris pH = 8.5 as reaction buffer. The reaction products were desalted with ZipTip µ-C18 (Millipore) mixed with a saturated α-cyano-hydroxycinnamic acid (Sigma) solution and immediately spotted onto a stainless steel target plate. Spectra were acquired on a Voyager DE STR workstation (Applied Biosystems). Integration of the signals corresponding to each peptide (isotopic cluster area) was automatically performed by the Data Explorer software (Applied Biosystems) excluding all the peaks whose signal-to-noise ratio was smaller than 4 (8,9).

MS/MS

For MS/MS analysis a third of the digest was typically injected in an Ultimate 3000 HPLC system (LC Packings Dionex). Samples were desalted on-line by a C18 micro column (300 µm i.d. × 5 mm, packed with C18 PepMap™, 5 µm, 100 Å by LC Packings), and peptides were separated with a gradient from 5% to 60% acetonitrile in 0.1% formic acid over 40 min at 300 nl/min on a C18 analytical column (75 µm i.d. × 15 cm, packed with C18 PepMap™, 3 µm, 100 Å by LC Packings). The effluent from the HPLC was directly electrosprayed into the LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). The MS instrument was operated in the data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 300–800 for H2B N-termini in Figure 1; m/z 572–590 H2B N-termini in Supplementary Figure S2; m/z 300–2000 for H3 in Figure 6) were acquired in the Orbitrap with resolution R = 30 000 (Figure 1 and Supplementary Figure S2) or 60 000 (Figure 6) at m/z 400. The three most intense peptide ions with charge states between two and five were sequentially isolated (window = 2 m/z) to a target value of 10 000 and fragmented in the linear ion trap by collision-induced dissociation (CID). Fragment ion spectra were recorded in the Orbitrap part of the instrument. For all measurements with the Orbitrap detector, three lock-mass ions from ambient air (m/z = 371.10123, 445.12002, 519.13882) were used for internal calibration as described (43). Typical mass spectrometric conditions were: spray voltage, 1.4 kV; no sheath and auxiliary gas flow; heated capillary temperature, 200°C; normalized collision energy, 35% for CID in linear ion trap. The ion selection threshold was 10 000 counts for MS2. An activation q = 0.25 and activation time of 30 ms were used.

Protein cloning and expression of Flag-dNTMT

Flag-dNTMT (accession number NP_610528) was amplified from the FMO4789 expression plasmid using oligonucleotides: fw: 5′-TTTGAATTC**GATTACAAGGATGATGATGATAAG**GGGACGACTACATTAGAAGAGCAGCTTTCAGAC (FLAG tag in bold); rev: TTTAAGCTTCTATTCCTTGGAGACGGGTTTGCAGG. The PCR product was cloned into pET28a (Novagen) using unique EcoRI and HindIII sites. For expression in SL2 cells we have used the pMCFHBD (FMO4789) vector that was acquired from the DGRC gold collection. Full length *Drosophila* dART8 (accession number NP_609478) was amplified and cloned into pETG-N-GST (gift of A. Baiker, University of Munich) for bacterial expression and in pMT-Flag-HA [derived from pMT-HA (44)] for expression in SL2 cells. Primer sequences for expression constructs and knockdowns of dARTs and dNTMT are available upon request.

Immunohistochemistry

Drosophila L2-4 cells (Schneider S2 derivative) were transiently transfected with the FMO4789 dNTMT expression plasmid (DGRC) using Effectene (Qiagen) according to the manufacturer's instructions. Expression was induced 2 days after transfection by the addition of copper sulfate (0.25 mM final). After an additional 48 h cells were fixed with formaldehyde, permeabilized with Triton X−100, incubated with anti-FLAG M2 antibody (Sigma). After incubation with fluorescently labeled anti-mouse secondary antibody and DNA counterstaining with ToPro-3, cells on coverslips were mounted with Slow Fade Gold reagent (Invitrogen). Images were recorded using a Zeiss 510 LSM confocal microscope and processed using ImageJ software.

Coimmunoprecipitation of dART8 and dNTMT

SL2 cells stably expressing N-terminal FLAG-HA-tagged dART8 under a metallothioneine promoter were induced with 0.25 mM copper sulfate 24 h before harvest. Cells were lysed in hypotonic buffer by the addition of Triton X-100 at a final concentration of 0.02%. After lysis, KCl concentration was raised to 110 mM and proteins were

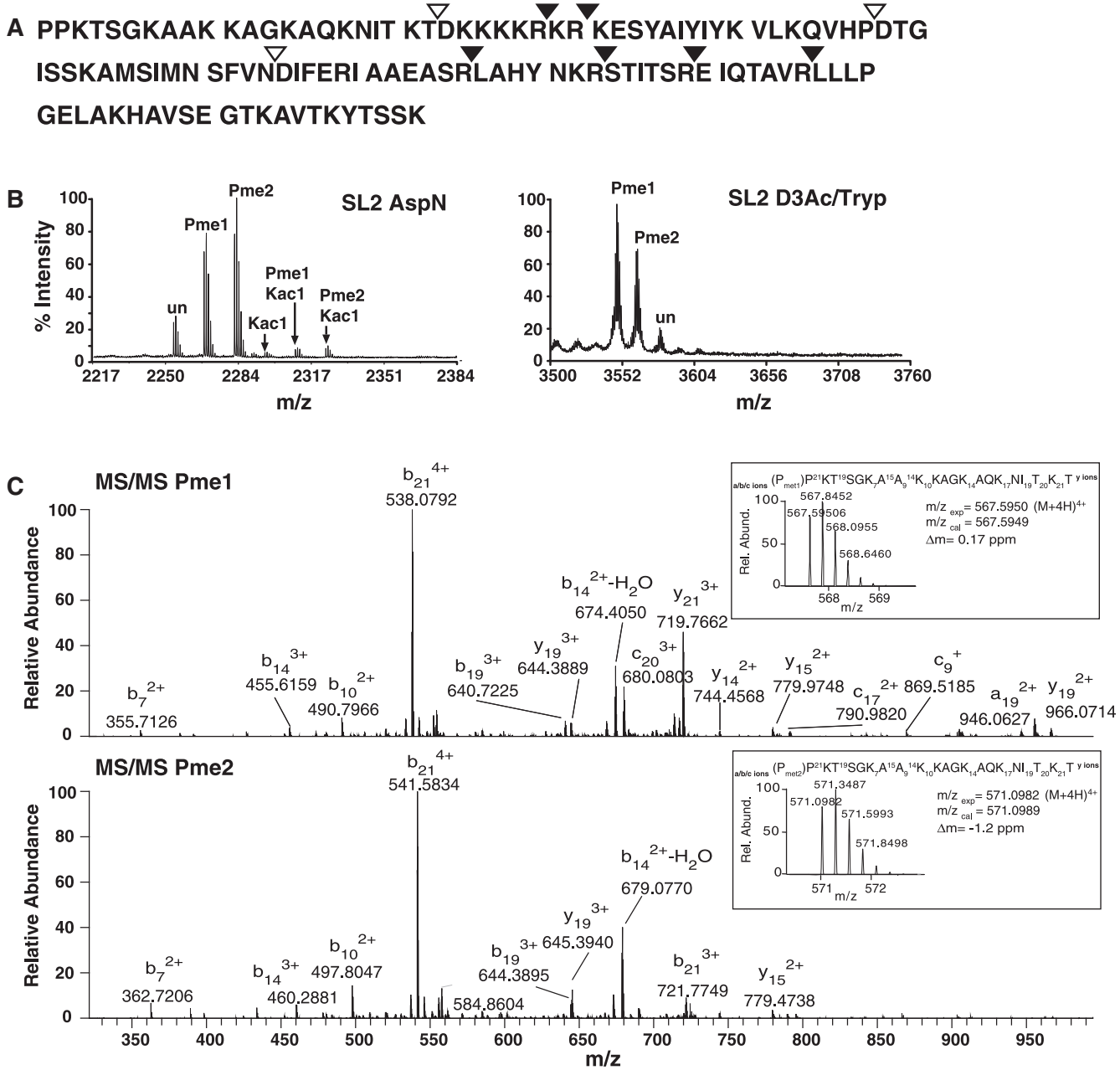

Figure 1. H2B in *D. melanogaster* is methylated at the N-terminus. (**A**) Sequence of the fruit fly's protein H2B. Open triangles show the cutting sites for the protease Asp-N, whereas the filled triangles label the cutting sites for trypsin after the free amino groups of the sample have been blocked by treatment with an anhydride (either D6-acetic or propionic anhydride). (**B**) MALDI-TOF spectra of digested H2B isolated from SL2 cells. The left panel shows the signals corresponding to the peptide 1–22, which are obtained after Asp-N digestion of H2B. The right panel displays the signals corresponding to the peptide 1–28, obtained after treatment of the protein with D6-acetic anhydride followed by trypsin digestion. un, unmodified proline; Pme1, monomethylated proline; Pme2, dimethylated proline; Ac1, one acetylated lysine. (**C**) Tandem MS spectra of the peptide 1–22 demonstrate that the observed mono- (upper panel) and dimethylation (lower panel) take place at P1. The mass of the parent ions and the fragment ions were measured with a resolution of 30 000 and 15 000, respectively. The inserts display the signals of the corresponding parent ions and the errors respect to the expected (calculated) values.

extracted by the addition of ammonium sulfate (1/10th volume of a 4 M stock). After centrifugation the supernatant was dialysed against a 150 mM KCl buffer, precipitate was removed by centrifugation yielding the extract used for coimmunoprecipitation. Extract from the equivalent of 5×10^8 cells was used for each immunoprecipitation. A mix of rat monoclonal antibodies of the subtype IgG2a specific for dNTMT (clones 3F4, 4B9, 9E5) was used for dNTMT IPs. A rat monoclonal antibody of equal subtype specific for the Xenopus laevis Smad Interacting Protein was used as a negative control. Sigma mouse anti-FLAG M2 agarose was used to precipitate FLAG-HA-dART8. The immunoblot was also performed with the above mentioned antibodies.

RESULTS

H2B is mono and dimethylated at the N-terminal proline

To determine the modifications present in H2B from *D. melanogaster*, the protein was isolated from asynchronously growing SL2 and Kc cells, separated using SDS–PAGE, digested and analyzed by MS. As H2B is rich in arginine and lysine residues, we either used trypsin after acylation of lysine residues (42) or Asp-N as proteases of choice (Figure 1A). By the combination of the two proteases we could achieve a sequence-coverage of 95% (Table 1). We clearly detected a large proportion of the N-terminal peptide generated by an Asp-N or trypsin after acylation ($MH^+ = 2253.34$ for Asp-N and 3532.22 for trypsin after acetylation with D6-acetic anhydride) in a mono- or dimethylated form (Figure 1B).

Additionally, a part of the N-terminal tail is mono or diacetylated (Figure 1B). In order to determine the exact positions of the modifications, Asp-N digested H2B was submitted to a MS/MS analysis. Fragmentation patterns allow the assignment of the mono and dimethylation to position 1, which is in agreement with previous reports that described a methylation of the N-terminal proline *Drosophila* (35) (Figure 1C). The acetylation resides predominantly at position K11 (mono) or K11 and K17 (di) with minor acetylations at K10 and 14 (Supplementary Figure S1).

Methylation of H2B increases during development

In order to study the biological function of H2B N-terminal methylation we measured its level during different developmental stages of *D. melanogaster*. We isolated and analyzed the modification pattern of H2B from embryos at different time points after egg laying (a.e.l.), 3rd instar larvae and adult flies (Figure 2). Interestingly, we observed mostly unmodified H2B in embryos (0–24 h a.e.l.) whereas in adult animals mono- as well as dimethylations were very abundant (Figure 2A and B). This finding suggests a relationship between H2B methylation and cell proliferation, senescence or differentiation. We therefore wondered whether levels of H2B N-terminal methylation are indicative of the proliferative capacity or the differentiated state of cells.

The methylation pattern in cultured cells depends on cell density

To investigate this, we measured the N-terminal methylation of H2B in *Drosophila* tissue culture cells that were initially established from late embryos (45,46). Due to their embryonic origin we expected H2B from these cell lines to have a low level of methylation. However, when we analyzed H2B from SL2 or Kc cell lines we consistently detected a higher proportion of methylated H2B molecules compared to the developmental stage they were derived from (compare Figure 2 and Figure 3). This suggests that higher amounts of H2B methylation either reflect an adaptation to tissue culture conditions or an outgrowth of a highly methylated minor cell clone during the establishment of the cell line. In culture, the proportion of methylated H2B depends on the cell density, as the methylation levels increase when cells are seeded at higher densities (Figure 3A). As SL2 and Kc cells accumulated in G2/M when seeded at such high densities (Figure 3B and data not shown) we wondered whether we could simulate the increase in methylation by treatment of the cells with rapamycin, a drug that inhibits the highly conserved nutrient-sensing TOR signaling pathway (47) and led to a similar effect on the cell cycle distribution of Kc and SL2 cells (Figure 3D and data not shown). Although the cell cycle distribution was clearly affected under the conditions we used (Figure 3D), we could not observe significant changes in the methylation pattern of H2B (Figure 3C) even after an extended period of treatment. These experiments suggest that neither the cell cycle arrest nor inhibition of TOR signaling is sufficient to alter the N-terminal methylation of H2B. It had been shown previously that the methylation of the N-terminal proline is stimulated by heat shock (35). To check if this is also the case in our system, we subjected SL2 cells to a 1-hr heat shock at 37°C, isolated the histones via acid extraction and analyzed the levels of H2B N-terminal methylation. In accordance with data previously published (35,37) we detected an increase of H2B methylation after heat shock, which is similar to the one seen when growing cells at higher density. In summary, we concluded from these experiments that the N-terminal methylation of H2B is able to integrate several external signals such as proliferative or physiological stress.

dNTMT (CG1675) is the enzyme responsible for H2B methylation

We next asked which enzyme is responsible for the N-terminal methylation of H2B in *Drosophila*. Recently the enzyme that methylates the N-terminus of RCC1 was identified in human and yeast (36,39) and therefore the *Drosophila* ortholog (CG1675) was a good candidate

Table 1. Predicted and detected peptide fragments of dH2B after digestion with proteases

Asp-N	$[M+H]^+$	Detected	Modifications	Trypsin/D3-acetylated	$[M+H]^+$	Detected	Modification
1-22	2253.34	+	me1, me2, ac1, ac2	1–28	3532.22	+	me1 me2
23-47	3146.8	–		29–30	348.25	–	
48-64	1801.58	+		31–69	4702.45	+	
65-122	6412.4	–		70–76	717.39	+	
				77–83	946.53	+	
				90–96	664.36	–	
				97–122	2908.66	+	

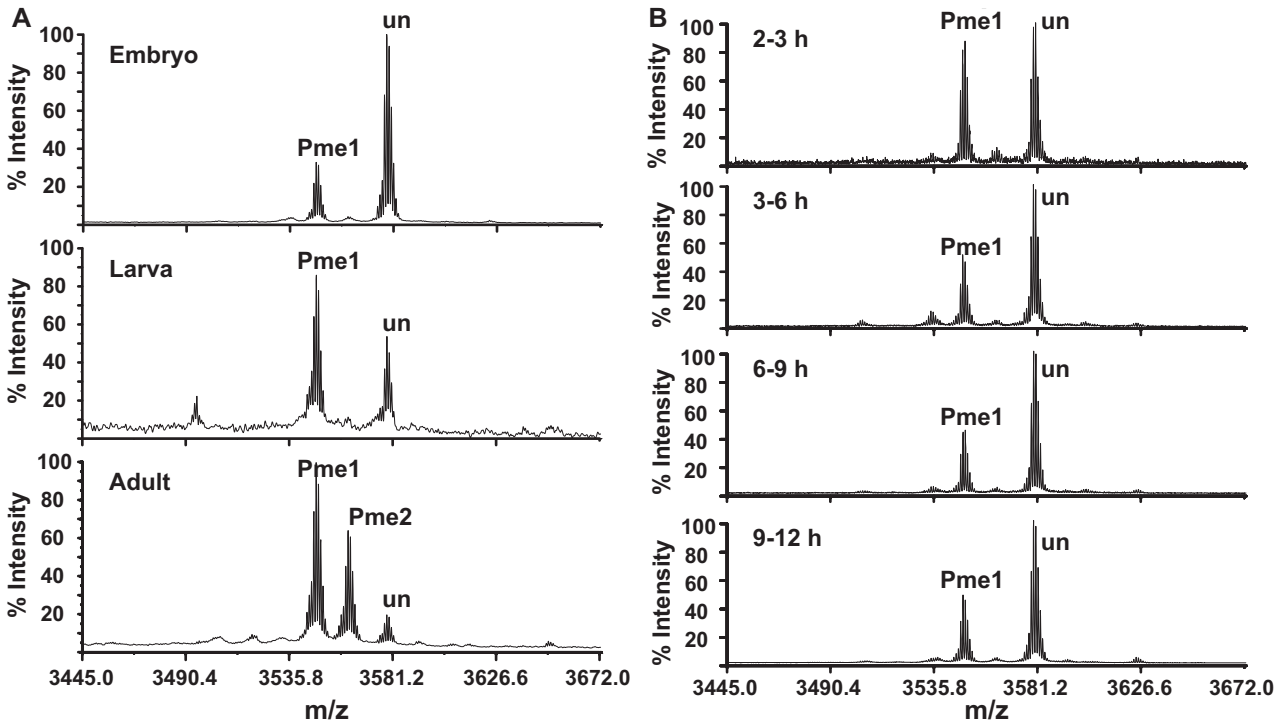

Figure 2. The methylation pattern changes during development. MALDI-TOF spectra of the peptide 1–28 of H2B isolated from a variety of developmental stages. Prior to MS, histone H2B was treated with deuterated acetic anhydride and subsequently digested with trypsin. (**A**) Methylation levels of H2B in 0–24 h after egg laying (a.e.l.) embryos (upper panel), 3rd instar larvae (middle panel) and unsorted adult flies (bottom panel). (**B**) Methylation levels in staged embryos do not significantly change in the first hours a.e.l. From top to bottom, 2–3, 3–6, 6–9 and 9–12 h a.e.l. un, unmodified proline; Pme1, monomethylated proline; Pme2, dimethylated proline.

for carrying out this function in the fly. To test the hypothesis, we expressed the protein in bacteria and tested its ability to methylate recombinant H2B *in vitro* (Figure 4). When incubated with radioactive *S*-adenosyl-methionine and different recombinant histone molecules, the enzyme specifically modified H2B but not the other three histones (Figure 4A). In order to better characterize the enzyme we determined the appearance of mono and dimethylated dH2B during the dNTMT catalyzed reaction (Figure 4B). The transient accumulation of monomethylated H2B quickly after the reaction had been started suggests that the enzyme is not processive. However, a more detailed analysis would be necessary to exclude other explanations for this observation. As the N-terminal methionine is only partially cleaved when the protein is produced in bacteria, a portion of the H2B molecules we have used in the assay still carried it at the N-terminus. The H2B that still contained the methionine was not methylated by CG1675 (Figure 4C) whereas the one carrying a proline residue was, suggesting that the enzyme uses the N-terminal proline as its substrate. This is in good agreement with the recognition site for the mammalian orthologs, which has been reported to be (Ala/Ser/Pro)-Pro-Lys (39). Within *Drosophila*, 36 proteins carry such a recognition site (Table 2), which is a similar amount compared to what has been reported for mammals (39). Surprisingly only eight of them are evolutionary conserved. Most notably, all ribosomal subunits that have been suggested to serve as substrates for NRMT1 were also potential targets in the fruit fly. *Drosophila* RCC1, however, has a different N-terminal sequence making it unlikely as a substrate for CG1675. Therefore we termed CG1675 dNTMT for *Drosophila* N-terminal methyltransferase. A knockdown of dNTMT in tissue culture cells leads to a substantial decrease of H2B methylation, suggesting that it is a major enzyme responsible for the modification in cells (Figure 4D). When expressed in SL2 cells, tagged-dNTMT mainly localizes to the nucleus (Figure 5A) pointing to a function in this organelle. Unfortunately the low transfection efficiency of <10% did not allow us to detect an increased level of total H2B isolated from a pool of transfected cells. However, the model of dNTMT acting mainly in the nucleus is further supported by our finding that over 95% of chromatin-bound H2B is methylated (Figure 5B and C) whereas we consistently detect about 15–18% of acid extracted (total) histones in an unmethylated form (Figure 1B).

dART8 interacts with dNTMT and regulates H2B methylation

In order to get more insights into the function of the methylation of H2B, we studied the possible regulation of dNTMT. Yeast two-hybrid screenings (48) had reported seven interaction partners for this methyltransferase: CG1324 and Zasp66, whose molecular function is unknown; Cpr47Ef, a structural protein of the cuticle;

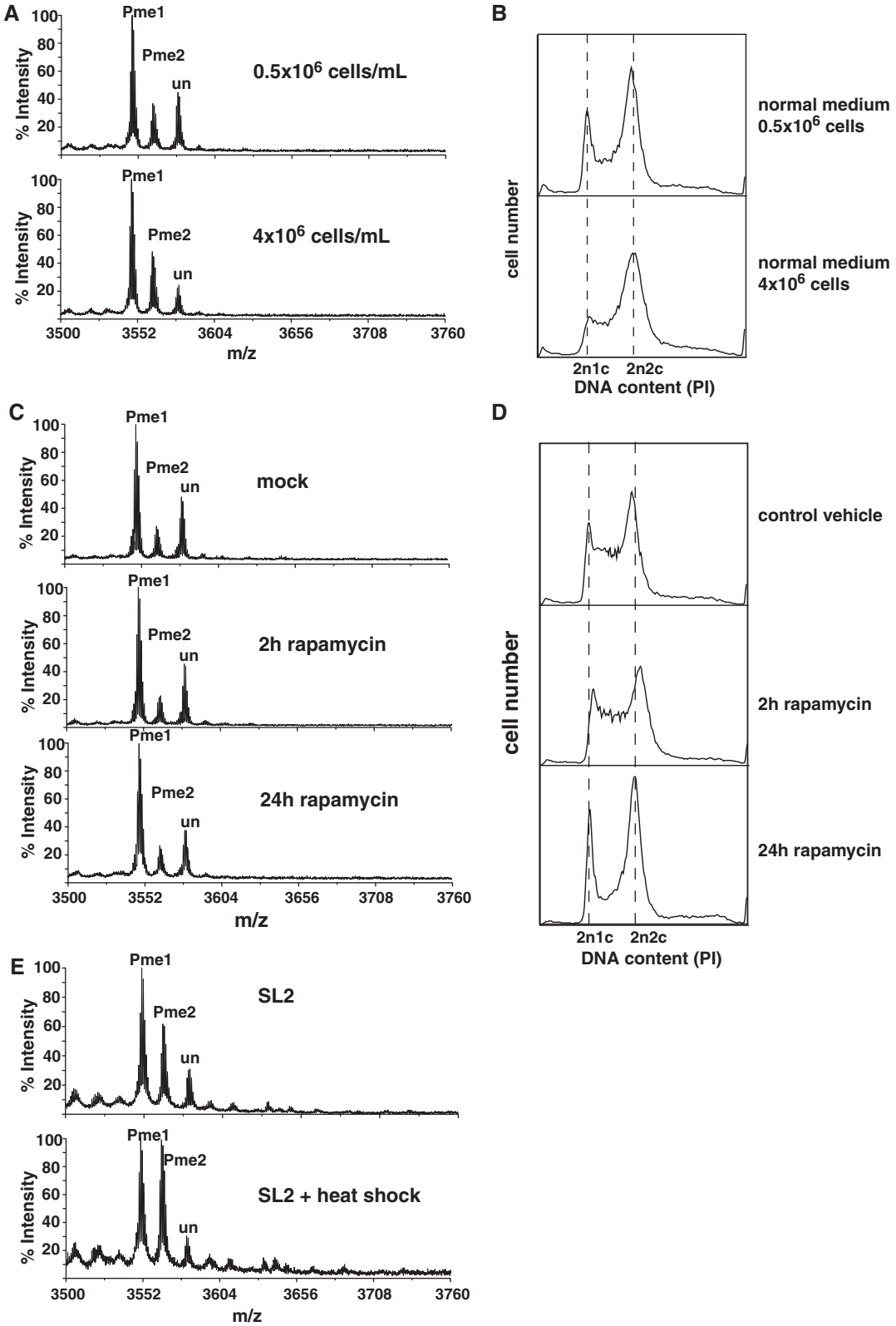

Figure 3. Cell density and heat shock, but not rapamycin influence the methylation levels. MALDI-TOF spectra showing the region of the peptide 1–28 of H2B after treatment with deuterated acetic anhydride and trypsin digestion (**A, C** and **E**) and cell cycle histograms (**B**) and (**D**) of the corresponding samples. (**A**) and (**B**) Kc cells were seeded in fresh medium at 0.5 (upper) and 4.0×10^6 (lower) cells/ml, collected 24 h later and used either for H2B or cell cycle analysis. (**C**) and (**D**) Kc cells were seeded in fresh medium at 0.5×10^6 cells/ml, 24 h later were treated with 20 nM rapamycin, harvested at the indicated time points (0, 2 and 24 h) and prepared for subsequent analyses. (**E**) SL2 cells were incubated for 1 h at 37°C and subsequently harvested. The upper panel shows the result for the mock-treated sample and the lower, for the heat shocked one. un, unmodified proline; Pme1, monomethylated proline; Pme2, dimethylated proline.

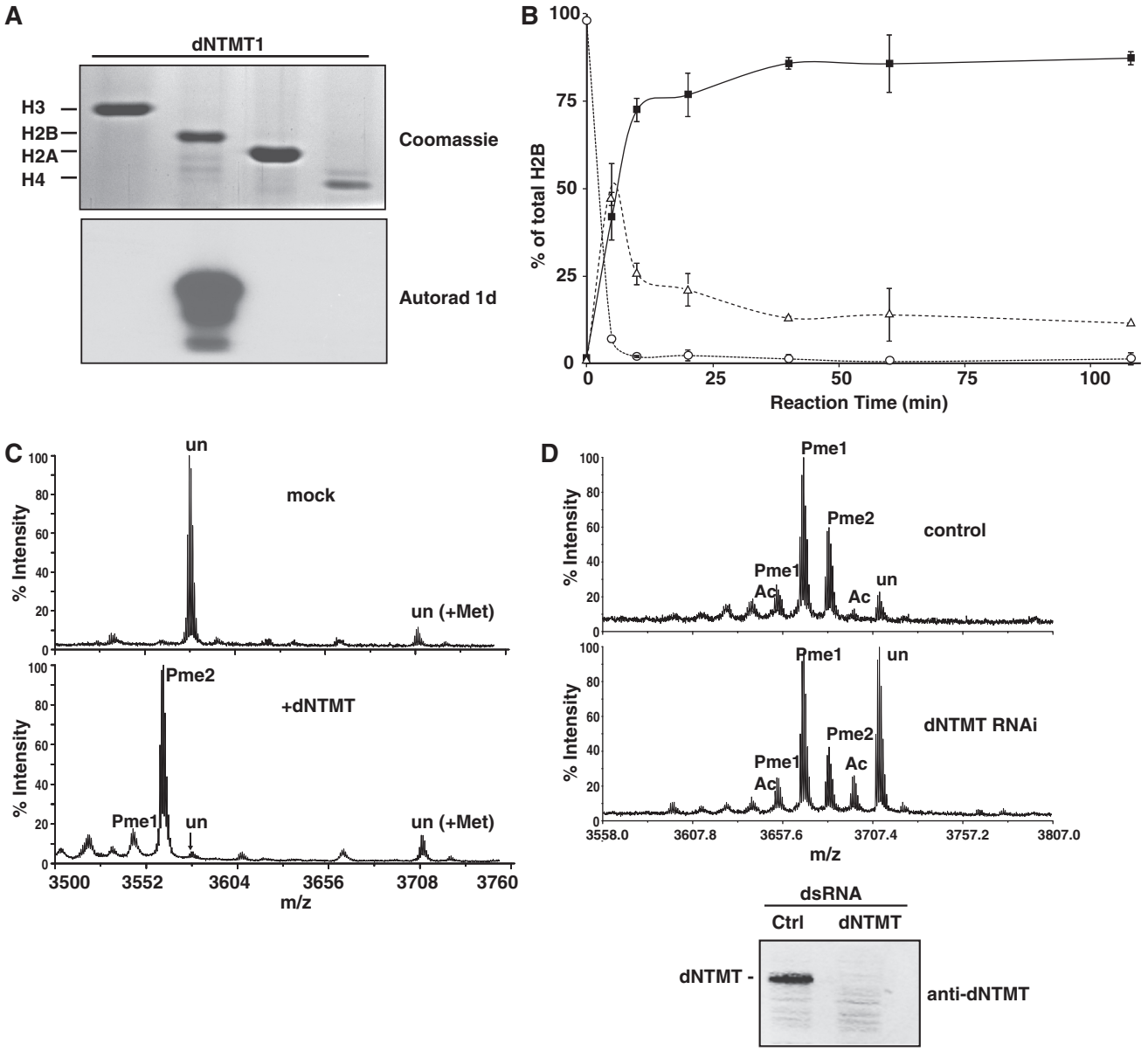

Figure 4. dNTMT methylates P1 of H2B *in vitro* and *in vivo*. (**A**) After incubation of different recombinant core histones (H3, H2B, H2A and H4) with recombinant dNTMT in the presence of ^{3}H-S-Adenosyl Methionine (SAM), only H2B became methylated. Upper panel, Coomassie stained protein gel; lower panel, corresponding autoradiography. (**B**) *In vitro* methylation kinetics of dH2B N-terminus by dNTMT. Open circle, unmodified proline; open triangle, monomethylated proline; filled square, dimethylated proline. $N = 3$. (**C**) MALDI-TOF spectra of Asp-N digested H2B shows that the N-terminal peptide becomes methylated after incubation of the histone with dNTMT and SAM (right panel) but not after incubation with the enzyme alone (left). (**D**) MALDI-TOF spectra of propionylated, trypsin digested H2B reflect the loss of methylation upon knockdown of dNTMT. Upper panel: control (GST RNAi); middle and lower panel: knockdown of dNTMT, using two different dsRNA molecules. un, unmodified proline; Pme1, molecules with monomethylated proline and/or molecules with dimethylated proline and one acetylated lysine; Pme2, dimethylated proline; Ac, one acetylated lysine; Pme1 Ac, monomethylated proline and one acetylated lysine and/or peptides with dimethylated proline and two acetylated lysine.

sec24, a cytoplasmatic protein; CG36329, a putative ATP-dependent peptidase; CG6745, which has putative pseudouridine synthase activity; and dART8, a putative arginine methyltransferase. We therefore decided to investigate a possible effect of dART8 levels on H2Bmethylation.

When we knocked down dART8 using specific dsRNA, we reproducibly observed an increase in H2B dimethylation. This effect was highly specific for dART8, as a knock down of other members of this family of arginine methyltransferases did not influence H2B methylation levels (Figure 6A). *Drosophila* Art8 had not been shown to have arginine methyltransferase activity. We therefore tested whether the *Drosophila* protein has a similar specificity to its closest human ortholog PRMT6, which methylates R2 in histone H3. We expressed dART8 in bacteria as a GST-fusion protein and performed an *in vitro* methyltransferase assay using the four recombinant core histones (Supplementary Figure S2A). As expected dART8 mainly

Table 2. Predicted targets of dNTMT

Accession number	Protein name	N-terminal sequence	N-terminus conserved in mammals
PPK			
AAZ66546	H2B	PPKTSGKA	No
AAM68298	Ribosomal protein L12	PPKFDPTE	Yes
AAF51420	Testis specific Tetratricopeptid containing protein	PPKKAKK	No
AAM7112	Testis-specific ankyrin repeat protein	PPKRRRRF	No
AAF48293.3	Bth-D selenoprotein	PPKRNKKAE	No
EDP27932	Na-K ATPase	PPKKKNKEH	No
AAF49907	Sticky protein kinase	PPKMEPISVR	No
AAF47545	Ribosomal protein L23a	PPKKPTEKSAK	Yes
AAF51837.1	NADH-ubichinon oxidoreduktase	PPKPKHRDVA	Yes
AAF53216.1	SPATA5 spermatogenesis-associated factor	PPKSSSKKNQVT	No
AAF56433.1	Peptidyl-prolyl *cis*-trans isomerase	PPKKDAKSGKD	No
AAN13397.1	Putative membrane protein	PPKRPPPLVPKK	No
AAF59063.1	Zn-finger Protein CG8635	PPKKAPPGPSKK	Yes
AAF49042.1	CG17122	PPKVKKEKKDVNK	Yes
AAF58585.1	CG42554	PPKKPAKKKKDVDW	No
AAN09613.1	OBG like ATPase1	PPKKHDEPERKPLI	Yes
AAN13495.1	Ribosomal protein S25	PPKKDAKSSAKQPQ	Yes
AAF45877	Growth arrest-specific 8	PPKGKKGKKGKKLP	Yes
APK			
AAF51851.1	Maelstrom3	APKKHSGFMMFV	No
AAN10535.1	BMP receptor IA	APKSRKKKAHARSL	No
AAF56807.1	ABC-2 transporter	APKKEATLSQQQTQ	No
AAF49667	CTP synthase	APKKSTIVLNVEQF	No
AAF53210.1	TCP1	APKAAAVNIKPTAKA	No
SPK			
AAN12034	HP4	SPKTKKMIVKIPRH	No
AAS65294.1	Moesin	SPKALNVRVTTMDA	No
AAF48248.1	Dimethylarginase	SPKYTHAIVARIS	No
AAN09604.1	Phosphodiesterase 4D	SPKSMSRNSSIASE	No
AAF51492.1	ZN-finger CG4133	SPKYHNFEIALVYFL	No
AAF48383	CG15890 solute carrier protein	SPKDGSLGDAKFAK	No
AAF45682	CG14818	SPKNNHDPSSSGDS	No
AAN11992	Zasp66	SPKLHEFAVVLLRD	No
AAN11991.2	CG42826 solute carrier protein	SPKKCEPPPEDSL	No

All proteins that carry a (M)-A/P/S-P-K recognition site on their N-terminus within the predicted proteome of *D. melanogaster* are listed (67).

methylates H3 but is also able to methylate H2A and H4 albeit to a much lower level. The only methylation site we could detect on H3 was a mono and dimethylation of R2, which prevented a cleavage of trypsin at H3R2 leading to a fragment carrying amino acids 1–8. The fragmentation spectra also showed that dART8, like its mammalian counterpart (49), catalyzes the formation of an asymmetrically methylated arginine. (Supplementary Figure S2B).

As a knock down of dART8 leads to an increase of H2B methylation, we wondered whether an increased amount of dART8 would in turn lead to decreased N-terminal H2B methylation. We therefore expressed dART8 in SL2 cells under an inducible promoter and investigated the degree of H2B methylation after the induction of expression (Figure 6B). Consistent with a role of dART8 in the regulation of H2B methylation we detected a strong reduction of H2B methylation in cells where dART8 expression had been induced. As the human dART8 ortholog PRMT6 has been shown to act as a transcriptional repressor, we tested whether the reduction of dART8 leads to an increased expression of dNTMT. However, we did not detect significant changes of dNTMT levels dependent on the amount of dART8 (Figure 6C and Supplementary Figure S3). We next wanted to test whether dART8 might in fact methylate dNTMT thereby inhibiting its activity. However, we could neither observe an effect of dART8 on the ability of dNTMT to methylate H2B when the three proteins were incubated together in an *in vitro* methyltransferase assay nor did we detect a methylation of dNTMT by dART8 or vice versa (Supplementary Figure S4).

Since both dNTMT and dART8 localize to the nucleus (Figures 5A and 6D), we wanted to test whether they physically interact with each other. To do this we expressed the two recombinant proteins in *Escherichia coli* carrying two different tags, mixed them and purified the complex using either immobilized glutathion or an anti-FLAG resin (Figure 6E) To test whether the two proteins also interact *in vivo*, we expressed flag tagged dART in SL2 cells and purified it using a monoclonal antibody specific for dNTMT. This interaction was verified using the reciprocal purification of dNTMT using an anti-FLAG resin (Figure 6F). Based on the observed interactions, we assume that the negative regulation of H2B methylation by dART8 is likely to be due to its interaction with the enzyme responsible for this modification.

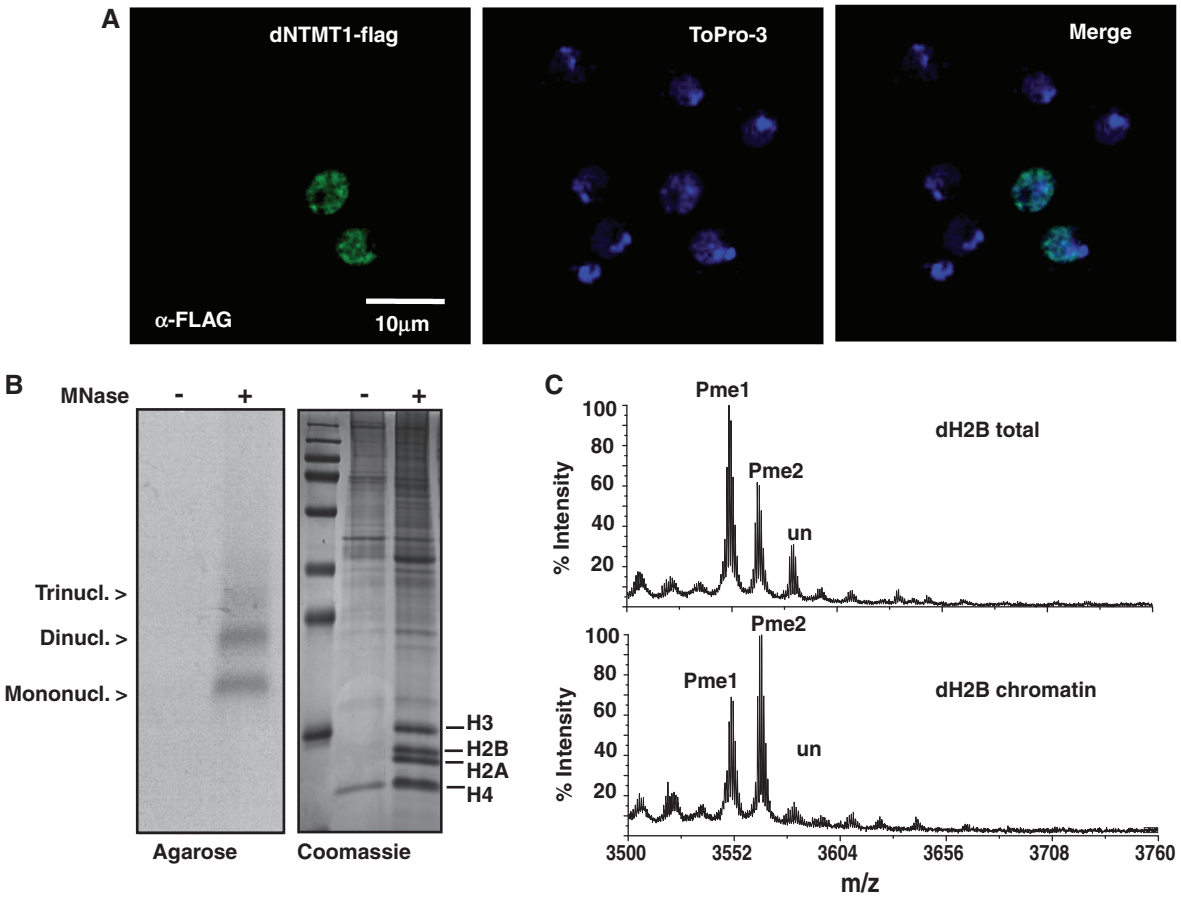

Figure 5. dNTMT is a nuclear protein and generates highly methylated chromatin in the nucleus. (**A**) Immunolocalization of FLAG-tagged dNTMT in *Drosophila* L2-4 cells transiently transfected with the FMO4789 dNTMT expression plasmid. DNA was stained with ToPro-3 to visualize the nuclear volume. (**B**) Isolation of chromatin using micrococcal nuclease (MNase). Chromatin fragments were released from isolated nuclei using MNase and the DNA was analyzed by agarose gel electrophoresis (left panel) or SDS–PAGE followed by a Coomassie staining (right panel), respectively (**C**) MALDI-TOF spectra showing the peptide 1–28 of H2B after acylation and trypsin digestion. The top spectrum is derived from acid extracted H2B whereas the bottom one is a result of the analysis of chromatin bound H2B. 'un' = unmodified proline, 'Pme1' = monomethylated proline, 'Pme2' = dimethylated proline.

DISCUSSION

Post-translational modifications in histones play a major role in setting up specific chromatin structures. In the last years it became evident that histone modifications can strongly affect each other thereby generating complex modification patterns. Several evidences for a crosstalk among different tails have been reported, suggesting a complex network and an interdependency of modifications and the accompanying enzymes. While studying the post-translational modifications of H2B in *D. melanogaster* we found the methylation of its N-terminus as the major modification of this histone. In contrast to many other known N-terminal modifications, the methylation is highly regulated. We identified the enzyme responsible for establishing this modification (dNTMT/CG1675) and confirmed that it physically interacts with another histone methyltransferase (dART8) specific for H3R2 thereby constituting a bifunctional methyltransferase complex. The two enzymes not only interact with each other physically but also have opposing functions as the reduction of dART8 protein levels results in an increase of N-terminal methylation and the overexpression in a strong decrease. This effect of the modulation of dART8 concentration on H2B methylation may be due to a negative crosstalk between the methylation of H3R2 and the N-terminal methylation of H2B or due to a competition of the two enzymes for the common cofactor SAM.

N-terminal methylation is a rare modification in eukaryotic proteins and has only recently been addressed functionally. The methylation of the N-terminus of human RCC1 has been shown to be important for its stable interaction with chromatin (38) and its disturbance leads to mitotic defects *in vivo* (38). This regulation is in accordance with the structure of the nucleosome bound RCC1 molecule, where a N-terminal loop has been suggested to interact with the nucleosomal DNA (50). Interestingly, despite the strong conservation of RCC1 function in metazoans, the N-terminal methylation site of RCC1 is not conserved in the *Drosophila* ortholog of RCC1, suggesting that the stabilization of RCC1 on chromatin is either accomplished by a different mechanism or not required in *Drosophila*.

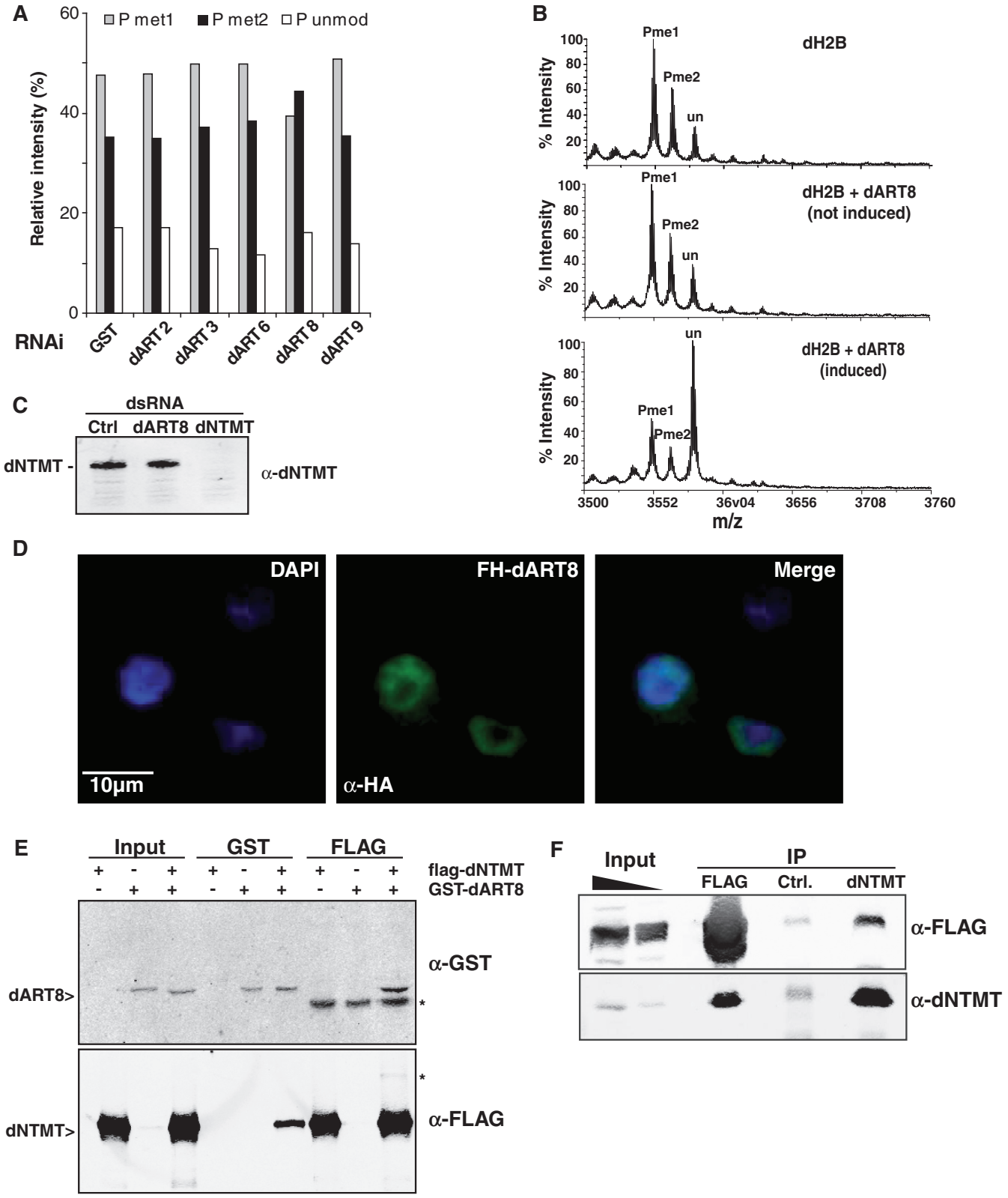

Figure 6. *Drosophila* ART8 interacts with dNTMT and regulates H2B N-terminal methylation. (**A**) H2B methylation measured by MALDI-TOF MS after knockdown of different dART enzymes. The relative intensities of the signals assigned to unmodified (white bars), mono- (gray bars) and dimethylated proline of H2B were determined after a knockdown of the corresponding enzyme. (**B**) MALDI-TOF spectra showing the peptide 1–28 of H2B after acylation and trypsin digestion. The spectra are derived from acid extracted H2B from untransfected cells (top), or cells transfected with an inducible dART8 expression construct before (middle) or after (bottom) induction. un, unmodified proline; Pme1, monomethylated proline; Pme2, dimethylated proline. (**C**) Western blot of a whole cell extract after knockdown with dsRNAi against GST (Ctrl.), dART8 (dART8) and dNTMT (dNTMT). dNTMT was detected by a monoclonal antibody against dNTMT. (**D**) Immunolocalization of FLAG-HA tagged dART8 in *Drosophila*

(continued)

Eight proteins out of 36 that carry a recognition site for a presumptive N-terminal methyltransferases are conserved between humans and fruit flies. Those are three ribosomal subunits, two proteins associated with stress response and/or growth arrest, two with an unknown function and one member of the respiratory chain. Considering the conservation of the enzyme responsible for this modification, the low degree of overlap is very surprising. However, it is striking that frequently the human orthologous of proteins that carry a recognition site only in *Drosophila* can be found associated with putative targets in humans. So is for example RCC1 (methylated in humans but not in *Drosophila*) in a complex with H2B (methylated in *Drosophila* but not in humans) (50) and many factors that are predicted to become N-terminally methylated are expressed in a testis specific manner in humans as well as in *Drosophila* [compare (39) to Table 2]. This suggests that the N-terminal methylation exerts its function on a protein complex as long as one subunit carries the modification.

What may be the function of H2B methylation in *Drosophila*? The observation that it increases during temperature stress as well as during differentiation points towards a role of H2B methylation in stabilizing chromatin. In both circumstances (heat shock and aging) the overall transcription is reduced and becomes restricted to a limited number of active genes (51,52). At the same time, we detect only low levels of methylation at early stages of embryonic development, where chromatin has been reported to be hyper-dynamic in other systems (53). In general, histones have been shown to have a higher turnover in dynamically transcribed regions compared to non-transcribed domains (54) therefore lowering this turnover may have a repressive effect on general transcriptional activity. As N-terminal modifications have been shown to regulate protein turnover (55), H2B methylation might similarly stabilize the protein and contribute to a reduced overall transcription in differentiated or stressed cells. Alternatively, H2B methylation could also be a consequence of low transcriptional activity, which is the supported by the observation that it also increases when cells are treated with transcriptional inhibitors or with inhibitors of TopoII (35,56).

N-terminal modification of histones is not restricted to H2B in flies but has also been detected in H4, which is N-acetylated in virtually all eukaryotes (57–59), H2A, which is N-acetylated in human tissue culture cells (60) and H2B from yeast, which is also acetylated at its N-terminal residue (61). None of the N-acetylations have been associated with a particular function and are thought to be constitutive modifications following histone synthesis. Our description of a developmentally and stress-induced regulated N-terminal modification of H2B sheds new light on the potential function of this modification in chromatin metabolism.

The relative expression levels of dNTMT during different developmental stages correlate very well with the relative levels of H2B methylation [compare Figure 2 with Supplementary Figure S5 (62)]. This suggests that the proportion of H2B methylation is regulated by the amount of enzyme present in the cell. However, in SL2 cells we also observe that the level of dART8 regulates H2B methylation, which suggests a second layer of control for H2B methylation. Although the two proteins interact physically, they do not methylate each other and the interaction does not lead to an alteration of dNTMTs activity. The observed interference of dART8 expression with H2B methylation can therefore not be explained by a direct effect mediated by the simple interaction of the two polypeptides. Expression studies show that the dNTMT expression is not downregulated by dART8 expression or upregulated in cells that lack dART8.

Recently local SAM concentrations within the nucleus have been suggested to play an important role in regulating the activity of histone methyltransferases (63). The regulation of H2B methylation we observe *in vivo* by modulating dART8 levels may therefore be due to a competition of the two enzymes residing in the same complex for the limiting cofactor SAM. Several nuclear complexes have been shown to contain multiple methyltransferases activities (64–66) that could potential be regulated by a similar mechanism. Alternatively, as dART8 methylates R2 at H3, a crosstalk between the two histone-tails where methylation of H3R2 inhibits H2B N-terminal methylation could also be an explanation for the reciprocal activities of the two enzymes. Based on our data, future studies are necessary that distinguish the possible mechanisms of how dART8 modulates dNTMTs activity within a single protein complex and analyze the role of the striking increase in H2Bmethylation during fly development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5, Supplementary Reference [63].

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Figure 6. Continued
SL2 cells transfected with an inducible FHdART8 expression plasmid. DNA was stained with DAPI to visualize the nuclear volume. (**E**) *In vitro* interaction of flag-dNMT and GST–dART8. Recombinant proteins were mixed and incubated for 10 min at room temperature the complexes were subsequently either purified by glutathione-(GST) or M2-(FLAG) agarose., separated via SDS–PAGE, blotted and detected using either an anti-GST (top panel) or an anti-FLAG (bottom panel) antibody. (**F**) Interaction of dART8 and dNTMT. Whole cell extracts from cells expressing Flag tagged dART8 were either purified by M2 (FLAG) agarose, an immobilized monoclonal dNTMT antibody (dNTMT) or a non-related immobilized antibody (Ctrl.). 1 and 5% of Input as well as the immunoprecipitated material were separated by SDS–PAGE, blotted and detected by either an anti-FLAG (top panel) or an anti-dNTMT antibody (bottom panel).

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