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A Mitotic GlcNAcylation/Phosphorylation Signaling Complex Alters the Posttranslational State of the Cytoskeletal Protein Vimentin

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O-linked β-*N*-acetylglucosamine (O-GlcNAc) is a highly dynamic intracellular protein modification responsive to stress, hormones, nutrients, and cell cycle stage. Alterations in O-GlcNAc addition or removal (cycling) impair cell cycle progression and cytokinesis, but the mechanisms are not well understood. Here, we demonstrate that the enzymes responsible for O-GlcNAc cycling, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) are in a transient complex at M phase with the mitotic kinase Aurora B and protein phosphatase 1. OGT colocalized to the midbody during telophase with Aurora B. Furthermore, these proteins coprecipitated with each other in a late mitotic extract. The complex was stable under Aurora inhibition; however, the total cellular levels of O-GlcNAc were increased and the localization of OGT was decreased at the midbody after Aurora inhibition. Vimentin, an intermediate filament protein, is an M phase substrate for both Aurora B and OGT. Overexpression of OGT or OGA led to defects in mitotic phosphorylation on multiple sites, whereas OGT overexpression increased mitotic GlcNAcylation of vimentin. OGA inhibition caused a decrease in vimentin late mitotic phosphorylation but increased GlcNAcylation. Together, these data demonstrate that the O-GlcNAc cycling enzymes associate with kinases and phosphatases at M phase to regulate the posttranslational status of vimentin.

INTRODUCTION

The posttranslational modification O-linked β-*N*-acetylglucosamine (O-GlcNAc) is an essential cellular regulator in multicellular eukaryotes that influences many processes, such as signaling pathways, stress protection, nutrient sensing, transcription, and cell cycle progression (Hart *et al.*, 2007). In contrast to complex extracellular glycosylation, GlcNAcylation consists of the dynamic cycling of a single GlcNAc residue on serine and threonine hydroxyls of intracellular proteins (Dias and Hart, 2007; Hart *et al.*, 2007). In many ways, O-GlcNAc is analogous to phosphorylation. GlcNAcylation of proteins is highly dynamic and is responsive to multiple cellular stimuli, much like phosphorylation (Kearse and Hart, 1991; Hart *et al.*, 2007). The addition of the sugar residue is catalyzed by the enzyme uridine diphospho-*N*-acetylglucosamine:peptide β-*N*-acetylglucosaminyltransferase (O-GlcNAc transferase [OGT]; Haltiwanger *et al.*, 1990). O-GlcNAc is essential for life in mammals because OGT knockout mice are embryonic lethal (Shafi *et al.*, 2000). The removal of the sugar is catalyzed by a neutral family 84 *N*-acetyl-β-D-glucosaminidase (O-GlcNAcase [OGA]) localized mainly to the cytoplasm (Dong and Hart, 1994; Gao *et al.*, 2001; Cetinbas *et al.*, 2006).

Although increased protein GlcNAcylation is protective under multiple forms of cellular stress (Zachara *et al.*, 2004) and prolonged increases in GlcNAcylation contributes to insulin resistance and glucose toxicity (Vosseller *et al.*, 2002; Wells *et al.*, 2003; Hu *et al.*, 2005; Dentin *et al.*, 2008; Housley *et al.*, 2008; Whelan *et al.*, 2008; Yang *et al.*, 2008), the roles of GlcNAcylation in cell cycle progression and cytokinesis are only now being fully explored. A growing body of evidence suggests that dynamic cycling of O-GlcNAc is necessary for cellular growth. When OGT is knocked out of mouse embryonic fibroblasts using cre-lox technology, the cells become growth arrested with increased amounts of cyclin inhibitor p21 (O'Donnell *et al.*, 2004). Further studies in *Xenopus* oocytes showed delayed progesterone induced maturation when OGA or OGT was inhibited (Slawson *et al.*, 2002; Dehennaut *et al.*, 2007). These data suggest that O-GlcNAc cycling is of critical importance during oocyte maturation; furthermore, this hypothesis is supported by galactose-capping experiments in which β-galactosyltransferase, which caps terminal GlcNAcs and prevents cycling, was microinjected into oocytes and maturation was blocked (Fang and Miller, 2001). Studies in mammalian cells further corroborated these findings. Increases in O-GlcNAc either with pharmacological agents or with OGT overexpression delayed cell cycle progression, especially at M phase (Slawson *et al.*, 2005). Cells overexpressing OGT showed mitotic exit defects and a substantial increase in polyploidy (Slawson *et al.*, 2005). Interestingly, OGT localized to the mitotic spindle and moved to the midbody during cytokinesis (Slawson *et al.*, 2005). The OGT midbody localization was

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Abbreviations used: GFP, green fluorescent protein; GT, O-GlcNAc-thiazoline; OGA, O-GlcNAcase; O-GlcNAc, O-linked β-*N*-acetylglucosamine; OGT, O-GlcNAc transferase; PP1, protein phosphatase 1; ZM, ZM447439.

strikingly similar to localization of key components of the midbody, such as Aurora kinase B (Terada *et al.*, 1998).

Aurora kinase B is an essential regulator of mitotic progression (Carmena and Earnshaw, 2003). The enzyme phosphorylates histone H3 at Ser10 (Adams *et al.*, 2001) as well as midbody substrates vimentin and MgcRacGAP (Goto *et al.*, 2003; Minoshima *et al.*, 2003; Fuller *et al.*, 2008). Both knockdown and overexpression of the kinase led to an increase in polyploidy (Tatsuka *et al.*, 1998; Adams *et al.*, 2001; Ota *et al.*, 2002). Additionally, multiple tumor cell lines overexpressing Aurora B demonstrate an increase in polyploidy (Tatsuka *et al.*, 1998). The anaphase-promoting complex tightly controls the half-life of Aurora B. Disruption in Aurora B degradation also leads to an aneuploid phenotype (Nguyen *et al.*, 2005). Therefore, regulation of Aurora B expression and activity is important for proper mitotic exit.

Aurora B strongly associates with inner centromere protein (Adams *et al.*, 2001) and survivin (Honda *et al.*, 2003). This complex, referred to as the passenger protein complex, is essential for Aurora B interaction with tubulin (Wheatley *et al.*, 2001) and targeting to substrates (Wheatley *et al.*, 2004). This targeting of the complex to distinct cellular locations, such as the midbody, allows the cell to control the accessibility of substrates for Aurora B. Additionally, Aurora B interacts with protein phosphatase 1 (PP1), which acts to oppose the actions of the kinase (Sugiyama *et al.*, 2002; Goto *et al.*, 2006; Emanuele *et al.*, 2008). Together, the cell uses these mechanisms to regulate Aurora function; however, GlcNAcylation could potentially add an additional level of cellular control to this complex.

In this study, we demonstrate that Aurora B localizes in a transient complex with OGT, OGA, and PP1 at the midbody during cytokinesis. Proper cellular localization of this complex is dependent upon Aurora kinase activity because inhibition of the kinase disrupts formation of the complex at the midbody. Furthermore, this complex regulates the posttranslational modifications on vimentin. Together, these data suggest the existence of an O-GlcNAc/phosphate regulatory complex that cooperatively regulates the posttranslational status of proteins.

MATERIALS AND METHODS

Reagents

Antibodies used in the study are the following: AIM-1 (1:1000, 611083, BD Transduction Laboratories, Lexington, KY) Aurora B kinase (1:200 600-401-465, Rockland Immunochemicals, Gilbertsville, PA), PP1c (1:1000, sc-7482 and sc-443, Santa Cruz Biotechnology, Santa Cruz, CA), green fluorescent protein (GFP; 1:5000, sc-9996, Santa Cruz Biotechnology), vimentin (1:5000, V 6630, Sigma, St. Louis, MO), pSer82 vimentin (1:2000, MO82, Medical and Biological Laboratories, Nagoya, Japan), pSer71 vimentin (1:2000, TM71, Medical and Biological Laboratories), pSer55 vimentin (1:1000, 3877, Cell Signaling Technology, Beverly, MA), and O-GlcNAc (1:5000, 110.6; available from Sigma or Covance Laboratories, Madison, WI; Comer *et al.*, 2001). Polyclonal antibodies for OGT (1:1000, AL-28) and OGA (1:1000, 345) were raised in rabbit and chicken, respectively. Protein A/G beads were from Santa Cruz (sc-2003) and donkey anti-IgY agarose was from Gallus Immunotech (Wildwood, MO; DAIgY-AGA). Horseradish peroxidase–conjugated secondary antibodies were purchased from GE Healthcare (Waukesha, WI; rabbit: NA934V, mouse: NA931V, rat: NA935V) or Sigma (chicken: A 9046 and anti-mouse IgM: A 8786). Fluorescently labeled secondary antibodies Alexa Fluor 647 and Alexa Fluor 488 (A11029, A21449, and A21245) and propidium iodide (P1304MP) were from Invitrogen-Molecular Probes (Eugene, OR). Blots were developed using ECL reagent (GE Healthcare) on Hyper Film (GE Healthcare). Nocodazole and thymidine were from Sigma (M1404 and T1895). ZM447439 (ZM) was from Tocris Bioscience (Ballwin, MO; 2458), dimethylsulfoxide (DMSO) was from Sigma (D 8418), and GlcNAc-thiazoline (GT) was synthesized as described previously (Knapp *et al.*, 1996). β-1,4-Galactosyltransferase was from Sigma (48279), and uridine diphosphate-lsqb]^{3}H]galactose was from American Radiolabeled Chemicals (St. Louis, MO; ART 0131).

Cell Culture and Synchronization

HeLa cells were maintained in Dulbecco's modified Eagle's medium (5 mM glucose, Mediatech, Herndon, VA) with 10% (vol/vol) fetal bovine serum (Cell Gro, Mediatech) and 1% (vol/vol) penicillin-streptomycin (Invitrogen, Carlsbad, CA). Double thymidine block was performed as previously described (Slawson *et al.*, 2005). Eight hours after release from the second thymidine block, ZM (20 μM) and GT (10 μM) were added. Cells were harvested at 12 h. For a late mitotic extract, cells used in the experiment were treated with nocodazole (80 ng/ml) overnight (18 h). The cells were released from the plate by mitotic shake-off, washed twice with sterile phosphate-buffered saline (PBS), and replated. The cells were then harvested 1 h after release. For adenoviral infection, cells were infected at an MOI of 100 for GFP, OGT, and OGA (Slawson *et al.*, 2005). Cells were harvested 2 d after infection and synchronization.

Analysis of Proteins

Cell pellets were washed and lysed as described previously with the addition of 10 mM sodium molybdate (Zachara *et al.*, 2004). Briefly, protein concentrations were determined by the Bio-Rad assay (Bio-Rad, Richmond, CA). For immunoprecipitation studies, antibodies were added to 2 mg/ml protein overnight at 4°C on a rotator. Protein A/G beads (30 μl of slurry) were added to each tube for an additional 2 h the next day. Beads were washed five times in lysis buffer after gentle mixing and then denatured in Laemmli buffer. Protein extracts were separated by SDS-PAGE on Criterion Tris-glycine gels (Bio-Rad), transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then blocked with 3% wt/vol bovine serum albumin (Sigma) in Tris-buffered saline. After detection, blots were stripped for 1 h at 25°C in 200 mM glycine (Sigma; pH 2.5) and reprobed using different antibodies.

Confocal Microscopy

HeLa cells were plated and fixed for staining as previously described (Slawson *et al.*, 2005). After fixation cells were washed two time for 10 min in PBS/Mg^{2+} containing 100 mM glycine (pH 7.4). Next, cells were permeabilized in PBS/Mg^{2+} containing 1% (vol/vol) Triton X-100 (Sigma) for 20 min, washed, and then blocked for 1 h in Tris-buffered saline with 0.05% Tween 20 (Sigma) containing 3% bovine serum albumin. Slides were sequentially incubated overnight at 4°C with primary antibody (1:200 Aurora B, 1:1000 OGT/OGA, 1:100 vimentin, 1:200 pSer 55 and pSer71 vimentin), washed as before, incubated with secondary antibody (1:1000 Invitrogen-Molecular Probes) for 1 h at 25°C, and washed. Before mounting, slides were incubated in PBS/Mg^{2+} containing 0.1% Triton X-100 and propidium iodide (1 μg/ml) for 1 min and washed as before. Fluorescent images were obtained on the 3i Spinning Disk Confocal microscope using Olympus Slidebook software (Melville, NY) at the Johns Hopkins University School of Medicine core microscopy facility. 3D-Tiff images procured using Olympus Slidebook software were analyzed using Zeiss LSM 510 AIM software (Thornwood, NY) for quantification.

Galactosyltransferase Labeling

Before immunoprecipitation of vimentin, protein A/G beads (50 μl of slurry) and vimentin mAb (1 μg, V 6630 Sigma) were incubated overnight at 4°C in galactosyltransferase labeling buffer (10 mM HEPES, pH 7.2, Sigma, H 3375), 10 mM galactose (Sigma, G 0750), 5 mM $MnCl_2$, (Sigma, M 8054), 200 μM uridine diphosphate-galactose (Sigma, U 4500), and 1 U of β-1,4-galactosyltransferase to prelabel terminal GlcNAc on immuglobulin chains. The beads were then washed in lysis buffer and added to extract (2 mg/ml) overnight at 4°C. Beads were washed as before in lysis buffer and then five times in labeling buffer. Immunoprecipitated vimentin was then labeled as before except with radioactive UDP-galactose (3 μCi). Beads were washed twice in lysis buffer and denatured for SDS-PAGE. The gel was then stained overnight with Coomassie brilliant blue G-250 (Bio-Rad, 161-0406), destained, and then incubated for 1 h at 25°C in En^{3}Hance (Perkin-Elmer Cetus, Norwalk, CT; 6NE9701). The gel was then incubated for 1 h at 25°C in a 1% (vol/vol) solution of glycerol, dried, and placed with film at −80°C for a minimum of 4 d.

RESULTS

OGT Localizes with Aurora Kinase B during Cytokinesis

Previously, we showed that OGT localized to the midbody during cytokinesis (Slawson *et al.*, 2005). Interestingly, the localization of OGT is nearly identical to the mitotic kinase Aurora kinase B (Murata-Hori *et al.*, 2002). Therefore, we performed colocalization studies of OGT with Aurora kinase B in HeLa cells fixed 12 h after double thymidine release (Slawson *et al.*, 2005). Localization of endogenous OGT and Aurora B was evaluated using immunofluorescence microscopy. During midbody formation and cytokinesis, OGT and

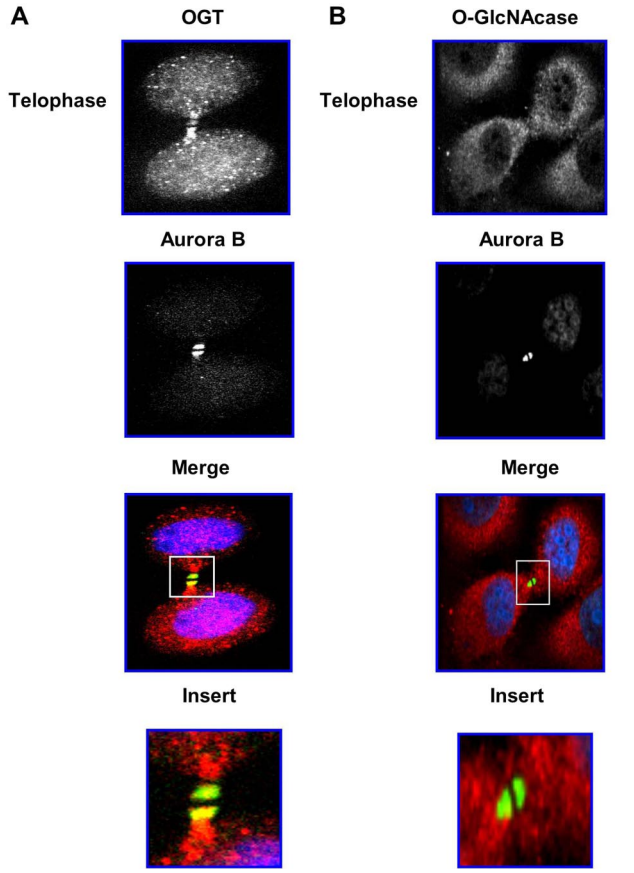
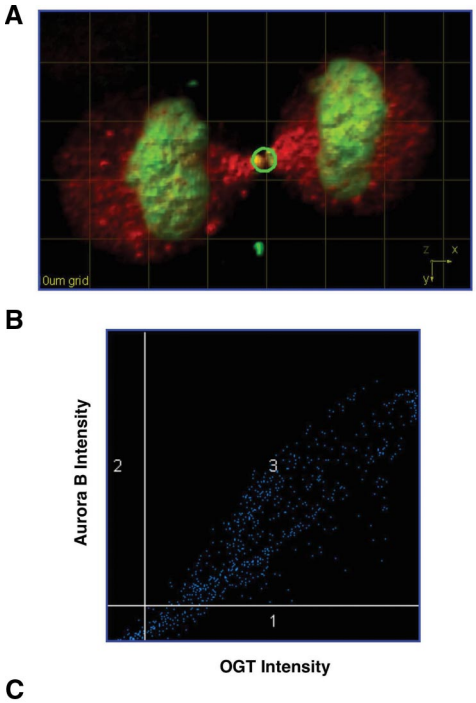


Figure 1. O-GlcNAc transferase localizes with Aurora B at the midbody. (A and B) HeLa cells were synchronized into G1/S by double thymidine block and released. Cells were fixed at 12 h after release. Cells were triply labeled at telophase. OGT is red, Aurora B green, and DNA blue. (A) Endogenous OGT and Aurora B strongly costain at the midbody. (B) Endogenous OGA stains diffusely throughout the cell, and only a small amount was found at the midbody compared with Aurora B. All costaining experiments were performed a minimum of three times.



C

Scatter Region	Colocalization Coefficient Ch1	Colocalization Coefficient Ch2	Weighted Coloc. Coefficient Ch1	Weighted Coloc. Coefficient Ch2	Overlap Coefficient	Correlation R
3	0.875	1	0.951	1	1	0.84

Figure 2. Quantification of three-dimensional colocalization of Aurora B and O-GlcNAc transferase. (A) Three-dimensional image of a cell at telophase captured on 3i-spinning disk confocal microscope. Aurora B staining is green and OGT is red. The green circle is the area used in the quantification analysis. (B) Scatter diagram of the signal from both Aurora B and OGT. Area 3 of the diagram is the area of colocalization with a linear distribution of spots indicating greater colocalization. (C) The statistical analysis of fluorescent signals showed a high degree of colocalization. The overlap coefficient was 1, whereas the more stringent Pearson's analysis (Correlation R) has an overlap of 0.84 (a perfect correlation is 1, whereas no correlation is −1).

Aurora B localized together at the midbody (Figure 1A). No colocalization was seen during metaphase (data not shown), because in metaphase, Aurora B localizes near the central spindle (Minoshima *et al.*, 2003), whereas OGT is enriched near the centromere (Slawson *et al.*, 2005). Furthermore, using Zeiss LSM 510 AIM software, we quantified the colocalization of OGT and Aurora B (Figure 2). On a representative image (Figure 2A), the relative fluorescent intensities of the two proteins showed a linear distribution on a scatter diagram (scatter region 3), suggesting a high degree of pixel overlap (Figure 2B). Analysis using both the overlap coefficient and the Pearson's r (R coefficient) demonstrated nearly perfect colocalization of the two proteins in the selected region (Figure 2C). This data strongly suggests the proteins are localized together at the midbody. OGA stained diffusely throughout the cell during M phase without a concentration increase at the midbody. Little OGA colocalized with Aurora B (Figure 1B).

OGT and OGA Purify Together with Aurora B and PP1 at M Phase

To further verify the interaction between OGT and Aurora B, we performed a series of immunoprecipitation experiments. To show endogenous interactions between the OGT and Aurora B, we synchronized cells by double thymidine block and gathered mitotic cells 10 h after release by mitotic shake-off. These cells were replated and harvested 1 h later to make a late mitotic extract. This extract was compared with an asynchronously growing extract in the coimmunoprecipitation (co-IP) studies.

Although Aurora B protein level was increased in the double thymidine extracts, the enrichment compared with an asynchronous extract was only about twofold (Figure 3A). When Aurora B was immunoprecipitated from these extracts, OGT was found to copurify (Figure 3B). Additionally, several other proteins were also pulled down with Aurora B. The enzyme PP1c, which interacts with Aurora B, was coprecipitated in the mitotic extract (Sugiyama *et al.*, 2002; Goto *et al.*, 2006). OGA was purified in this complex, although the levels are not significant above background (Figure 3B). Aurora B, OGT, and OGA all copurified with PP1 when PP1 was immunoprecipitated (Figure 3C) Previously, OGT was demonstrated to interact with PP1c (Wells *et al.*, 2004), but the interaction of PP1c with OGA is novel.

Immunoprecipitation experiments with OGT and OGA demonstrated that both enzymes were capable of precipitat-

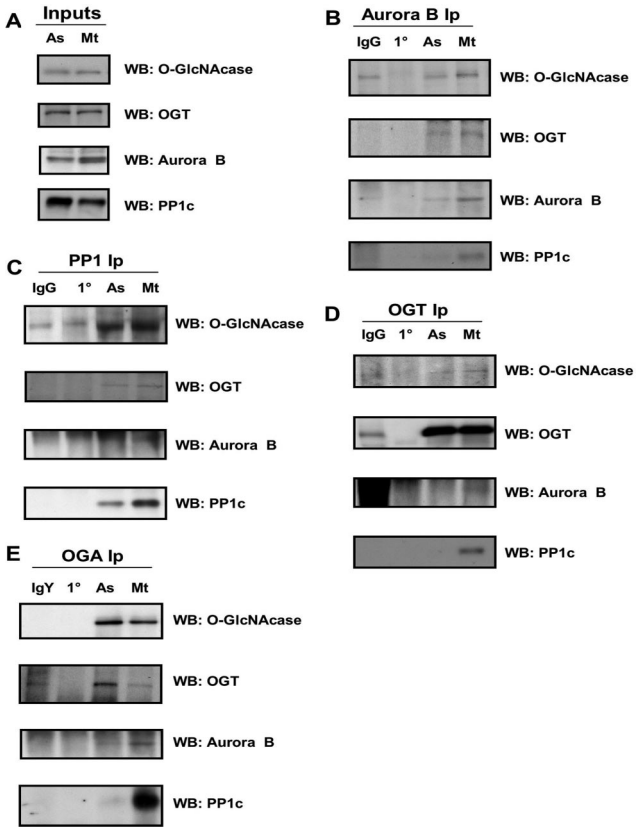


Figure 3. Endogenous Aurora B, PP1, OGT, and OGA all precipitate together in mitotic cells. (A–E) HeLa cells were synchronized by double thymidine block into G1/S and serum released. At 10 h after release, mitotic cells were collected by shake-off and replated. The cells were harvested 1 h after replating to produce a late M phase extract. Cells were lysed and used in coimmunoprecipitation studies. (A) Protein expression of OGA, OGT, Aurora B, and PP1 (30 μg of lysate per lane) as determined by Western blotting (As, asynchronous; Mt, mitotic). (B–E) Endogenous Aurora B, OGA, OGT, and PP1 precipitate each other from synchronized cells (3 mg/ml lysate was used in each precipitation, PP1 IPs were performed with the sc-443 antibody, whereas Aurora B Western blots were performed with the Rockland antibody). Nonspecific IgG and primary antibody (1°) were used as antibody controls. All immunoprecipitations were repeated a minimum of three times.

ing each other and that these interactions were not M phase dependent (Figure 3, D and E). This finding is in agreement with other published results (Whisenhunt *et al.*, 2006). Endogenous OGT was capable of precipitating PP1c (Wells *et al.*, 2004), although this interaction appeared strongest at M phase. Endogenous OGA was also capable of precipitating PP1c both in asynchronous and mitotic cells, although, like OGT, the interaction appeared stronger in the mitotic cells (Figure 3E). Both OGT and OGA were capable of precipitating Aurora B (Figure 3, D and E).

Next, because overexpression of either OGT or OGA cause mitotic exit defects (Slawson *et al.*, 2005), we wanted to determine if overexpression of the O-GlcNAc processing enzymes would potentially disrupt the these interactions. Therefore, HeLa cells were infected with OGT or OGA adenovirus. A GFP adenovirus was used as a control for infection. Because enrichment of a mitotic sample compared with an asynchronous was poor using the double thymidine block mitotic shake-off method, we mitotically arrested cells overnight with nocodazole (80 ng/ml). Cells were then washed twice with PBS to remove the nocodazole and replated for 1 h to make a late M phase extract with or without cytokinesis defects. The cells were then harvested and lysed in a detergent buffer for co-IP studies. To verify that the cells were released into late M phase by nocodazole treatment, a complementary set of samples were analyzed for cell cycle stage by flow cytometry (Supplemental Figure S1A). Expression of Aurora B was 3–4 times higher in these cells compared with asynchronous cells (Figure 4A or 5A)

Aurora B immunoprecipitated little to no OGT from asynchronous cells; however, in the mitotic GFP control sample, endogenous OGT precipitated with Aurora B (Figure 4B). Additionally, endogenous OGA also precipitated with Aurora B in the GFP control sample. When either OGT or OGA was overexpressed, more of both enzymes precipitated with Aurora B, but no interactions were disrupted. Interestingly, increased expression of OGT decreased the overall protein levels of Aurora B (Figures 4A and 5A). Aurora B, OGT, and OGA again all precipitated with PP1 (Figure 4C). These interactions were all higher in mitotic cells compared with asynchronous, although overexpression of OGT or OGA had little effect on the total amount of coprecipitated protein.

Aurora B and PP1 precipitated with both endogenous OGT and OGA and this interaction was M phase dependent (Figure 5, B and C, GFP control sample). Again, OGT and OGA purified each other and this interaction was not M phase dependent. Overexpression of either enzyme did little to disrupt these protein interactions. The immunoprecipitations were not saturating; therefore, the differences seen in each lane were inconsistent.

The Aurora Kinase Inhibitor ZM447439 Alters O-GlcNAc Levels and Disrupts OGT Localization to the Midbody during Cytokinesis

Next, we focused on how inhibition of Aurora B or OGA activity would affect GlcNAcylation and the interactions of the enzyme complex. A late mitotic HeLa extract was generated by nocodazole treatment followed by washing and replating as before. On replating, cells were treated with Aurora B inhibitor ZM447439 (ZM, 20 μM), OGA inhibitor GlcNAc-thiazoline [GT, (3a*R*,5*R*,6*S*,7*R*,7a*R*)-5-acetoxymethyl-6,7-diacetoxy-2-methyl-5,6,7,7a-tetrahydro-3a*H*-pyrano[3,2-*d*] thiazole, 10 μM], or both. An equivalent amount of DMSO was used as a vehicle control. To verify that the arrested cells were in fact mitotic, a complementary set of samples was counted by flow cytometry (Supplemental Figure S2A). Mitotic cells generated in this manner show less GlcNAcylation than asynchronous cells, which had previously been seen (Slawson *et al.*, 2005); however, cells treated with ZM had increased GlcNAcylation, compared with the DMSO-treated controls (Figure 6A). Increased GlcNAcylation was seen on specific protein bands around 80, 120, and 160 kDa. The addition of GT raised O-GlcNAc levels as expected, but the combination of ZM and GT dramatically raised O-GlcNAc levels globally. Treatment of cells with OGA inhibitors is known to raise the expression level of OGA and lower the expression level of OGT (Slawson *et al.*, 2005), which was the result seen with the GT treatment (Figure 6B). ZM treatment lowered Aurora B expression levels, whereas OGT expression increased to levels similar to those of asynchronous cells. OGA levels did not change with ZM treatment. The protein levels of PP1c are not known to change during the cell cycle or after treatment with ZM and GT. PP1c protein levels did not change after treatment and therefore are an internal loading control on the Western blots.

Next, we looked at the cellular localization of Aurora B, OGT, and OGA after inhibitor treatment. Mitotic cells were

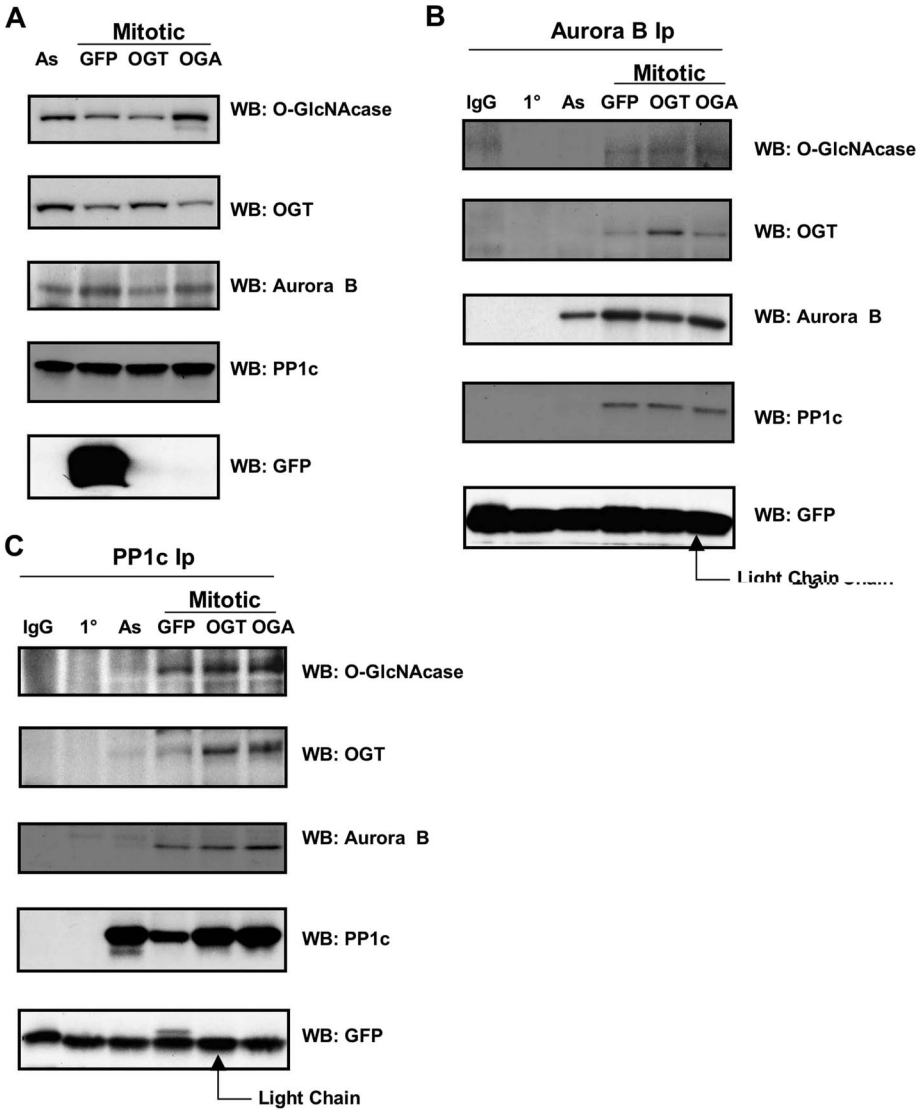


Figure 4. Overexpression of OGT and OGA does not alter the ability of Aurora B and PP1 to precipitate the mitotic complex. (A) Input lanes from samples used in immunoprecipitation studies. Cells were synchronized into M phase by nocodazole treatment and harvested 1 h after nocodazole washout. Protein expression of OGA, OGT, Aurora B, and PP1c (30 μg of lysate per lane) was determined by Western blotting (As, asynchronous). (B and C) Aurora B or PP1c were immunoprecipitated from 2 mg/ml lysate (PP1 IPs were performed with the sc-7482 antibody). Endogenous OGT and OGA copurify with Aurora B and PP1c (GFP lane) in M phase extracts but not in asynchronous extracts. Overexpression of OGT or OGA did not alter their ability to copurify with Aurora B or PP1c. GFP was used as an infection control, whereas nonspecific IgG and primary antibody (1°) alone were used as antibody controls. All immunoprecipitations were repeated a minimum of three times.

prepared by double thymidine block instead of nocodazole treatment because the block method maintains cellular morphology better for immunostaining studies. At 8 h after release, ZM, GT, or both, were added to cells. At 12 h after release, the cells were fixed for immunofluorescent microscopy. Cell cycle stage was verified by flow cytometry (Supplemental Figure S2B). Control cells treated with DMSO demonstrated costaining of OGT and Aurora B matching Figure 1A, but treatment of cells with ZM caused a dramatic reduction in the amount of OGT at the midbody (Figure 6C). The inhibitor treatment also disrupted midbody formation, DNA segregation, and, to some extent, Aurora B localization, all effects that have been previously reported (Ditchfield *et al.*, 2003; Gadea and Ruderman, 2005). Treatment with the OGA inhibitor GlcNAc-thiazoline (Knapp *et al.*, 1996; Macauley *et al.*, 2005) did not impair localization of either Aurora B or OGT. When the two inhibitors were used in concert, the cells were similar in phenotype to the ZM-treated cells. Treatment with ZM and GT had little effect on OGA localization (Supplemental Figure S3). The combination of inhibitors again caused a phenotype similar to the ZM-treated cells with no change in OGA localization.

The Cytoskeletal Protein Vimentin Is a Substrate for Both GlcNAcylation and Phosphorylation at M phase

The mitotic complex of Aurora B, OGT, PP1c, and OGA may regulate both the GlcNAcylation and phosphorylation state of target molecules at M phase; therefore, we looked at possible target proteins for this complex. The cytoskeletal protein vimentin contains at least one mitotic GlcNAcylation site (Wang *et al.*, 2007) and multiple mitotic phosphorylation sites that disrupts filament structure and allows for the proper segregation of the protein to the daughter cells (Tsujimura *et al.*, 1994; Goto *et al.*, 1998; Kosako *et al.*, 1999; Yasui *et al.*, 2001; Goto *et al.*, 2003; Yamaguchi *et al.*, 2005). First, we confirmed the M phase increase in vimentin GlcNAcylation by immunoprecipitating vimentin from cells treated with nocodazole and released as before and galactosyltransferase labeling the O-GlcNAc residues with [^{3}H]UDP-galactose (Figure 7A). When vimentin was galactosyltransferase labeled in cells overexpressing OGT, a new GlcNAcylated form appeared slightly higher than the normal form at ~58 kDa. Under these conditions, it was impossible to determine whether the gel shift was due to GlcNAcylation on unknown sites or to phosphorylation (Figure 7B). The possibil-

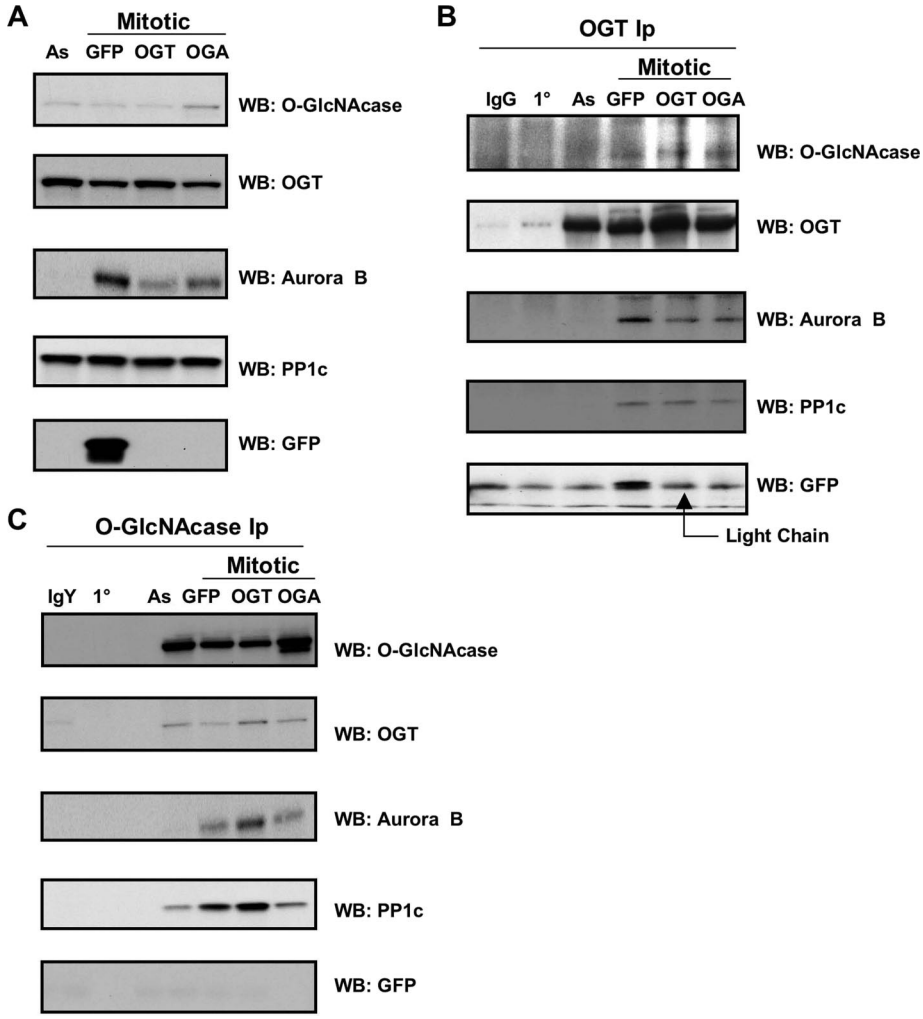


Figure 5. Overexpression of OGT and OGA does not alter the ability of OGT and OGA to precipitate the mitotic complex. (A) Input lanes from samples used in OGT/OGA immunoprecipitation studies. Cells were synchronized into M phase by nocodazole treatment and harvested 1 h after nocodazole washout. Protein expression of OGA, OGT, Aurora B, and PP1c (30 μg of lysate per lane) was determined by Western blotting (As, asynchronous). (B and C) Endogenous OGT and OGA precipitate both Aurora B and PP1c in mitotic extracts (GFP lane; 2 mg/ml). No change in purification was seen in OGT/OGA overexpressing cells (OGA/OGT lanes). GFP was used as an infection control, whereas nonspecific IgG or IgY and primary antibody (1°) alone were used as antibody controls. All immunoprecipitations were repeated a minimum of three times.

ity also exists that the labeled band is an unknown protein that is GlcNAcylated and strongly interacts with vimentin even under stringent precipitation conditions.

When cells were infected with either adenoviral OGT or OGA, there were expected changes in the total GlcNAcylation (Figure 7C). However, when using three different antibodies to specific mitotic phosphorylation sites on vimentin, we also saw major differences in mitotic phosphorylation after adenoviral treatment. Early in G2/M, vimentin is phosphorylated by CDK1 at Ser-55 (Tsujimura *et al.*, 1994). We saw a reduction in Ser-55 phosphorylation in the control GFP-adenoviral late mitotic extract compared with the asynchronous cells (Figure 7C). This observation was not surprising as Ser-55 phosphorylation is a transient early M phase phosphorylation and is absent in telophase cells (Tsujimura *et al.*, 1994) An asynchronous extract would contain a higher population of cells in G2/M than a late mitotic extract. This phosphorylation site is adjacent to the mitotic GlcNAcylation site at Ser-54 (in some sequences of vimentin this site is referred to as Ser-55; Wang *et al.*, 2007). However, the OGT overexpressing cells demonstrated a slightly higher level of Ser-55 phosphorylation than the GFP controls. These cells also had a higher amount of GlcNAcylated vimentin, but the increased O-GlcNAc did not concomitantly reduce the addition of phosphate at this site.

Subsequently, we examined vimentin Ser-82 phosphorylation. Ser-82 is phosphorylated by PLK1 after recruitment to the Ser-55 phosphorylated vimentin (Yamaguchi *et al.*, 2005). As expected, mitotic control GFP extracts showed significantly higher levels of Ser-82 phosphorylation than asynchronous cells. Interestingly, both overexpressed OGT and OGA caused a reduction in phosphorylation at Ser-82 (Figure 7C). Lastly, in cytokinesis, vimentin is phosphorylated at Ser-71 by Rho kinase (Goto *et al.*, 1998) and at Ser-72 by Aurora B (Goto *et al.*, 2003). When we used the pSer-71 vimentin antibody, not only was the 55-kDa phospho-vimentin band increased compared with GFP control in both the OGT- and OGA-overexpressing cells, but an additional band also appeared at ~60 kDa (Figure 7C). This band was of a higher molecular weight compared with the higher glyco-vimentin band seen in Figure 7B.

Next, we treated cells with ZM and GT to see how these inhibitors affected vimentin GlcNAcylation and phosphorylation. Using galactosyltransferase labeling to probe for O-GlcNAc (Haltiwanger and Philipsberg, 1997; Slawson *et al.*, 2002), we found an increase in GlcNAc-vimentin in the cells treated with GT or with ZM and GT (Figure 7D), although the increase in GlcNAc-vimentin with the inhibitor combination was not as robust. The Aurora B inhibitor alone had little effect on vimentin GlcNAcylation. The changes in vimentin posttranslational state after all treatments are summarized in Table 1.

By using the phospho-vimentin antibodies as before, we saw an increase in pSer-55 staining in the ZM-treated cells

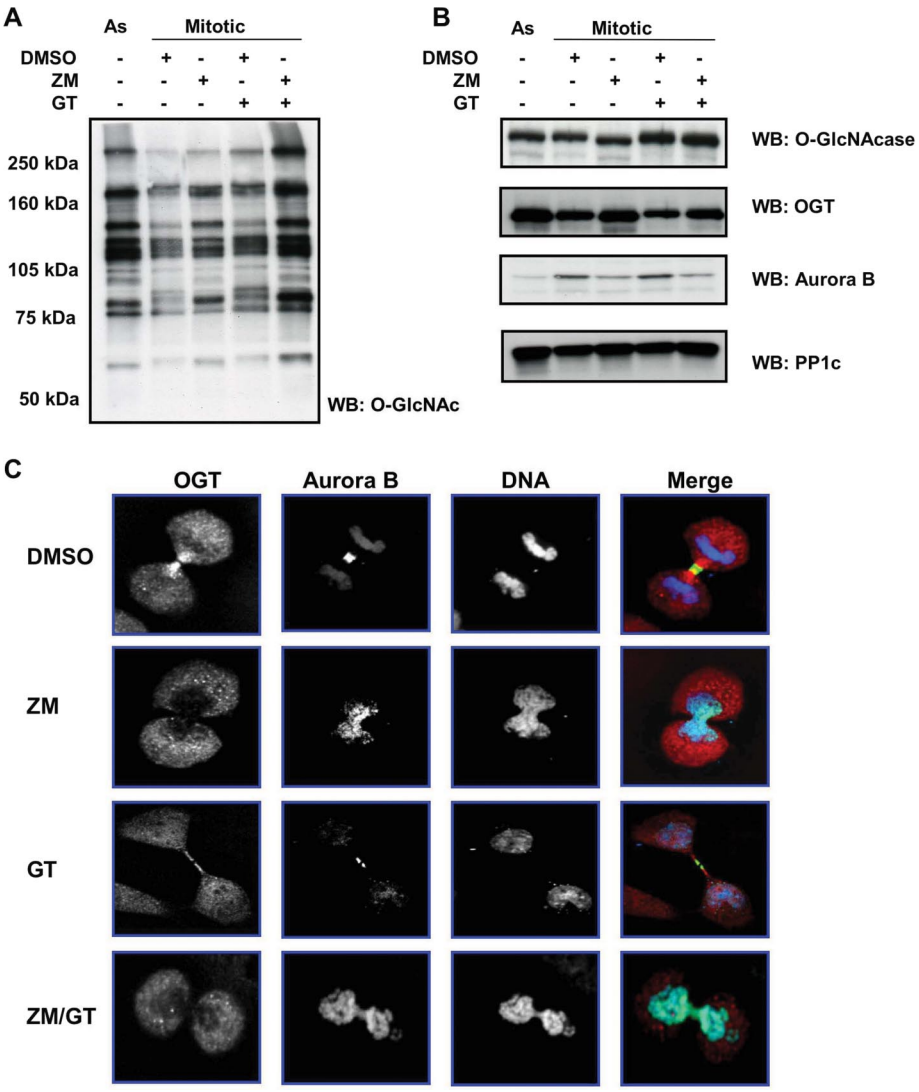


Figure 6. Aurora B inhibition disrupts GlcNAcylation and OGT localization at M phase. Cells were synchronized by nocodazole and released for 1 h. Inhibitors were added at the time of release. (A) Western blot analysis of GlcNAcylated proteins after treatment with DMSO (control), ZM (20 μM), and GT (10 μM). O-GlcNAc levels were elevated in response to ZM, GT, and ZM/GT treatment compared with DMSO controls. (B) Western blot analysis of mitotic proteins (30 μg per lane). ZM treated cells showed a decrease in Aurora B levels and an increase in OGT levels. GT treatment decreased OGT levels and raised OGA protein levels. PP1c was unchanged by treatment. (C) Confocal staining of OGT and Aurora B after double thymidine block and release. Cells were fixed after 12 h. Inhibitors were added after 8 h of release. OGT stained red, Aurora B was green, and DNA was blue. ZM-treated cells caused a marked reduction in OGT staining at the midbody. GT appeared to have little to no effect on localization. Each experiment was done a minimum of three times.

(Figure 7E). The GT-treated cells resembled the DMSO controls. Interestingly, phosphorylation at both Ser-82 and Ser-71 was markedly reduced in the cells treated with GT. Phosphorylation of vimentin at Ser-82 was slightly increased in the ZM-treated samples, whereas pSer-71 was similar to DMSO controls.

Next, we looked at how the different treatments affected the cytolocalization of vimentin and the phosphorylated forms of vimentin. Cells were synchronized by double thymidine block and serum released. The cells were fixed 12 h after release. For adenoviral infection, cells were infected 1 d before release. Cell cycle stage after infection was determined on a complimentary set of samples by flow cytometry (Supplemental Figure S1B) and were similar to previously published data (Slawson *et al.*, 2005). Inhibitors were added 8 h after serum release at previously used concentrations and harvested as before. When control cells were stained for vimentin, a diffuse staining pattern was seen with a lack of filament structure, agreeing with previous reports (Supplemental Figure S4; Tsujimura *et al.*, 1994; Goto *et al.*, 1998, 2003; Kosako *et al.*, 1999; Yasui *et al.*, 2001; Yamaguchi *et al.*, 2005). Cells overexpressing OGT did show a slight increase of filament-like structure near the cleavage furrow. OGA overexpression stained similar to control. Inhibitor treatment also slightly increased the amount of filament structure.

DISCUSSION

In this study, we found that OGT and OGA are in a transient complex during late M phase with the mitotic kinase Aurora B and with PP1. The Aurora kinase inhibitor ZM447439 ablated Aurora B staining at the midbody and colocalization with OGT. Furthermore, overexpression of OGT or OGA or the addition of ZM or the OGA inhibitor GlcNAc-thiazoline disrupted the mitotic GlcNAcylation and phosphorylation of the cytoskeletal protein vimentin. Together, these data suggest that OGT and OGA form complexes with mitotic kinases and phosphatases and that this complex controls the balance of phosphorylation and GlcNAcylation on mitotic substrates, such as vimentin, leading to proper segregation of the proteins into the daughter cells (Figure 8).

Aurora B forms a tight complex with survivin and inner centromere protein (INCENP; Carmena and Earnshaw, 2003), which then rides along the mitotic tubulin network interacting with substrates and other proteins (Carmena and Earnshaw, 2003). PP1 interacts with Aurora B to negatively regulate kinase activity (Sugiyama *et al.*, 2002; Emanuele *et*

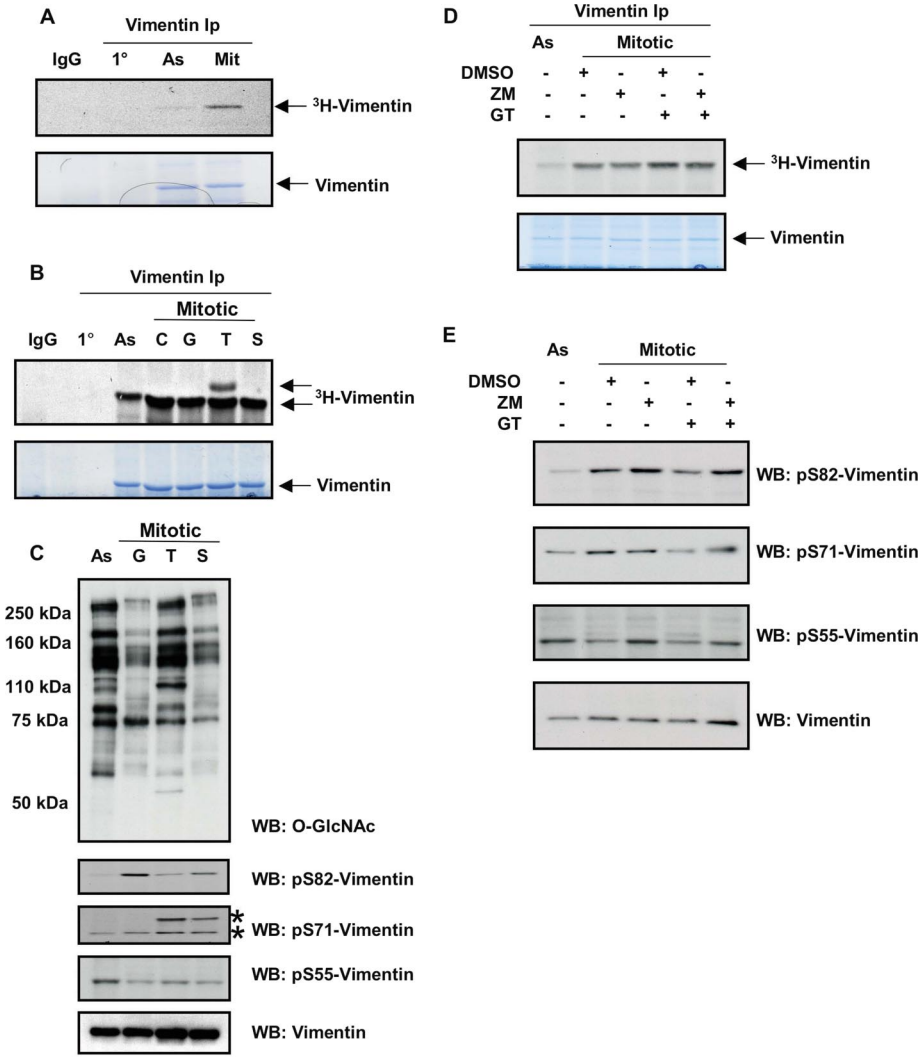


Figure 7. Vimentin is a mitotic substrate of an O-GlcNAc/phosphate complex. (A) Galactosyltransferase labeling of precipitated vimentin from asynchronous (As) and nocodazole released mitotic extracts (Mit) with [^{3}H]galactose. Vimentin was significantly more GlcNAcylated at M phase (top), whereas the amount precipitated was the same (bottom). (B) Vimentin was precipitated and radiolabeled as before from extracts overexpressing OGA and OGT (As, asynchronous; C, uninfected mitotic control; G, GFP overexpression control; T, O-GlcNAc transferase overexpression; S, OGA overexpression). OGT overexpression caused a new GlcNAcylated band to appear. (C) Overexpression of OGT or OGA altered the GlcNAcylation of mitotic proteins and disrupted specific mitotic phosphorylations of vimentin in cells nocodazole released. Asterisk denotes position of pS71-Vimentin. (D) GT treatment increased mitotic GlcNAcylation of vimentin (top), whereas ZM had no effect. Vimentin precipitated equally under all conditions (bottom). (E) Both ZM and GT treatment altered the mitotic phosphorylation of vimentin as judged by Western blot analysis on cell extracts (30 μg loaded per lane). Each experiment was repeated a minimum of three times.

al., 2008). OGT interacts with PP1 (Wells *et al.,* 2004) as well as with OGA (Whisenhunt *et al.,* 2006). Therefore, at mitosis, a transient complex forms in which Aurora B, OGT, OGA, and PP1 interact synergistically to modulate the posttranslational state of given substrates. What is unclear from the coprecipitation studies is whether these enzymes are acting together in one large complex or are existing as mini-complexes of OGT-Aurora B, PP1-OGA, etc, or whether INCENP and survivin bridge the enzymes together by indirect interactions.

OGT contains a large N-terminal tetratricopeptide repeat domain (TPR), which targets OGT to complexes and substrates (Kreppel and Hart, 1999; Jinek *et al.,* 2004). The transcriptional corepressor protein mSin3A targets OGT to complexes through the TPR (Yang *et al.,* 2002) and OGT-interacting protein 106 kDa (OIP-106) interacts with this domain on OGT to target OGT to RNA polymerase II (Iyer *et al.,* 2003; Iyer and Hart, 2003). Interestingly, OIP106 interacts with the microtubule-based motor protein kinesin (Brickley *et al.,* 2005). This interaction might target an OIP106, OGT, and Aurora B complex to kinesin for transport. Therefore, a possible role of the OGT interactions could be in substrate or in complex targeting. Recently, OGT was found to interact with p38 MAP kinase during cellular stress (Cheung and Hart, 2008). The kinase directed OGT to neurofilament H, an intermediate filament protein similar to

Table 1. Summary of M phase GlcNAcylation and phosphorylation states after viral or inhibitor treatment

Treatment	p55 Vimentin	p71 Vimentin	p82 Vimentin	GlcNAc-Vimentin
Viral OGT	Increase	Increase	Decrease	Increase
Viral OGA	No change	Increase	Decrease	No change
ZM	Increase	No change	No change	Decrease
GT	No change	Decrease	Decrease	Increase
ZM/GT	Increase	No change	No change	Increase

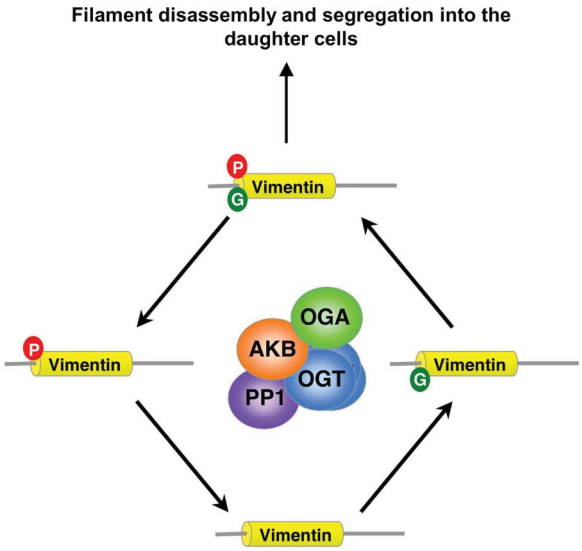


Figure 8. An O-GlcNAc/phosphate complex forms in M phase to regulate the posttranslational state of proteins. A model representing the protein complex of Aurora kinase B (AKB), Protein Phosphatase 1 (PP1), O-GlcNAc transferase (OGT), and O-GlcNAcase (OGA) is proposed; this complex potentially regulates the posttranslational state of target proteins such as vimentin at M phase (P, phosphorylation; G, GlcNAcylation).

vimentin, increasing the proteins GlcNAcylation state and solubility (Cheung and Hart, 2008). Therefore, one can envision Aurora B interacting with OGT and targeting OGT to vimentin (Figure 8) or other potential mitotic substrates. Clearly, OGT substrate targeting is of critical importance in understanding the function of this enzyme, because a single catalytic subunit modifies hundreds of different proteins.

Aurora kinase activity is needed for the proper localization of OGT to the midbody at mitosis. Reduction in Aurora activity caused a significant decrease in midbody localization of OGT. Although Aurora B was still present at the midbody after ZM treatment, a more dispersed staining pattern was evident. OGT or OGA overexpression or treatment with GT did not alter the localization of Aurora B to the midbody. Therefore, Aurora B localization was not predicated on either the amount of OGT or OGA or on levels of cellular GlcNAcylation.

The actions of the inhibitors or overexpressed proteins altered the overall protein levels of Aurora B, OGT, and OGA. The protein levels of OGT and OGA are in equilibrium with total O-GlcNAc levels. Cellular concentrations of each enzyme change reciprocally with increased or decreased GlcNAcylation. For example, as GlcNAcylation increases, OGT decreases and OGA increases. (Slawson *et al.*, 2006). Interestingly, Aurora B protein levels were reduced with increased O-GlcNAc. At this time, the data do not clearly indicate whether Aurora B is an in vivo substrate for OGT and, therefore, a potential target for O-GlcNAc cycling. This decrease in Aurora B could be caused by changes in Aurora B protein stability, translation, or transcription. Conversely, Aurora B inhibition increased OGT protein levels to that of asynchronous cell levels. The expression changes seen with Aurora B and OGT could be symptoms of delayed cell cycle progression in which the cells are aberrantly mitotic or still at G2/M.

Vimentin, a type III intermediate filament protein, undergoes a series of phosphorylations in the head domain during mitosis (Izawa and Inagaki, 2006; Omary *et al.*, 2006), which destabilizes the filament and allows for the proper segregation of the filaments in the daughter cells (Izawa and Inagaki, 2006). A mitotic GlcNAcylation site at Ser-54 (Wang *et al.*, 2007) maps directly next to the CDK1 phosphorylation site at Ser-55 (Tsujimura *et al.*, 1994). The mitotic GlcNAcylation site was determined from cells mitotically arrested with nocodazole followed by chemo-enzymatic tagging and enrichment, chemical derivatization (BEMAD, Beta-Elimination followed by Michael addition by DTT), and mapping by ion-trap mass spectrometer (Wang *et al.*, 2007). This mapping strategy does not preclude the possibility that vimentin is GlcNAcylated at other times of the cell cycle or after cellular stimulus or stress.

Several other cytoskeletal proteins such as neurofilaments or keratins contain O-GlcNAc in the head domain (Chou and Omary, 1993; Dong *et al.*, 1993; Ku and Omary, 1995). The type I intermediate filament proteins keratin 8 and 18 contain up to three GlcNAcylation sites and multiple phosphorylation sites in the head domain (Chou and Omary, 1993; Haltiwanger and Philipsberg, 1997). Using nocodazole to generate mitotic extracts in HT29 cells, keratins were found to have both increased GlcNAcylation and phosphorylation, although, this observation was not seen in HeLa cells treated identically (Chou and Omary, 1993). Interestingly, using other synchronization reagents or isolation of floater mitotic cells for mitotic enrichment did not lead to the observed changes in keratin mitotic GlcNAcylation as seen with the anti-microtubule agents such as nocodazole (Chou and Omary, 1993, 1994). These data suggest that disruption of the tubulin network is key to the observed increase in keratin GlcNAcylation. However, these other synchronization methods do not yield a population of mitotic cells as synchronized as nocodazole treatment (Chou and Omary, 1993). We also saw an increase in a highly synchronous M phase population after nocodazole treatment compared with double thymidine block. Because in our vimentin experiments we also synchronized cells by nocodazole, we cannot determine if the changes in vimentin GlcNAcylation was due to disruption of the microtubulin network or M phase–specific changes. More detailed studies using different M phase enrichment techniques, varying concentrations of nocodazole, and possibly vimentin glyco-site specific antibodies are needed to address this question.

Vimentin GlcNAcylation was increased after OGT overexpression and OGA inhibition, but what remains unclear is how vimentin function is altered. The increased GlcNAcylation did not disrupt phosphorylation at the adjacent CDK1 site, but late M phase phosphorylation was altered. The decrease in pSer-82 was dependent on the overexpression of either OGT or OGA or GT inhibition of OGA. Because OGA overexpression caused a decrease in the late M phase Ser-82 phosphorylation, then the cells are likely exhibiting problems progressing through M phase and less likely that the GlcNAc is preventing Ser-82 phosphorylation by blocking the accessibility of the kinase for that site. Interestingly, OGT and OGA overexpression caused an increase in Ser-71 phosphorylation and an appearance of a higher molecular weight band but GT decreased phosphorylation. The GT data argues for reciprocity between glyco-phospho states on filament proteins such as seen with Neurofilament M (Deng *et al.*, 2007). In the case of vimentin, however, the M phase regulation is much more complicated than just a matter of GlcNAcylation and phosphorylation being antagonistic to each other, because overexpression of both OGT and OGA led to increased GlcNAcylation.

Importantly, ZM treatment skewed the population of pSer-55 toward G2/M phosphorylation levels instead of an

M phase level, suggesting that Ser-55 phosphorylation occurs before GlcNAcylation. This observation explains why the two inhibitors in combination produce an effect phenotypically like that of ZM alone at this site. The actions of GT in locking the O-GlcNAc in place appeared after Ser-55 phosphorylation/dephosphorylation. Possibly, the Ser-55 acts as a priming phosphorylation site for an OGT/PP1 complex to interact with vimentin and add the O-GlcNAc to the adjacent serine while dephosphorylating Ser-55.

Alternatively, inhibitor treatment disrupted the cycling of the modifications on vimentin, locking the protein in specific posttranslational states. Staining of vimentin under these conditions showed a slight increase in filament structure. Potentially, increases in GlcNAcylated vimentin or disruption of the removal of O-GlcNAc from the protein might block filament disassembly. These data suggest that the timing of phosphorylation and GlcNAcylation of vimentin are connected and dependent on each other. Disruption of this timing could potentially disrupt the proper segregation of vimentin in the two daughter cells.

These observations suggest that O-GlcNAc processing by OGT and OGA is coupled to phosphorylation processing during M phase. Linking mitotic kinases and phosphatases with OGT and OGA generates a signaling complex that could potentially fine-tune the regulation of target proteins. This study demonstrates the potential for dynamic regulation of a large number of mitotic proteins by this complex. However, more targets of this complex need to be identified before a clear picture of mitotic regulation is possible. The use of large-scale proteomic methods to map mitotic GlcNAcylation sites would greatly aid in determining substrates for this complex.

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