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Somatic Cell Cycle Regulation By Histone H3 Modifications: Action of OGT and Kinases

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Abstract.- Histone H3 is amongst the most evolutionarily conserved proteins, and is located along with histone 2A, 2B and 4 in the core of the nucleosome. The N-terminal tails of the histone protrude the chromatin structure and become accessible to various enzymes for post translational modifications (PTMs). Phosphorylation of H3 has been found to have an impact on progression of the cell cycle, especially during mitosis. Another equally abundant PTM is the glycosylation at serine/threonine by *O*-GlcNAc (*O*-linked glycosylation) that occurs on the same or neighboring Ser or Thr residues, which also are accessible to kinases (Yin Yang sites). *O*-GlcNAc is added by *O*-GlcNAc transferase (OGT), and is found exclusively in the nucleus or cytoplasm of the cell. By using computational methods like Netphos 2.0 and Yinoyang 1.2 we found that OGT, Aurora B kinase and OGT, Death-associated protein (DAP)-like kinase, work together in a Yin Yang way, and thereby control specific checkpoints during mitosis. Bio-informatics tool, thus, are very helpful to elucidate the function of the protein by predicting the PTMs in proteins.

Key words: Mammalian Histone 3, phosphorylation, *O*-linked Glycosylation, cell cycle, mitosis

INTRODUCTION

Post-translational modifications (PTMs) of histone proteins play a key role in regulation of chromatin structure and its contact with DNA. These PTMs serve as a specific code (histone code) and facilitates diverse cellular responses like induction of specific gene expression and orderly progression of the cell cycle (Cheung *et al.*, 2000a; Cosgrove and Wolberger, 2005).

Histones, evolutionarily conserved proteins (Cheung *et al*., 2000a), assemble with the DNA to form nucleosomes that are the basic building blocks of the chromatin. Amino terminal tails of histone proteins are located outside the compact chromatin. This portion of the peptide is accessible for structural modifications, such as phosphorylation. Histone H3 phosphorylation is cell cycle dependent, and is associated with different cellular processes. H3 phosphorylation on Ser 10 during interphase does not affect the whole genome, but only affects a subset of genes. This specific phosphorylation is associated with transcriptional activation of specific genes, such as hyperphosphorylation of Ser 10 and activation of PKA in follicle stimulating hormone induced cellular differentiation and phosphorylation of Ser 10 in Fos gene during mitogen induced transcriptional activation (Prigent and Dimitrov, 2003). Phosphorylation is also necessary for the initiation of chromosome condensation in mammalian cells (Van Hooser *et al.*, 1998). During mitosis histone phosphorylation begins in the pericentric heterochromatin region followed by its extension throughout the genome during the G2/M transition phase (Hendzel *et al.*, 1997).

Phosphorylation of Ser 10, in particular, seems to be crucial for progression of mitosis. During mitosis phosphorylation of Ser 10, catalyzed by Aurora B, is highest during prophase and metaphase, and decreases during anaphase and telophase as Ser 10 is dephosphorylated by protein phosphatase 1 (PP1)) in vertebrates (Goto *et al.*, 2002). Therefore, it is apparent that phosphorylation of Ser 10 may play a role in chromatin condensation (Sauvé *et al.*, 1999). Serine 28 also becomes phosphorylated during mitosis and akin to Ser 10 is a highly conserved event playing a role in

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chromosome condensation (Goto *et al.*, 1999). While in the centromeric region H3 is much less present and is replaced by one of its variant known as a centromere-specific H3 variant-A (CENP-A). The CENP-A does not contain Ser 10 in its N-terminal, whereas Thr 11 in centromeric region is phosphorylated by Death-associated protein (DAP)-like kinase (Dlk) (Preuss *et al*., 2003). Since Dlk is a centromere specific kinase Thr 11 in chromosomal arms is not phosphorylated. Dlk has been found to phosphorylate core histones H3, H2A and H4 (Kögel *et al.*, 1998). Preuss and co-workers (2003) proposed phosphorylated Thr 11 of H3 in centromeric region as a recognition code for kinetochore proteins.

Several PTMs of H3 have been documented and have been found to play a prominent role in gene expression. *O*-linked glycosylation (*O*-GlcNAc modification) parallel to phosphorylation is a highly dynamic modification, and occurs on the same or neighboring residues which also are substrates of kinases (Wells *et al.*, 2003). *O*-linked glycosylation (O-GlcNAcylation) is regulated by *O*-GlcNAc transferase (OGT) (adds *O*-GlcNAc to protein backbone) and *O*-GlcNAcase (OGN which removes *O*-GlcNAc from protein backbone). Glycosylation is an important PTM, and is known to influence protein folding, localization and trafficking, protein solubility, antigenicity, biological activity and half-life, as well as cell-cell interactions (Kreppel and Hart, 1999). Unlike other *O*-linked carbohydrate modifications, *O*-GlcNAc is no further modified and is found almost exclusively in the nucleus and cytoplasm. Interplay between these PTMs on the same or neighboring residues has been observed in several nuclear and cytoplasmic proteins (Wells *et al.*, 2003). Computational methods like Netphos 2.0 (Blom *et al.*, 1999) and YinOYang 1.2 (unpublished) are useful for assessing the modification potential (glycosylation and phosphorylation) of a given protein. The amino acids, important for maintaining the 3D structure of a protein and/or its function(s) such as catalytic activity, and binding to ligand, DNA or other proteins, have often been found to be highly conserved residues evolutionarily. These residues are of biological importance. We have identified false negative Yin Yang prediction sites by the above two said methods coupling the conservation status with the modification potential. In several cases potential for glycosylation and phosphorylation on Ser and Thr residues is predicted correctly. But in certain cases Ser and/or Thr residues show a very high potential either for *O*-GlcNAcylation or for phosphorylation but for the other modifications they show a potential very close to the specific threshold value. For instance, when a residue shows a very high potential for phosphorylation and a potential for *O*-GlcNAcylation very close to the threshold value or *vice versa* it appears to be a false negative Yin Yang prediction site as both kinase and OGT compete for the same site. In this study we have identified Yin Yang sites in H3 of *Mus musculus*, and we propose here the involvement of these identified Yin Yang sites for their functional importance.

MATERIALS AND METHODS

Sequence data

The sequence data used to predict phosphorylation and glycosylation potentials of H3 protein in *Mus musculus* was retrieved from the Swiss Prot databases with the entry name H3 histone family, member 1 and primary accession no. P68433. BLAST search was carried out by using NCBI database of non-redundant sequences using all default parameters. The NCBI Blast searches databases and finds regions of local similarity among the sequences of proteins or nucleotides, which can be used to elucidate evolutionary relationships (Altschul *et al.*, 1997). The searches were performed on known species and were divided into vertebrates and invertebrates. The sequences of different species selected for multiple alignment of *Mus musculus* H3 in vertebrates were from Human (RefSeq. CAB02546.1), *Xenopus laevis* (African clawed frog) (RefSeq. CAA51455), *Gallus gallus* (RefSeq. AAA48795) and *Xenopus tropicalis* (RefSeq. CAJ81662); and for multiple alignment of *Mus musculus* H3 with sequences from invertebrate members were *Caenorhabditis elegans* (RefSeq. P08898), *Mytilus chilensis* (RefSeq. AAP94665), *Drosophila pseudoobscura* (RefSeq. BAD02413), *Lytechinus pictus* (RefSeq. AAA30003) and *Styela plicata* (RefSeq. AAB27669).

The chosen sequences were multiply aligned using ClustalW using all default parameters. ClustalW is a multiple sequence alignment program for DNA or proteins. Multiple alignments are used to characterize protein families, to distinguish between new sequences and existing families of sequences, to help predict the secondary and tertiary structures of new sequences and are essential in evolutionary analysis (Thompson *et al.*, 1994).

Prediction methods

Neural network computational methods are composed of a large number of highly interconnected processing elements (neurones) working in parallel to solve a specific problem. An artificial neuron has many inputs and one output. Netphos 2.0 is a neural network prediction method assessing the potential phosphorylation sites on Ser, Thr and Tyr, and is trained to recognize 9-11 amino acids around these phosphorylation sites (Blom *et al.*, 1999). This method is developed by training the neural networks with phosphorylation data from the phosphobase 2.0, which comprise of 414 phospho-protein entries covering 1052 phosphorylatable Ser, Thr and Tyr residues (Kreegipuu *et al.*, 1998). The YinOYang 1.2 server produces neural network predictions for *O*-ß-GlcNAc attachment sites in eukaryotic intracellular/ nuclear protein sequences. This server uses NetPhos to identify possible phosphorylation sites and thus identify Yin-Yang sites. YinOYang 1.2 employed the sequence data to train a jury of neural networks on 40 experimentally determined *O*-GlcNAc acceptor sites for recognize-ing the sequence context and surface accessibility.

For prediction of potential phosphorylation in human histones Netphos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was used. A threshold value of 0.5 is used by Netphos 2.0 to determine possible potential for phosphorylation. All sites that cross the threshold value are determined of possessing positive potential for phosphorylation.

For prediction of *O*-linked glycosylation YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) was used. Similar to Netphos 2.0 a threshold value is also used by YinOYang 1.2 that is varying depending upon surface accessibility of different residues the residues possessing higher potential than the threshold are determined as possible *O*-linked (*O*-β-GlcNAc) glycosylations sites. To determine the feasible interplay between phosphorylation and glycosylation, Yin Yang sites were determined in *Mus musculus* H3. YinOYang 1.2 predicts potential Yin Yang sites in protein. False negative sites were also identified coupling conservation status and modification potential results of the two methods.

RESULTS AND DISCUSSION

Phosphorylation and *O*-linked glycosylation (*O*-β-GlcNAc modification) are two equally important modifications, and have a profound impact on proteins structure and function. In this study we show that the dynamic intracellular *O*-GlcNAc glycosylation by OGT along with phosphorylation plays a key role in progression of the cell cycle.

All of the potentially predicted phosphorylation sites (Fig. 1) were found to be fully conserved in vertebrates as well as in invertebrates. Furthermore, all of the positively predicted phosphorylations sites in *Mus musculus* H3 were found to have potential for *O*-GlcNAc modification and thus were potential Yin Yang sites (Ser 57 was predicted false negative Yin Yang site) (Table I, Fig. 2). No Tyr residues were predicted potential for phosphorylation. The predicted phosphorylation sites in *Mus musculus* are of functional importance as these Ser/Thr residues can be modified by *O*-β-GlcNAc as well providing possible phosphorylation interplay sites. This possible interplay of glycosylation and phosphorylation may regulate the condensation of the chromatin and progression of mitosis.

Zhang and co-workers (2003) identified PTMs in different parts of bovine H3. They also observed phosphorylation at Ser 10 and Ser 28 as described earlier. Using peptide mass finger printinting trypsin lysed fragments, Zhang *et al.* (2003) identified two main Thr phosphorylation sites in H3 on Thr 6 or 11 and 118. These findings are similar to our predicted results for phosphorylation (Table I). Serine 10 and 28, and Thr 6, 11 and 118 were predicted as positive O-GlcNAc modification sites, as well as Yin Yang sites (Table I). All these predicted Yin Yang sites

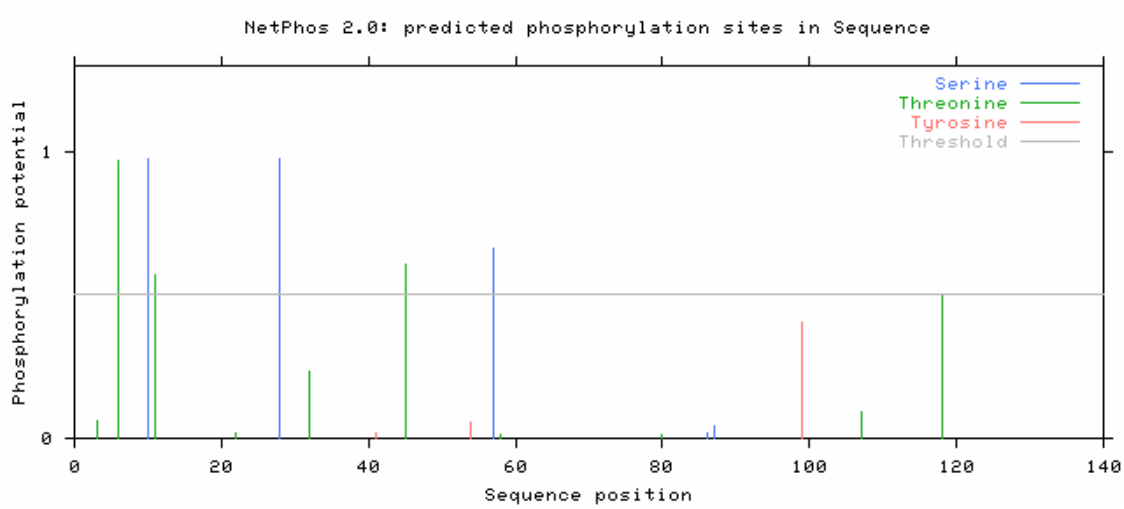

Fig. 1. Graphic presentation of the potential phosphate modification on Ser and Thr residues in *Mus musculus* histone 3. The blue vertical lines show the potential phosphorylated Ser residues; the green lines show the potential phosphorylated Thr residues; the red line show the potential phosphorylated Tyr residues. The light blue horizontal wavy line shows threshold for modification potential

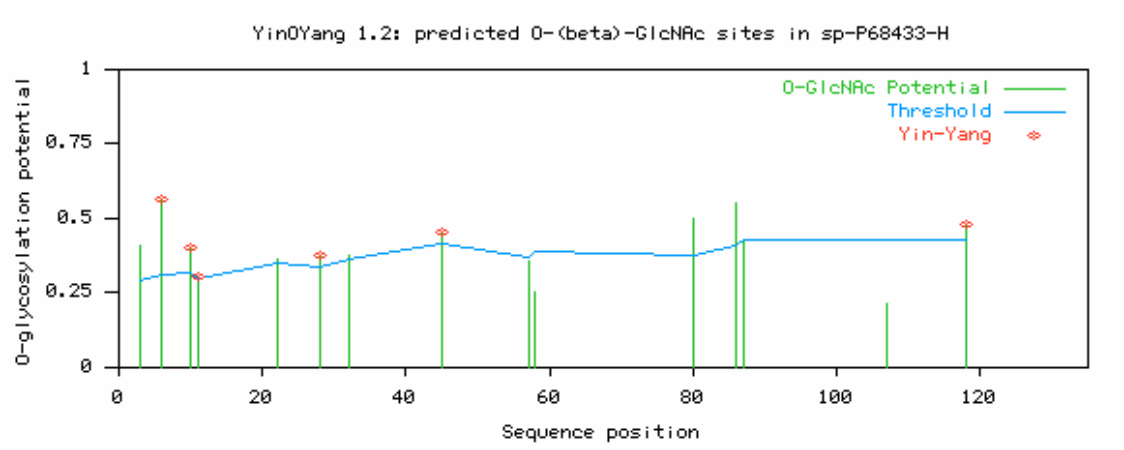

Fig. 2. Graphic representation of potential for *O*-GlcNAc modification of Ser and Thr residues in *Mus musculus* histone 3. The green vertical lines show the *O*-GlcNAc potential of Ser/Thr residue and light blue horizontal wavy line shows threshold for modification potential. The red ○ shows potential yin-yang sites, where phosphorylation and glycosylation is predicted to occur in *Mus musculus* histone 3.

are found in conserved region. Two additional phosphorylation sites have been predicted at Ser 57 and Thr 45 that are located in between the N- and C-terminal of H3 (Table I) and both of these sites are conserved in different animal groups. Amongst them Thr 45 also was a predicted Yin Yang site whereas Ser 57 was identified as false negative Yin Yang site (Table I). Phosphorylation at Ser 10 has been described as the most important site, because it has been found to play a crucial role in the progression of mitosis (Prigent *et al.*, 2003). The Ser 10 is located in N-terminal region that contains several PTM sites. Lys 9, an important functional amino acid can be acetylated or methylated, has been found to play a role in gene silencing. We have predicted Thr 11 as potential phosphorylation

site and acetylation of Lys 14 is linked to Ser 10 phosphorylation in EGF stimulated cells (Cheung *et al*., 2000b). During interphase, acetylation of Lys 9 or Lys 14 influences Ser 10 phosphorylation (Lo *et al*, 2000). Ser 10 of H3 tail becomes a better substrate for its kinase (Aurora B) when either residue (Lys 9 or 14) is previously acetylated (Rea *et al*., 2000). Methylation of Lys 9 prevents phosphorylation of Ser 10 (Rea *et al.*, 2000). This suggests an interplay may exist between different PTMs in H3.

Table I.- Predicted phosphorylation-, glycosylation- and Yin Yang sites in *Mus musculus* of H3.

Predicted phosphorylation sites	Predicted glycosylation sites	Predicted Yin Yang sites
Ser 10, Ser 28, Ser 57 Thr 6, Thr 11, Thr 45, Thr 118	Ser 10, Ser 28, Ser 86 Thr 3, Thr 6, Thr 11, Thr 22, Thr 32, Thr 45, Thr 80, Thr 118	Ser 10, Ser 28, Ser 57* Thr 6, Thr 11, Thr 45, Thr 118

*Predicted false negative Yin Yang site.

H3 phosphorylation during mitosis is essential for chromatin condensation during cell division in mammals (Van Hooser *et al*., 1998). H3 phosphorylation on Ser 10 is linked to transcriptional activation as well as chromosome condensation in mitosis and meiosis. H3 has also been shown to become phosphorylated on Ser 10 on condensed mitotic chromosomes (Hendzel *et al.*, 1997; Prigent and Dimitrov, 2003) and appears more persistent, commencing in pericentric hetero-chromatin during late G2 phase, becoming extensive by prophase, and continue until telophase (Hendzel *et al.*, 1997). H3 is heavily phosphorylated on Ser 10 and possibly on Ser 28 as well during metaphase. Prigent and Demitrov (2003) proposed that phosphorylation at Ser 10 of H3, that commenced in interphase, was a label for the cell to acknowledge that the chromosome was ready for anaphase, where the paired chromatids separated at the kinetochores. Wei *et al.* (1999) described in *Tetrahymena* strain that non-phosphorylated Ser 10 of histone H3 could lead to abnormal segregation and chromosome loss. Phosphorylation of Ser 10 and 28 during mitosis on the H3 N-tail is proposed to be mediated by Aurora B kinase (Goto *et al.*, 2002). But Ser 28 becomes phosphorylated later than that of Ser 10 and is only detectable from prophase to metaphase. Aurora B kinase has been found to destabilize improper microtubule attachments, thereby keeping checkpoint signaling active, and has been found to have a role in the spindle assembly checkpoint (Hauf *et al.*, 2003). Kelly and Hart (1989) showed that chromatin was enriched in *O*-GlcNAc, and countless chromatin proteins were *O*-GlcNAc modified in *Drosophila* polytene chromosomes, meaning thereby that *O*-GlcNAc-modified proteins were concentrated in condensed chromatin and not in transcriptional active regions. Several microtubule associated proteins like TAU and high molecular weight microtubule-associated proteins (MAP 1, 2 and 4) have been found to be modified by *O*-GlcNAc catalyzed by OGT (Arnold *et al*., 1996; Ding *et al*., 1996). OGT has also been found to interact with a histone deacetylate complex by binding to a core repressor mSin3A, repressing transcription and eukaryotic gene silencing (Yang *et al.*, 2002). Toleman and co-workers (2004) showed that OGN possesses intrinsic acetyltransferase activity *in vitro*, and thereby diminish glycosylation and increase the rate of acetylation on histones. Together these data suggest that OGT/OGN may affect DNA synthesis by altering chromosomal structure. Slawson and co-workers (2005) established that progression of the cell cycle is controlled by kinases and OGT/OGN. Furthermore, they showed that during metaphase, OGT is located at the mitotic spindle during anaphase, within the central spindle assembly, and during cytokinesis in the midbody (analogous to Aurora B kinase). According to Murata-Hori and co-workers (2002) Aurora B is localized at chromosomal centromeres during prometaphase, and subsequently relocates to midzone microtubules and midbodies during anaphase and telophase. Furthermore Aurora B kinase is required for appropriate succession of cytokinesis in mammalian cells (Terada *et al.*, 1998). The present results suggest that Aurora B kinase and OGT may work together to control progression of the mitosis. Chromatin-associated PP1 has been found to dephosphorylate Ser 10 of

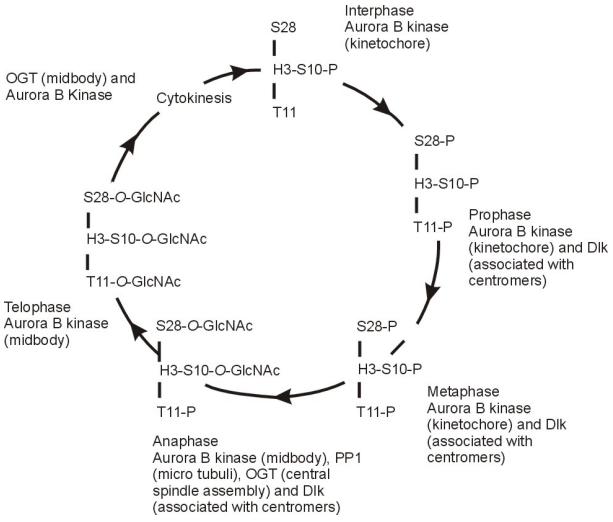

Fig. 3. The complex interplay between OGT and Aurora B kinase, and OGT and Dlk. Aurora B kinase is present during progression of mitosis, and is found in the centromeric region from interphase to metaphase, and then it is located to the midbody. Dlk is found to be associated in the centromeric region at least from prophase to anaphase. OGT is associated with the central spindle assembly in anaphase, from where it dynamically works in a yin yang way with above (and other) kinases. S, T and P are abbreviations for serine, threonine and phosphate, and numbers on these abbreviations show the number of the amino acid sequence position in the Histone 3.

H3 and regulate Aurora B kinase (Goto *et al.*, 2002; Murnion *et al.*, 2001). Wells *et al.* (2004) showed that OGT can exist in a Yin Yang complex with PP1β and PP1γ, and thereby regulates the function of the corresponding kinase. PP1β isoform is found predominantly associated with microtubules (Strack *et al.*, 1999), and is therefore the most likely phosphatase dephosphorylating H3. We propose that Aurora B kinase phosphorylates Ser 10 of H3 from late G2 phase to metaphase, and then is inactivated by PP1, making way for OGT to dynamically add *O*-GlcNAc on Ser 10 thereafter (Fig. 3).

Phosphorylation of Ser 10 initiates at pericentromeric heterochromatin, and then progresses along the chromosomal arms until it spreads to the whole chromosome. H3 is present in the centromers to some extent, but mainly it is substituted by another H3 variant CENP-A, which also is a substrate of Aurora B kinase (Blower *et al.*, 2002). Phosphorylation on Thr 11 of H3 is concentrated in the centromeric region, and occurs from prophase to early anaphase (Preuss *et al.*, 2003). According to Preuss and co-workers (2003) phosphorylation of Thr 11 and CENP-A which also can be phosphorylated by Dlk, might act as a recognition code for kinetochore proteins rather than centromere assembly, because phosphorylation of these residues occurs well after establishment of centromere complexes. Dlk may also work with OGT in a Yin Yang way by phosphorylating Thr 11 until early anaphase, then after dephosphorylation of Thr 11 the *O*-GlcNAc moiety may be added by OGT.

The presence of OGT at the spindle assembly suggests that OGT works together with mitotic kinases in a Yin Yang relationship and regulates the cell division. Aurora B kinase regulates spindle assembly checkpoint (Hauf *et al.*, 2003) which restrains cells from entering anaphase until all replicated chromatids are correctly attached to the bipolar spindle. When PP1 inactivates Aurora B kinase, the metaphase chromosome is ready to enter anaphase. In anaphase, where the pulling of chromosome begins Ser 10 becomes dephosphorylated. OGT then steps in and glycosylates this residue, thereby preventing Ser 10 from getting phosphorylated until required (Fig. 3). We propose that OGT and PP1 together with Aurora B kinase may control the cell's entry into anaphase.

Dlk phosphorylates Thr 11 in the centromere region, where it may contribute labeling centromere-specific chromatin for subsequent mitotic processes (Preuss *et al.*, 2003). Phosphorylation of Thr 11 is affected after phosphorylation of Ser 10, implying that centromere region is complete and kinectochore proteins have started binding to the outer region of centromer, suggesting that this modified residue may play another equally important role in mitosis.The kinetochore is important, because it is responsible for attachment of spindle microtubule, the transition from metaphase to anaphase and the poleward movement. Unattached kinetochores, diminishes microtubules, and unattached or improperly attached kinetochores lead to wait in the metaphase and anaphase signal (Rieder *et al.*, 1995). When Thr 11

becomes phosphorylated it might lead to attachment of kinetochore proteins, and thereby enhance microtubule attachment. In early anaphase when the paired chromatids start separating Thr 11 becomes dephosphorylated and OGT present in central spindle assembly might dynamically add *O*-GlcNAc to this residue (Fig. 3).

Our findings demonstrate that a complex interplay between *O*-linked glycosylation and phosphorylation of mammalian H3 occurs during mitosis (Fig. 3). During interphase, where Aurora B kinase is present in the centromeric/kinetochore region, only Ser 10 of H3 becomes phosphorylated. Then in prophase Ser 28 and Thr 11 are phosphorylated as well. This means that Aurora B kinase is present throughout interphase to telophase, and is active until early anaphase, where PP1 recruited by OGT inactivates Aurora B kinase. When Aurora B kinase is inactivated OGT dynamically adds *O*-GlcNAc on Ser residues, which might persist until telophase. Same is observed with Thr 11, which becomes phosphorylated in late prophase, where Dlk is activated in the centromeric region. During anaphase this residue becomes dephosphorylated and OGT may add *O*-GlcNAc in early telophase. It appears that prior to cytokinesis *O*-GlcNAc is removed and cell becomes ready to enter cell cycle.

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