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MODIFICATION OF HISTONES BY THE SUGAR β-N-ACETYLGLUCOSAMINE (GlcNAc) OCCURS ON MULTIPLE RESIDUES, INCLUDING H3 SERINE 10, AND IS CELL CYCLE REGULATED

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Running title: Histone O-GlcNAcylation in the cell cycle
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Abbreviations: O-GlcNAcylation, phosphorylation, histones, cell cycle.

The monosaccharide, β-N-acetyl-glucosamine (GlcNAc), can be added to the hydroxyl group of either serines or threonines to generate an O-linked-β-N-acetyl-glucosamine (O-GlcNAc) residue (1-2). This post-translational protein modification, termed O-GlcNAcylation, is reversible, analogous to phosphorylation and has been implicated in many cellular processes. Here we present evidence that in human cells all four-core histones of the nucleosome are substrates for this glycosylation in the relative abundance, H3, H4/H2B and H2A. Increasing the intracellular level of UDP-GlcNAc, the nucleotide sugar donor substrate for O-GlcNAcylation, enhanced histone O-GlcNAcylation and partially suppressed phosphorylation of histone H3 at serine 10 (H3S10ph). Expression of recombinant H3.3 harboring an S10A mutation abrogated histone H3 O-GlcNAcylation relative to its wild-type version, consistent with H3S10 being a site of histone O-GlcNAcylation (H3S10glc). Moreover, O-GlcNAcylated histones were not detected in H3S10ph immunoprecipitates, whereas immunoprecipitation of either H3K4me3 or H3K9me3 (active or inactive histone marks, respectively) resulted in co-immunoprecipitation of O-GlcNAcylated histones. We also examined histone O-GlcNAcylation during the cell cycle progression. Histone O-GlcNAcylation is high in G1 cells, declines throughout S phase, increases again during late S/early G2 and persists through late G2 and mitosis. Thus, O-GlcNAcylation is a novel histone post-translational modification regulating the chromatin conformation during transcription and cell cycle progression.

O-GlcNAcylation, analogously to protein phosphorylation occurs as a reversible posttranslational modification of proteins involved in cell signaling, stress responses and during cell growth (reviewed in refs 1-2). This glycosylation involves the addition of a single sugar molecule, β-N-acetylglucosamine, to the hydroxyl group of serine or threonine (termed O-GlcNAc). This may lead to a reciprocal regulation of protein function since phosphorylation and O-GlcNAcylation may compete with each other for the same amino acid residue. O-GlcNAcylation has also been reported to regulate protein ‘turn-over’ by altering the activity of the proteasome or substrates targeted for degradation (3).

The level of O-GlcNAcylation is controlled within the cell by two evolutionarily conserved enzymes, a transferase (O-linked-β-N-acetylglucosamine transferase, or OGT) and a hydrolase (O-linked-β-N-acetylglucosaminidase, alternatively O-GlcNAcase or OGA), which add or remove, respectively, O-GlcNAc (4). OGT is present in mammals as a single copy gene on the X-chromosome and its deletion in the mouse results in early (day E5 post fertilization) embryonic lethality (5). In C. elegans, OGT plays a role in insulin resistance as its deletion suppressed the induction of dauer formation in a genetic background defective for insulin signaling (6). Whereas under similar conditions, knockout of OGA promoted dauer formation (7), supporting an involvement of O-GlcNAcylation in metabolic regulation in this animal.

OGT in human cells interacts with phosphoinositide at the plasma membrane, where O-GlcNAcylation is believed to attenuate insulin signaling by suppressing the phosphorylation of protein kinase Akt (8). Moreover, O-GlcNAcylation may control cell growth by...
altering the stability of the tumor suppressor p53 (9), as well as the NFkB-IkB signaling pathway (10-11). In response to various cellular stresses, an increased level of protein O-GlcNAcylation was observed (12). O-GlcNAcylation may have an effect on the formation of protein complexes, as has been observed for ribosomal proteins and which, in this case, leads to an alternation of translational activities or RNA decay (13).

Modification of proteins by O-GlcNAcylation has been implicated in chromosome biology since the determination that this glycosylation is enriched in the condensed and repressed regions of *Drosophila* polytene chromosomes (14). Chromosomal proteins modified by O-GlcNAc are exemplified by many transcriptional factors, such as Sp1 (15) and c-Myc (16), as well as p53 (9). A further role for this modification in regulating transcription is suggested by the reciprocal relationship between O-GlcNAcylation and elongation-dependent phosphorylation at the C-terminal domain (CTD) of RNA polymerase II (17). A direct role in transcriptional repression is suggested by the observation that the N-terminal TPR (tetratricopeptide repeat) domain of OGT interacts with a transcriptional repressor, mSin3, which exists in a complex with a histone deacetylase (HDAC) involved in gene silencing (18). Even more intriguingly, the C-terminus of OGA contains a domain resembling a HAT (histone acetyltransferase) domain that has been shown to be capable of acetylating nucleosomal histones *in vitro* (19). Clearly, the associations between OGT and HDAC and OGA and HAT activities suggest reciprocal and reversible control of gene expression.

Two recent observations further highlight the potential for O-GlcNAcylation in the regulation of transcription. Firstly, the gene encoding OGT in *Drosophila* has been mapped to a polycomb group (PcG) gene locus termed, *super sex combs* (sxc), which is involved in the repression of homeotic genes during the development of this organism (20-21). Secondly, O-GlcNAcylation of MLL5, a histone lysine methyltransferase, has been shown lead to dimethylation of H3K4 required for retinoic acid-induced transcription during promyelocyte differentiation (22).

However, despite increasing evidence supporting the involvement of O-GlcNAcylation in multiple aspects of chromosome biology, the possibility that the histones proteins may also be the targets for O-GlcNAcylation remained to be investigated. This might relate to difficulty in mapping sites of O-GlcNAcylation via mass spectrometry due to the labile glycosidic bond and/or low stoichiometry of this modification on some proteins (1-2). Nevertheless, recent data based on *in silico* analysis of histone posttranslational modifications predicts the occurrence of histone O-GlcNAcylation (23). We therefore used both antibodies and a lectin (wheat germ agglutinin, WGA) with specificity for O-GlcNAc to examine histones from human cells for O-GlcNAcylation. Here we report that histones are indeed substrates for O-GlcNAcylation. Using both one and two-dimensional electrophoresis we determined that all four nucleosomal core histones are O-GlcNAcylated in the relative order, H3, H4 or H2B and H2A. Furthermore, we provide multiple lines of evidence that serine 10 of histone H3 is indeed a site of O-GlcNAcylation (H3S10glc). Our data are compatible with the possibility of either an O-GlcNAc or an O-phosphate modification at serine 10 of histone H3. Increased global O-GlcNAcylation of all three isoforms of histone H3 resulted in a global loss of a modification indicative of active chromatin (H3K9Ac) and a corresponding global increase of a modification indicative of repressed chromatin (H3K9me3). Finally, histone O-GlcNAcylation correlated with progression of cells through the cell cycle. Specifically, histone O-GlcNAcylation was high in G1, globally decreased during S phase but re-emerged in late S/early G2 and persisted during mitosis. These findings suggest that regulation of histones by O-GlcNAcylation plays a regulatory role in chromosome metabolism during the cell cycle, with histone H3S10 being a major site of O-GlcNAcylation, and global histone O-GlcNAcylation being specifically depleted during DNA replication.

**EXPERIMENTAL PROCEDURES**

*Antibodies*: The mouse monoclonal antibodies CTD110.6 and RL2 specific to O-GlcNAc, the rabbit polyclonal antibodies against histone H3K9me3, H3K4me3, H3K9Ac and
H3S28p were obtained from Abcam (Cambridge, UK). The rabbit polyclonal IgG specific to histone H3S10ph was supplied by Upstate Biotechnology (Lake Placid, NY). All antibodies were used according to the manufacturers recommendations.

**Cell culture:** Human HeLa and HEK 293 epithelial cells were grown in Dulbecco’s modified Eagle’s medium (Lonza Walkersville, Inc., MD), supplemented with 10% fetal calf serum (Lanza) and cultured at 37 °C in a humidified atmosphere with 5% CO₂. Human CCRF-CEM, acute T lymphoblastic leukemia cell line, and K562, erythroleukemia cells, (both from ATCC) were grown in RPMI (Lonza) supplemented with 10% FCS (Lonza) at 37 °C in a humidified atmosphere with 5% CO₂. Treatment with glucosamine was performed with actively growing cells by adding it to the cell culture medium at concentrations of 0, 2.5, 5 and up to 15 mM. The cells were harvested after overnight incubation (>16 hours) and whole cell lysates or histones proteins were prepared as described below.

**Acid extraction of histones:** Histone preparation was based on an acid extraction using hydrochloric acid (HCl). The extraction was performed at 4 °C with all buffers supplemented with a protease inhibitor cocktail (Roche, Mannheim), 1 mM PMSF (phenylmethanesulphonylfluoride); 1 mM DTT; 1 mM NaF; 0.1 mM sodium orthovanadate (Na₃VO₄) and 80 µM PUGNAc [O-(2-Acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenylcarbamate, (Sigma)], an inhibitor of O-GlcNAcase. The cultured cells were harvested and washed once with PBS, followed by a suspension of cell pellets in 50 mM MES [2-(N-morpholino)ethanesulfonic acid, pH 6.0]; 1 M sorbitol; 5 mM MgCl₂ and then centrifuged at 1000 g for 5 min. The cell pellets were lysed by suspending them in 50 mM MES, pH 6.0; 75 mM KCl; 0.5 mM CaCl₂ and 0.1% Nonidet P-40 (NP-40) with repeated pipetting, followed by centrifugation at 12,000 g for 5 min. The resulting pellets were washed in high salt buffer containing 10 mM MES, pH 6.0; 430 mM NaCl and 0.5% NP-40 to dissociate weakly interacting proteins and centrifuged at 15,000 g for 5 min. This high salt wash was repeated twice to minimize contamination with non-histone proteins. Histone proteins in the pellets were then extracted by treatment with 0.25 N HCl (in dH₂O) and vigorous vortexing for 15 min, followed by centrifugation at 12,000 g for 5 min to obtain the histone-enriched supernatants. This acid extraction was repeated twice and both supernatants were combined, followed by centrifugation at 3000 g for 5 min to clear the histone extracts. Eight volumes of acetone were then added per one volume of clarified histone extract and incubated at -20°C overnight to precipitate proteins. Acetone precipitated histones were harvested by centrifugation at 5000 g for 5 min, washed once with 0.1 N HCl (in acetone) and twice with acetone alone. Histone pellets were air-dried and finally dissolved in a minimal volume of sterile distilled water. Histone preparations were aliquoted and stored at -80°C until required.

**Two-dimensional gel electrophoresis of histones:** Analysis of histones by two-dimensional gel electrophoresis was performed using the protocol described by Shechter, D. et al (24). The first dimension of histone separation was obtained by TAU electrophoresis in a short (approximately 10 cm) gel, with a final gel volume of 10 ml that contained 3.6 g urea; 2.5 ml of a 60%:0.4% acrylamide: bisacylamide solution; 500 µl glacial acetic acid; 370 µl Triton X-100; 60 µl N,N,N,N- tetra-methylethylene-diamine (TEMED) and 140 µl 10% ammonium persulfate (APS). Histones to be loaded onto the TAU gel were mixed with a freshly prepared sample buffer generated as follows: 0.36 g urea; 100 µl 0.2% pyronin Y; 50 µl glacial acetic acid and 500 µl of 25 mg/ml protamine sulfate in a final volume of 1 ml. TAU gel electrophoresis was performed at 200 V for approximately 90 min in a buffer of 5% glacial acetic acid. After TAU gel electrophoresis, conventional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the second dimension to resolve individual histones and certain isoforms. For the second dimension, one lane from the TAU gel after electrophoresis of the histones was excised, incubated in a buffer containing 2% SDS; 60 mM Tris/HCl, pH 6.8 and 5% β-mercaptoethanol with three changes, each of 5 minutes duration. The equilibrated gel slice was then placed on top of a standard 15% SDS-PAGE gel, followed by an overlay of stack gel solution. After polymerization of the stacking gel, electrophoresis was performed as usual for SDS-PAGE.
Western Blotting: After electrophoresis of histones on TAU gels, histones were transferred to PVDF membranes at 30 V overnight (>16 h) in a buffer of 0.7% acetic acid. Prior to transfer, TAU gels were incubated with 0.7% acetic acid for 15 min to equilibrate with the transfer buffer. After electrophoresis of histones by SDS-PAGE, transfer of proteins to nitrocellulose membranes was performed using standard conditions. For detection of O-GlcNAc, the membranes were blocked in 3% BSA and then incubated with the mouse antibody RL2 or CTD110.6, both diluted at 1 to 1000. Alternatively, a lectin probe specific to O-GlcNAc, Wheat Germ Agglutinin (WGA), coupled with horseradish peroxidase (WGA-HRP, from Alpha Diagnostic, TX) was used at a dilution of 1 in 1000. Membranes for all other immunodetections were blocked in 3% milk powder with a dilution of antibody 1 to 2000 for the rabbit polyclonal antibody against H3K4me3. Rabbit polyclonal antibody against H3K9me3 and polyclonal antibody 1 to 2000 for the rabbit polyclonal antibody 1 to 2000 for the rabbit polyclonal antibody were used. Detections were blocked in 3% milk powder with a dilution of 1 in 100. Subsequently, protein G-agarose (20 µl bed, GE Healthcare, Uppsala) was added to each mixture followed by a further incubation for 1 h. The protein G-agarose beads were pelleted by brief centrifugation at 15,000 g for 1 min, washed three times with a buffer containing 20 mM Tris/HCl, pH 7.5; 500 mM NaCl; 3 mM MgCl₂; 0.2 mM EDTA; 10% glycerol and 0.1% NP-40. Immunoprecipitates were finally suspended in a sample buffer for SDS-PAGE, heated at 95 °C for 5 min and analyzed by SDS-PAGE and Western blotting.

Histone Immunoprecipitation: Nuclear extracts of HEK293 cells were used for histone immunoprecipitations. All steps were performed at 4 °C with a supplement of protease inhibitor cocktail in all the buffers. After washing with PBS, the collected cell pellets were resuspended in a hypotonic buffer composed of 10 mM Tris/HCl, pH 7.5; 10 mM NaCl; 1.5 mM MgCl₂; 0.1% Triton X-100 and protease inhibitor cocktail. After centrifugation at 3000 g for 5 min, the nuclei pellets were suspended in a buffer of 50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.5 mM DTT; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS and protease inhibitor cocktail, followed by sonication for 6 pulses, each of 6 seconds duration at an output of 40% in a Branson-250 digital sonifier. After centrifugation of the sonicated nuclear extracts at 20,000 g for 5 minutes, supernatants were divided for incubation with antibodies specific to different histone posttranslational modifications. Subsequently, protein G-agarose beads were pelleted by brief centrifugation at 15,000 g for 1 min, washed three times with a buffer containing 20 mM Tris/HCl, pH 7.5; 500 mM NaCl; 3 mM MgCl₂; 0.2 mM EDTA; 10% glycerol and 0.1% NP-40. Immunoprecipitates were finally suspended in a sample buffer for SDS-PAGE, heated at 95 °C for 5 min and analyzed by SDS-PAGE and Western blotting.

In vivo expression of recombinant histones- A plasmid vector, pOZ FH-C, for expression of Flag and HA- tagged wild-type human histone H3.3 in mammalian cells was a kind gift from Prof. Tsuyoshi Ikura, (Radiation Biology Centre, Kyoto University). Wild type H3.3 and its mutant S10A or T11V were synthesized by the gene synthesis service from GENEART (Regensburg), and subcloned into XhoI and NotI sites of plasmid pOZ FH-C. For in vivo expression, plasmids were transfected into HEK293 cells via the CaCl₂ method. Cells were harvested 48 hours after transfection to prepare whole cell lysates. Immuno-precipitation of recombinant H3.3 was performed as described...
Expression of recombinant OGT and in vitro assays- A pET plasmid encoding full-length human OGT (116 kDa) was kindly supplied by Prof. Hanover, J. A. (Laboratory of Cell Biology and Biochemistry, National Institutes of Health, Bethesda). The full-length OGT gene was then subcloned into a donor plasmid pFastBac™ at the BamH1 site for its expression from baculovirus (bacmid, Invitrogen), with a 6x His tag at its N-terminus for affinity purification on Nickel (Ni²⁺)-agarose. Insect cells (High 5) 48 h after infection with bacmid expressing OGT were harvested and disrupted with acid extracted histones, a putative OGT substrate. The reaction mixture (10 µl) contained 50 mM Tris/HCl, pH 7.5, 1 mM DTT, 12.5 mM MgCl₂, 100 µM ATP, 100 µM UDP-GlcNAc (donor substrate of OGT, Sigma), recombinant histone H3 (0.1 µg/µl), and different amounts of Aurora B or OGT. When used, γ³²P-ATP as a phosphate donor was included at 10 µCi/assay (3000 Ci/ mmol, PerkinElmer) in addition to the unlabeled ATP given above. After incubation at 37 °C for 30 min, the reaction mixture was mixed with SDS-PAGE loading buffer and then heated at 95 °C for 5 min, followed by electrophoresis and autoradiography, or Western blotting with a phospho-epitope specific antibody to examine the phosphorylation.

RESULTS

Human histones are O-GlcNAcylated. We initially examined O-GlcNAcylation of human histones from whole cell lysates of HEK293 cells. Using an O-GlcNAc-specific antibody, CTD110.6, Western blot analysis revealed bands at positions corresponding to core histones (Fig. 1A). This putative identification of O-GlcNAcylated histone using CTD110.6 was confirmed using RL2 (Fig. 1B), an independent monoclonal antibody specific to O-GlcNAc. In this experiment we also enriched the histones using acid extraction and, as the O-GlcNAc modification is notoriously labile (1-2), we used PUGNAc, an inhibitor of the OGA enzyme that removes O-GlcNAc. We obtained highly similar data with the RL2 antibody, as well as demonstrating increased histone O-GlcNAcylation upon increasing concentration of the OGA inhibitor. These analyses also revealed an apparent order (which was similar with both antibodies) of histone O-GlcNAcylation, with histone H3 displaying the most O-GlcNAcylation, H4 and H2B being similarly O-GlcNAcylated and H2A O-GlcNAcylation only weakly apparent after prolonged exposure (and see below). To further confirm the authenticity of histone O-GlcNAcylation we used a non-immunological method to detect histone O-GlcNAcylation. The lectin wheat germ agglutinin (WGA) is specific to O-GlcNAc (1-2) and when coupled to horseradish peroxidase (WGA-HRP) can be used in Western analyses to detect O-GlcNAcylated protein. WGA-HRP revealed a similar pattern of histone reactivity to the antibodies RL2 or CTD110.6, although WGA-HRP preferentially detects H4 over H2B (Fig. 1C and see also Figure 2). In addition, in this experiment we also used a mild base treatment (up to 50 mM NaOH) of the acid-extracted histones, as this treatment is known to reduce O-linked rather than N-linked glycosylation due to the β-elimination reaction (28). This mild base treatment significantly diminished the WGA detection of histone O-GlcNAcylation by approximately 50% (see upper graph in Fig. 1C).

To obtain a higher resolution of individual histones modified by O-GlcNAc, and also to further confirm histone O-GlcNAcylation, we performed two-dimensional gel electrophoresis with acid extracted histones (Fig 2). The first dimension involves a separation based on both the positive charge and hydrophobic properties of histones, termed TAU gel electrophoresis (24). The second dimension separation proteins based
on molecular weight using conventional SDS-PAGE. This methodology resolved all histones from the core nucleosome including H2A, and its minor variants, all three H3 variants, H3.1, 3.2 and 3.3, H2B and H4 (Fig. 2A). Histones resolved on these 2D gels were transferred to nitrocellulose membrane, followed by detection with either the RL2 (Fig. 2B) or CTD110.6 (data not shown) antibodies, which both revealed that the O-GlcNAc signal was highest for histone H3 (the sum of all three of its variants), followed by H2B and H4, and finally H2A (Fig. 2 B). Similar results were also obtained using WGA-HRP to detect O-GlcNAcylated histones (Fig. 2C), although detection of H2B in these gels with this lectin reagent is relatively weaker than with either of the immunological reagents. Notably, the 2D gel technique greatly facilitates visualization of O-GlcNAcylated H2B and H2A that are otherwise difficult to resolve by the one dimensional SDS-PAGE, the latter technique has a lower resolution and more limited loading capacity that together prevent easy detection of weak O-GlcNAc signals (see Fig. 1).

*Increasing the cellular levels of UDP-GlcNAc results in enhanced histone O-GlcNAcylation.* Within cells the O-GlcNAc donor is the nucleotide-sugar UDP-GlcNAc that is used by OGT to add the GlcNAc moiety to serine or threonine residues in targeted proteins. UDP-GlcNAc is generated in the hexosamine biosynthetic pathway (HBP), with its level directly related to that of glucose or glucosamine (1-2). As a result, increasing the level of glucosamine in cells often increases the stoichiometry of O-GlcNAcylation on a modified protein. We therefore treated HEK293 cells with glucosamine at 5 mM during cell culture, followed by acid-extraction of histones and examination of histone O-GlcNAcylation. Western blot analysis of histones separated by SDS-PAGE revealed a significant enhancement of histone O-GlcNAcylation as detected using the RL2 monoclonal antibody (Fig. 3A). Quantification of this glucosamine-dependent increase in histone O-GlcNAcylation using 2D gels and the RL2 antibody demonstrated a parallel increase of all four core histones (Fig. 3B). The glucosamine stimulated increase in O-GlcNAcylation of the core histones further supports our observations on histone O-GlcNAcylation.

*Serine 10 of histone H3 is a site of modification by O-GlcNAc.* While treating cells with glucosamine to stimulate histone O-GlcNAcylation we noticed a reciprocal relationship between histone O-GlcNAcylation and phosphorylation of histone H3 on serine 10 (Fig. 4A). Increased O-GlcNAcylation of all forms of histone H3 detected on one-dimensional TAU gels using antibody RL2 correlated with decreased phosphorylation of H3 forms on serine 10 (H3S10ph). Intriguingly, phosphorylation of serine 28, which is found in a similar sequence context (ARKS) to serine 10, increased upon glucosamine treatment. The relative loss of H3S10ph but increase in H3S28ph upon stimulation of global histone O-GlcNAcylation points to a surprisingly distinct regulation of these related posttranslational modifications upon glucosamine treatment. We also examined modification of lysine 9 of histone H3, as it is the neighbouring residue to serine 10. Interestingly, increased histone H3 O-GlcNAcylation upon glucosamine treatment also correlated with a decreased level of H3K9Ac, but an increased level of H3K9me3, which correspond to posttranslational modification characteristic of active and repressed chromatin respectively (Fig. 4A).

The increased histone O-GlcNAcylation induced upon treatment with glucosamine observed with acid extracted histones (Fig. 4A) was also confirmed in whole cell extracts and with different concentrations of glucosamine (Fig. 4B.i). Once more, increasing histone O-GlcNAcylation correlated with diminishing levels of phosphorylation of histone H3 serine 10 (Fig. 4B.ii). Thus, the correlation between increasing histone O-GlcNAcylation and decreasing H3S10ph upon glucosamine treatment is dose-dependent and cannot be an artifact of the acid extraction protocol. Moreover, increasing histone O-GlcNAcylation also correlated with decreasing levels of H3K4me3 that is an active mark (Fig. 4B.iii) and increasing levels of H3K9me3, a repressive mark as seen above with the acid-extracted histones (Fig. 4B.iv).

The identification of specific sites of O-GlcNAcylation by mass spectrometry remains challenging due at least in part to the lability of the glycosidic bond once cells are lysed but also the observation that this modification is often present in cells at sub-stoichiometric levels (1-2).
Therefore, as an alternative approach to obtaining evidence directly supporting the modification of histone H3 on serine S10 by O-GlcNAcylation, we expressed N-terminally Flag- and HA-tagged histone H3.3 and its mutant form S10A and T11V in HEK293 cells. To examine O-GlcNAcylaton of recombinant H3.3 proteins, immunoprecipitation with an antibody against Flag-tag was performed with sonicated nuclear extracts (Fig. 4C.i). This led to an enrichment of Flag-tagged H3.3 proteins in the immunoprecipitates (Fig. 4C.ii). Probing these anti-Flag immunoprecipitates with WGA-HRP detected signal with the wild-type H3.3 or the T11V mutant, but less from its S10A mutant (Fig. 4C.ii, lower panel). Together with the reciprocal relationship between global histone O-GlcNAcylaton and H3S10ph, this genetic data supports serine 10 of histone H3 as a site of O-GlcNAcylaton and suggests an explanation for this reciprocal relationship. That is, serine 10 of histone H3 can either be phosphorylated or glycosylated but not both. Furthermore, the loss of signal in the histone H3.3-S10A mutant relative to the WT indicates that serine 10 is a major site of histone H3.3 O-GlcNAcylaton, while the residual signal in the H3.3-S10A mutant indicates that there are other sites of O-GlcNAcylaton in this histone on histone H3.3.

Association of histone O-GlcNAcylaton with active and inactive histone modifications. As a preliminary attempt to examine the possible biological role of histone O-GlcNAcylaton in the epigenetic regulation of chromosomes, we performed immunoprecipitations using HEK293 nuclear extracts and antibodies against specific histone posttranslational modifications (H3S10ph, H3K9me3 and H3K4me3). Consistent with histone H3 serine 10 being a major site of histone H3 O-GlcNAcylaton, H3S10ph-specific immunoprecipitates lacked appreciable signal when probed with either the anti-GlcnAc antibody, RL2, or the GlcNAc-specific lectin, WGA (Fig. 5A.i). O-GlcNAcylaton of H4 was also abrogated in these H3S10ph-specific immunoprecipitates, suggesting that the chromatin fragments precipitated may also have diminished H4 O-GlcNAcylaton. However, immunoprecipitates of both H3K9me3, a modification associated with inactive or repressed chromatin (see discussion), and H3K4me3, associated with transcriptionally active chromatin (see discussion), both contained O-GlcNAcylated histones as assessed by both WGA-HRP and the RL2 monoclonal antibody (Fig. 5A.i).

To more carefully discern an association of O-GlcNAcylaton with either of these histone marks, we repeated the immunoprecipitation of H3K4me3 and H3K9me3 with nuclear extracts subjected to increasing extents of sonication to minimize incomplete disruption of polynucleosomes. Despite this treatment, the association between H3K9me3 or H3K4me3 modifications with O-GlcNAcylated histone H3 was maintained even after extensive sonication (Fig. 5A.ii). Moreover, immunoprecipitation of micrococcal nuclease generated mononucleosomes (Fig. 5B.i) with the H3K4me3 or H3K9me3 specific antibodies fully supported the previous findings (Fig. 5B.ii). Thus, histone O-GlcNAcylaton can be coupled with posttranslational modifications associated with either active or inactive chromatin.

O-GlcNAcylaton of histones is cell cycle regulated. As a first step in determining the physiological relevance of histone O-GlcNAcylaton and given its reciprocal relationship with the cell cycle regulated phosphorylation of histone H3 on serine 10, we investigated histone O-GlcNAcylaton during cell-cycle progression. HeLa cells were arrested at the G1/S phase boundary by double thymidine block and then released into the cell cycle (see Materials and Methods). Synchrony of cell cycle progression was confirmed using FACS analysis (Fig. 6A), and also assessed by Western blot analysis using the cell cycle marker, cyclin A, (Fig. 6B) and the mitotic markers corresponding to phosphorylation of histone H3 on serine 10 and 28, i.e. H3S10ph and H3S28ph (Fig 6E).

After release from a double thymidine block, O-GlcNAcylaton of histone H3 exhibited an initially decreased level during S-phase and subsequently increased as cells progressed into G2 phase and mitosis (Fig. 6C). Although the signal corresponding to histone H4 was relatively weaker, we could also discern a regulated pattern of histone H4 O-GlcNAcylaton. This modification also increased as cells exited S phase, however, unlike H3 O-GlcNAcylaton, it then decreased concomitantly with the H3S10ph post-translational modification (Fig. 6E), which occurs at late stages of mitosis, but slightly before the
throughout S phase, with this modification regulation histones have a similar pattern of cell cycle O fractions. Although histone H3 is, as usual, more of histone O fractions by acid analysis of cyclins (Fig. 7C). The Western fractions by acid analysis of cyclins (Fig. 7C). The global pattern of histone O-GlcNAcylation in these fractions was very similar. All three histone H3 isotypes, H3.1, H3.2 and H3.3, H2A and H2B are similarly modified in the G1 (Figure 7C, Fraction 1), S (data not shown) and G2+M (Figure 7C, Fraction 7) cell populations. It is important to point out that examination of global histone O-GlcNAcylation during the cell cycle progression cannot rule out any cell cycle-specific modification of a specific histone residue. Therefore, it is possible that at the level of individual residues there may be other examples of cell cycle regulation that have been missed by our analyses.

Phosphorylation of histone H3 at serine 10 by Aurora B is suppressed by OGT in vitro. To determine whether a reciprocal relationship between phosphorylation and O-GlcNAcylation of histone H3 at serine 10 we established an Aurora B-specific in vitro kinase assay. Aurora B is known to phosphorlyate serine 10 of histone H3 and associates with OGT in a physical complex (see Discussion). It is likely that dynamic regulation of H3S10 by these two enzymes might contribute to the regulation of the cell cycle progression. Therefore, we expressed recombinant N-terminally 6xHis tagged OGT from baculovirus in insect cells and purified the protein on Nickel-agarose (Fig. 8A). Using radiolabeled ATP as the phosphate donor and recombinant histone H3 as the phosphate receiver, addition of OGT to the Aurora B-specific kinase reaction suppressed the phosphorylation of histone H3 (Fig. 8B.i). Western blotting with an antibody specific to phosphorylated H3S10 confirmed that OGT indeed suppressed phosphorylation at this residue by Aurora B (Fig. 8B.ii), an effect certainly involving an active enzyme since OGT after heat inactivation did not affect the phosphorylation of H3 by Aurora B (Fig. 8B.iii). These in vitro results support the existence in vivo of a reciprocal relationship between these two enzymes targeting serine 10 of histone H3 for distinct modifications.
DISCUSSION

This work presents primary evidence that serine and threonine residues in histone proteins are modified by the posttranslational addition of an O-linked monosaccharide, β-N-acetylglucosamine, (termed O-GlcNAc). Firstly, we find that glycosylation by O-GlcNAcylation occurs on all four-core histones to varying extents in the relative order, H3, H2B or H4 and H2A. Secondly, increased global O-GlcNAcylation of all three isoforms of histone H3 results in global loss of a modification indicative of active chromatin (H3K9Ac) and a corresponding global increase of a modification indicative of repressed chromatin (H3K9me3). Thirdly, serine 10 of histone H3 is a specific and major site of histone H3 O-GlcNAcylation that is in competition with phosphorylation of this residue. This reciprocal relationship between O-GlcNAcylation and phosphorylation at serine 10 (S10) of histone H3, may exemplify a “Yin-Yang” model involving these two posttranslational modifications competing with each other for the same amino acid to modulate protein function (30). Finally, histone O-GlcNAcylation is cell cycle regulated, being globally decreased in abundance during S phase but increasing from late S/early G2 into mitosis and persisting into the subsequent G1 phase.

Our observations that histone O-GlcNAcylation varied in level during cell cycle progression and overlapped with phosphorylation of histone H3 on S10 or S28 is suggestive of physiological significance for this modification. Phosphorylation of H3 at S10 and S28, as well as two threonine residues, T3 and T11, has been reported to be regulated during late G2 and mitosis, although the precise function of these histone phosphorylations remains mysterious (31). Two possibilities have been suggested. Firstly, phosphorylation of the N-terminal tail of histone H3 may be required for condensation and segregation of mitotic chromosomes. A second proposal is that these modifications serve as a mark indicating that chromosomes are ready for separation, the so-called “ready production” hypothesis (32). Adding further complexity, phosphorylation of H3 may work in combination with other histone modifications that ultimately result in the correct mitotic chromosome configuration required for the passage through the spindle assembly checkpoint (33).

The findings reported here indicate that replication of chromosomal DNA is accompanied by a global change of histone modification; specifically decreased O-GlcNAcylation. We hypothesize that removal of histone O-GlcNAcylation during S phase may lead to greater access of chromatin for the DNA replication machinery.

Modification of histone H3 serine 10 by phosphorylation has also been shown to negatively regulate modification of, or disrupt binding of effector proteins to, neighbouring residues. For example, methylation of the adjacent lysine 9 residue by the H3K9 methyltransferase, Suv3-9 (34), is inhibited by phosphorylation of serine 10, probably due to limited accessibility of K9 once serine 10 is phosphorylated (35). Phosphorylation of H3S10 also leads to dissociation of HP1 (heterochromatin binding protein 1) that is otherwise bound to methylated lysine 9 (36). Moreover, recent evidence has established that certain RNA splicing factors (SRp20 and ASF/SF2) that associate with interphase chromosomes, where they are proposed to have “chromatin-sensor” activity, are also lost from chromosomes upon phosphorylation of histone H3 on serine 10 (37). With respect to mitosis, H3S10 phosphorylation may, by a currently unknown mechanism, disrupt normal interphase chromatin structure, perhaps by causing the dissociation of factors such as SRp20 and ASF/SF2. H3S10ph-mediated dissociation of such factors might then promote recruitment of the chromosome condensing machinery for the compaction of mitotic chromosomes (38). We propose that antagonism of histone H3 phosphorylation at serine 10 by O-GlcNAcylation of the same residue may promote balanced condensation and/or segregation of chromosomes during mitosis, by locally and dynamically regulating the chromatin fiber.

A role for O-GlcNAcylation of non-histone proteins in the regulation of mitosis is indicated by the observation that OGT localizes to the midbody during telophase as part of a multicomponent complex. In addition to OGT, this complex also included OGA, Aurora B kinase and protein phosphatase 1 (39). It is likely that this complex may co-ordinate dynamic O-GlcNAc and
O-phosphate cycling at the mid-body to regulate cytokinesis and the exit from mitosis. O-GlcNAcylation of histone H3 at serine 10 may occur in a similar enzymatic context in which there is a reciprocal interplay between OGT/OGA and Aurora B kinase/PP1, the major kinase and phosphatase responsible for the mitotic phosphorylation and post-mitotic dephosphorylation of H3S10.

While H3S10 phosphorylation is associated with mitotic chromosomes that are in a highly condensed state, the same histone modification is also involved in transcription related to de-condensed chromosomes during interphase (40-41). Emerging evidence favours a role for OGT in transcriptional repression (18, 20-21), therefore it is reasonable to suggest that O-GlcNAcylation of H3S10 is involved in the formation of inactive or repressed chromatin by counteracting phosphorylation of the same residue (42). Phosphorylation of H3S10 has been shown to recruit the transcriptional activator and histone acetyltransferase, Gen5, leading to acetylation of H3K9 and K14 (43-44). Phosphorylated H3S10 has also been shown to recruit a phosphospecific binding protein, 14-3-3, which may in turn promote release of HP1 proteins or, alternatively, provide a platform for additional transcriptional factors, as has recently been shown for the H4K16 histone acetyltransferase, hMOF (45-47).

Moreover, rapidly inducible transcriptional responses, such as those at heat-shock loci, require H3S10 phosphorylation to target a serine 2-specific CTD kinase, termed P-TEFb (positive transcription elongation factor b), which in turn promotes the release of the paused RNA polymerase II into the elongation phase of RNA synthesis (48-49). We speculate that O-GlcNAcylation of histones at residues including H3S10 may function to counteract the varied effects of H3S10ph on transcription. With respect to genes such as those regulated by heat shock where regulation of RNA polymerase II occurs at the initiation-to-elongation transition, O-GlcNAcylation of H3S10 might also be related to earlier reports that O-GlcNAcylation of RNA polymerase II at serine residues of its CTD heptapeptide tandem repeats (17), inhibit phosphorylation of these repeats and thereby inhibits RNA polymerase II transcriptional cycling (50-51).

We found that histone O-GlcNAcylation was associated with both H3K9me3 and H3K4me3, marks delimiting repressed and active chromatin respectively (52). Thus, histone O-GlcNAcylation should not be considered as exclusively a repressive mark, indeed a recent study in Caenorhabditis elegans indicates that O-GlcNAcylation of proteins cycles dynamically at many promoters (53). One possibility is that the association of histone O-GlcNAcylation with histone modifications characteristic of both repressed and active chromatin may be indicative of functions in rapid switching between the inactive and active state of chromatin. In this respect, transcriptionally poised RNA polymerase II is already known to be associated with both an active mark (H3K4me3) and a repressive mark (H3K27me3) at certain promoters, the so-called ‘bivalent’ modification (54-55). If at some promoters GlcNAcylation of H3S10 can work synergistically with the repressive mark, H3K27me3, this might explain our observation that glucosamine-induced histone O-GlcNAcylation led to decreased phosphorylation at H3S10 but increased phosphorylation at H3S28. H3S28ph could antagonize the repressive H3K27me3 modification in order to compensate for increased H3S10glc-dependent repression.

The regulation of transcription through posttranslational modification of serine 10 of histone H3 is no doubt highly complex and involved in many signaling pathways that respond to multiple intra- or extracellular stimuli (56-57). For example, H3S10 phosphorylation has been implicated in expression of NF-kB responsive gene (58), stress responses (59), insulin signaling (60), development (61) and oncogenic transformation (62). Coincidentally, many of these processes are also regulated by O-GlcNAcylation (1-3, see introduction). The future development of reagents capable of selectively recognizing specific sites of O-GlcNAcylation will be required in order to determine the precise functions of specific sites of histone O-GlcNAcylation.

During submission of this study the Hart group reported O-GlcNAcylation of histones, with a specific focus on three sites of O-GlcNAcylation on histones H2A (T101glc), H2B (S36glc) and H4 (S47glc) (63). Our work is focused on O-GlcNAcylation of histone H3 and is complementary to the earlier report. Together
these studies strongly support the identification of O-GlcNAcylation as a novel posttranslational modification of histone proteins, further expanding the repertoire of histone modifications
REFERENCES


FIGURE LEGENDS

Fig 1. Human histones are posttranslationally modified by glycosylation, specifically O-GlcNAcylation. A. Whole cell lysates were prepared from cultured HEK293 cells that were immediately denatured in SDS-PAGE sampling buffer at 95°C for 5 min. After electrophoresis through a 15% SDS-
polyacrylamide gel, Western blotting was performed with the mouse monoclonal antibody CTD110.6, specific to O-GlcNAc. B. Histones were prepared by acid-extraction from HEK293 cells as described in Experimental procedures. PUGNAc was included at the indicated concentrations in all the buffers to inhibit O-GlcNAcase that removes O-GlcNAc. After SDS-PAGE of extracted histones, Western blotting was performed with the mouse monoclonal antibody RL2, also specific to O-GlcNAc, which is quantified 3 times via densitometry and presented in units normalized to the protein levels, with errors indicating standard deviation of the mean. C. Histone O-GlcNAcylation was examined for its sensitivity to mild base treatment leading to β-elimination of the O-linked GlcNAc. For this purpose, the acid-extracted histones were incubated without or with 25 and 50 mM NaOH for 30 min at 37°C, followed by SDS-PAGE and Western blotting with a lectin probe WGA-HRP that is also specific to O-GlcNAc. A rabbit polyclonal antibody against total histone H3 was used for presenting an input control. Quantifications are presented as above.

Fig. 2. Histone O-GlcNAcylation is further resolved using two-dimensional gel electrophoresis. Acid-extracted histones from HEK293 cells were separated first by TAU (Triton X-100-Acetic acid-Urea) gel electrophoresis, followed by a second dimensional separation through 15% SDS-PAGE. Histones after TAU or two dimensional gel electrophoresis were visualized by Coomassie blue (A), or transferred to a nitrocellulose membrane for Western blot with the antibody RL2 (B) or WGA-HRP (C), both of which revealed the levels of O-GlcNAcylation of individual histones and some of their isoforms or variants, as indicated.

Fig. 3. An in vivo treatment with glucosamine enhanced global levels of histone O-GlcNAcylation. A. HEK293 cells in culture were treated by glucosamine at 5 mM for 16hrs, followed by acid-extraction of histones, SDS-PAGE and Western blot with antibody RL2 to examine histone O-GlcNAcylation. B. The acid-extracted histones as shown in A were separated in parallel by two dimensional gel electrophoresis as shown in Figure 2, followed by Western blot with WGA-HRP for detection of individual histone O-GlcNAcylation. Quantifications based on densitometry were presented for a comparison of individual histone O-GlcNAcylation without or with the glucosamine treatment. Quantifications, in units normalized to the protein levels, are the average and the standard deviation of three experiments.

Fig. 4. Histone H3 serine 10 is a site of O-GlcNAcylation. A. Enhancement of histone O-GlcNAcylation by glucosamine is associated with a decreased level of phosphorylation of H3S10 (H3S10ph). Acid-extracted histones from HEK293 cells untreated (-) or treated (+) with glucosamine were separated by TAU gel electrophoresis, followed by Western blotting with antibody RL2 to detect histone O-GlcNAcylation and antibodies against various histone H3 posttranslational modifications, as indicated, as well as an antibody against total H3. B. Western blot of whole cell lysates after glucosamine treatment at the indicated concentrations. Quantifications based on densitometry are presented in units normalized to the protein levels and are the average and the standard deviations of three experiments. SDS-PAGE followed by Coomassie staining indicates total histones. C. Flag- and HA-tagged wild-type H3.3, its S10A and T11V mutant expressed from plasmid pOZ-FH-C transfected into HEK293 cells. Cells at 48 h after transfection were collected for preparing the nuclear extracts (i) and then immunoprecipitates (ii) with a mouse monoclonal antibody (M2) against the Flag-tag. Recombinant H3.3 proteins were examined by Western blot with WGA-HRP for detecting O-GlcNAc from recombinant H3.3. The WGA signals were quantified and normalized to the signals of anti-HA tag antibody examining recombinant H3.3 in the FLAG-tag pull down.

Fig. 5. O-GlcNAcylation is associated with both inactive and active marks of histone H3. A. (i) Histone immunoprecipitations using HEK293 nuclear extracts were performed with increasing amounts of antibody against H3S10p, H3K9me3 and H3K4me3 as indicated. Immunoprecipitated histone H3 was examined by Western blot with the antibody against total H3, WGA-HRP or antibody RL2 to detect
histone O-GlcNAcylation in the immunoprecipitates. (ii) Histone immunoprecipitations as above and as indicated were performed with nuclear extracts sonicated for indicated number of times, followed by Western blot of immunoprecipitated histone H3 and histone O-GlcNAcylation revealed by WGA-HRP.

B. Immunoprecipitation of mononucleosomes derived from micrococcal nuclease (MNase) digestion of the HEK293 nuclei (i) and Western blot showing histone O-GlcNAcylation associated with mononucleosomes immunoprecipitated with the indicated antibodies (ii).

Fig. 6. Histone O-GlcNAcylation is cell cycle regulated as assessed by double thymidine block and release. HeLa cells were synchronized by a double-treatment with thymidine to block cell cycle at the transition of G1 to S phase. Cell cycle was followed at the indicated time points after release from the thymidine block. A. FACS profiles showing a progression through the cell cycle. B. Western blot using whole cell lysates and an antibody against cyclin A. Cell cycle phases estimated from the FACS data, cyclin A and H3S10ph (see below) are indicated above the cyclin A blot. C. Histone O-GlcNAcylation examined with the antibody RL2. D. Histones visualized by Coomassie-staining and SDS-PAGE. E. Western blot showing the histone H3 posttranslational modifications H3S10ph, H3S28ph and H3K9me3. Coomassie-stained total histones and H3K9me3 serve as loading controls.

Fig. 7. Histone O-GlcNAcylation is cell cycle regulated as assessed by centrifugal elutriation. Asynchronously growing human acute T lymphoblastic leukemia cells, CCRF-CEM, were loaded into an elutriator rotor and the flow rate was increased in steps to elute small to large cells. A. FACS analysis of the seven eluted fractions indicates successful cell cycle fractionation. B. Western blot analyses of the indicated proteins after SDS-PAGE. Histones were prepared by acid extraction and detected using WGA-HRP and antibodies against H3S10ph, total H3, cyclin E, cyclin A and cyclin B1 as indicated. C. O-GlcNAcylation of histones prepared from G1 (fraction 1) and G2 + M (fraction 7) cells assessed by two dimensional gel electrophoresis and Western blotting with WBA-HRP. Note that the faint spot below and to the left of the H2A spot that is visible in part C, fraction 1 is normally not visible in these 2D gels (and see ref 24) and is either a gel artifact or possibly a minor histone variant specific to G1.

Fig. 8. OGT suppressed phosphorylation of histone H3 at serine 10 by Aurora B in vitro. A. Purification of baculovirus-expressed OGT on Ni²⁺-agarose. Proteins from whole lysate, flow-through, and fractions eluted at the indicated concentrations of imidazole were examined by SDS-PAGE followed by Coomassie stain. B. Effect of OGT on phosphorylation of histone H3 by Aurora B. For all the reactions UDP-GlcNAc was included in the assay mixtures as described in Experimental Procedures (i). Aurora B kinase assay performed with γ-32P-ATP as the phosphate donor. (ii). Aurora B kinase assay performed with unlabeled ATP (cold) and phosphorylation examined by Western blotting with antibody specifically recognizing phosphorylated serine 10 of H3. O-GlcNAcylation of histone H3 by OGT was examined with the anti-O-GlcNAc antibody CTD110.6. Histone H3 as substrate in the assays was presented by Western blot with an antibody against unmodified H3. (iii). As a control of OGT activity, the protein was heated at 95 °C for 5 min before addition to the assay mixtures.
Figure 1

A. Whole cell lysate

B. Acid-extracted histones

C. β-elimination

[Images and graphs showing Coomassie stains and Western Blots for each condition]
H2A and its variants

Figure 2
A.

![Coomassie WB: RL2](short exposure)

- +

H3
H2B
H2A
H4

B.

![Bar graph](glucosamine (5 mM))

- glucosamine
+ glucosamine

Unit normalized to Protein

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Figure 3
**A.**

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**B.** Whole cell lysate

- **i.)**
  - WB: WGA-HRP
  - Unit Normalized to Protein
  - 0 2.5 5 15 Glucosamine (mM)

- **ii.)**
  - WB: H3S10ph
  - Unit Normalized to Protein
  - H3

- **iii.)**
  - WB: H3K4me3
  - H3

- **iv.)**
  - WB: H3K9me3
  - H3

- **v.)**
  - WB: H3(iota)
  - H3
  - H2B
  - H2A
  - H4

**C.**

- **i.)** Nuclear extract (Flag-IP input)
  - WB: HA
  - FLAG/HA-H3.3

- **ii.)** Flag-IP
  - WB: WGA-HRP
  - FLAG/HA-H3.3

- **iii.)**
  - WB: WGA-HRP
  - Unit Normalized to Protein
  - 0.2 0.1 0 0.3 0.4

**Figure 4**
Figure 5

A.

1. Nuclear extract
2. IP
3. Antibody (µg)

i).

- H3S10ph
- H3K9me3
- H3K4me3

ii).

- Input
- IP: H3K9me3
- IP: H3K4me3

- Sonication times

B.

i).

- Marker
- mono-nucleosome

ii).

- Rb Ig
- H3K9me3
- H3K4me3

- Antibody

- bp

Figure 5
A.

**Figure 6**

Release from thymidine

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**G1**

**S**

**G2 + M**

**G1**

---

**B.**

Cyclin A

**C.**

RL2

(short exposure)

(long exposure)

**D.**

Coomassie

**E.**

H3S10ph

H3S28ph

H3K9me3

Figure 6
Figure 7

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B. Fraction Number

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C. SDS-PAGE

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Figure 7
Figure 8

A. Elution on Nickle-agarose

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i). (autoradiograph)

ii). WB

Antibodies for WB

H3 S10ph

iii). WB (heat-inactivated OGT)

H3 S10ph