ROLE OF GLYCOSYLATION IN REGULATING KERATIN 8/18 FUNCTIONS

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution or University.

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Dedicated to
My Family

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Abbreviations

BSA : Bovine Serum Albumin

CHX : Cycloheximide

CK : Cytokeratin

DAPI : Diamidino-2-phenylindole dihydrochloride

ECL : Enhanced Chemiluminiscense

EDTA : Ethylene Diamine Tetra Acetic acid

ECM : Extracellular matrix

EYFP : Enhanced YFP

FBS : Fetal Bovine Serum

FN : Fibronectin

gSer29 : *O*-GlcNAcylated Ser29

Hr : Hour

HRPO : Horse Radish Peroxidase

IF : Intermediate Filament

K : Keratin

kDa : Kilo Dalton

LB : Luria Bertani

Mf : Microfilament

MT : Microtubule

NP-40 : Nonidet P-40

OA : Okadaic Acid

O-GlcNAc : N acetyl-D-Glucosamine

O-GlcNAcase : *O*-GlcNAc selective β-*N*-acetylglucosaminidase

P : Phosphorylation

PBS : Phosphate Buffered Saline

PIPES : Piperazine-N,N′-bis(2-ethanesulfonic acid)

PMSF : Phenyl Methyl Sulfonyl Fluoride

PCR : Polymerase Chain Reaction

PUGNAc : *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate

PVDF : Poly Vinylene DiFlouride

SDS : Sodium Dodecyl Sulphate

TRITC : Tetramethyl Rhodamine Isothiocynate

TEMED : N, N, N', N',-Tetramethylethylenediamine

TM : Triple mutant

ABBREVIATIONS

TTBS : Tween- Tris Buffered Saline

WGA : Wheat germ agglutinin

WT : Wild type

YFP : Yellow Fluorescent Protein

ABBREVIATIONS

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Ph. D. PROGRAMME

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SYNOPSIS

Introduction

Keratins are the major Intermediate filament proteins of epithelia. They form 10 nm filamentous cytoskeleton made up of non-covalent obligate heteropolymers of type-I and type-II keratins and are expressed in a tissue specific and differentiation dependent manner (1,2). Keratin pair 8/18 forms the Intermediate filament scaffold predominantly in simple epithelia such as lining of the alimentary canal, liver and pancreas (2,3). The expression pattern of keratins is highly regulated among various epithelia suggesting a cell-type specific role for various keratins (3). Ectopic expression of K8/18 in other epithelia is highly associated with malignant phenotype (4). Keratins

8/18 provide mechanical support for cellular integrity and are central to various non-mechanical functions like protein biosynthesis, protection from apoptosis, regulation of cell cycle progression, motility, organelle transport etc. (5-10). Their biological roles are majorly dependent on their functional properties like solubility, filament organization and dynamics (11,12). Keratins 8/18 undergo several post translational modifications like phosphorylation, O-GlcNAcylation, acetylation, sumoylation and transamidation (13). Of these, site specific phosphorylation is very well characterized for its role in regulating the functional properties like solubility and filament organization of both keratins 8 and 18 (5,9,14-16). Recent evidences show that these functional properties are also regulated by other modifications like O-GlcNAcylation, sumoylation of keratins 8/18 and acetylation of Keratin 8 (17-19). However, the site specific roles of O-GlcNAcylation and its cross-talk with phosphorylation are yet to be uncovered.

The addition of a single N-acetyl glucosamine (GlcNAc) to serine and threonine residues to nuclear and cytoplasmic proteins, termed as O-GlcNAcylation, was first identified in 1984 by Torres and Hart (20). Two enzymes, O-GlcNAc Transferase (OGT) and O-GlcNAcase (OGA), regulate the cycling of O-GlcNAc on proteins (21,22). Unlike complex classical glycosylation which is static in nature; O-GlcNAcylation is dynamic modification similar to phosphorylation (23). A wide range of nuclear and cytoplasmic proteins get abundantly modified with this single sugar modification viz., transcription factors, nuclear pore proteins, enzymes and cytoskeletal proteins (21). On most proteins O-GlcNAcylation exhibits cross-talk with phosphorylation to regulate essential properties of proteins like protein-protein/DNA-protein interactions (24), sub cellular localization (25) and proteasome mediated protein degradation(26,27); thereby controlling cellular processes like transcription, signal transduction, stress response and cell cycle progression (28,29).

Aberrant O-GlcNAcylation is associated with several pathological conditions like cancer, diabetes and neurodegenerative diseases (30-33).

There is no consensus sequence for O-GlcNAc modification. However it is often observed on the same or proximal Ser/Thr residues that are phosphorylated, thereby negatively regulating phosphorylation (23). These two modifications can regulate each other by competitively blocking the site (34), sterically hindering the addition at adjacent or proximal site (26) or by influencing their respective enzymes (35,36). This type of reciprocal interplay between O-GlcNAcylation and phosphorylation to regulate protein functions is observed on several proteins including intermediate filaments like neurofilaments (NF-M) and cytoskeleton associated proteins like Tau (37,38). With recent advances in mass spectrometry, more complex and extensive cross-talk between O-GlcNAcylation and phosphorylation on many cellular proteins have been discovered (36).

Although O-GlcNAcylation (gS29, gS30 and gS48) (39) and phosphorylation (pS33 and pS52) (5,40) occur at proximal sites on keratin 18, their mutual interplay in regulating its functional properties is largely unexplored. Previous report from our laboratory has shown that total O-GlcNAcylation on keratins 8/18 can regulate solubility, filament organization and stability (17). Here, we aimed to understand the site specific role of K18 O-GlcNAcylation in regulating proximal phosphorylation and functional properties.

Key Questions

1. Do all sites of glycosylation on K18 regulate filament organization/stability or there are very specific sites which regulate these properties?
2. Does O-GlcNAcylation regulate phosphorylation on K18, if yes which residues are involved?

3. Do these sites of glycosylation regulate any of the properties essential for malignant progression?

Objectives

The following objectives were proposed to answer these questions:

1. To investigate which site/s of O-GlcNAcylation on K18 is/are responsible for maintaining filament organization and stability
2. To investigate whether O-GlcNAcylation regulates phosphorylation on K18, and if yes then which site of glycosylation regulates which site/s of phosphorylation
3. To investigate if O-GlcNAcylation regulates cellular processes such as cell adhesion, spreading and motility and which site of O-GlcNAcylation is critical for these functions.

Methodology

Cell culture- HHL17 cell line was routinely cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 0.03% glutamine, 10 units/ml penicillin G-sodium, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B, and 10% FBS at 37°C and 5% CO2. For different drug/inhibitor treatments, Cycloheximide, PUGNAc and Okadaic acid were used at concentrations of 200μM, 100μM and 400nM/ml respectively for various time points.

Plasmids, site directed mutagenesis, cloning and stable expression: Human K18 WT-YFP cDNA and K18 O-GlcNAcylation triple mutant (TM) (S29A/S30A/S48A) cDNA were used to generate site specific (Ser to Ala) single and double O-GlcNAc mutants and K18- S33A, K18-S52A, K18-S33D and K18-S30A/S33D phosphorylation mutants of K18 using Quick Change II site directed mutagenesis kit. K18-WT and all the mutants were further cloned into EYFP-lentiviral vector (41). Infectious lentivirus to express K18-WT and various mutants were produced

as previously described (41). Further K18-WT and various K18 mutants expressing stable cell lines were generated.

Protein isolation and immunoblotting: Total cell lysates were prepared by solubilizing HHL17 cells in 2% SDS cell lysis buffer (62.5 mM Tris and 2% SDS, pH 6.8). Differential extraction of soluble and filamentous keratin was performed as described previously (17).

Purification of the glycosylated form of K8 and 18: K8 and K18 were differentially extracted from cellular fractions on the basis of molecular weight. O-GlcNAcylated proteins from these fractions were extracted using WGA-Sepharose beads as previously described (17). Glycosylated K8 and 18 from these samples were immuno-precipitated using K8 and K18 specific monoclonal antibodies as previously described (17).

Immunostaining and fluorescence imaging of cells expressing EYFP-K18: HHL17 cells stably expressing EYFP-K18-WT, single, double or triple O-GlcNAcylation mutants and phospho-mutants were immunostained for K8, as previously described (17). Images were acquired on a Zeiss LSM510 META confocal microscope (Carl Zeiss Micro imaging, Inc., Thornwood, NY) using oil immersion 63X Plan Apochromat phase contrast objective (NA: 1.4) and processed using LSM510 imaging software, version 4.2.

Cell adhesion assay- Serum starved HHL17 cells expressing K18 O-GlcNAc and phosphorylation mutants were seeded on Fibronectin coated 96 well plate. After 1 hr incubation at 37^{o}c non-adherent cells were removed by PBS washes. Adhered cells on Fibronectin were detected by MTT assay.

Cell spreading assay- The extent of cellular spreading on fibronectin of HHL17 cells stably expressing O-GlcNAcylation and phosphorylation site specific mutants and wild type was done as per described (42).

Scratch wound healing assay- HHL17 cells stably expressing O-GlcNAcylation and phosphorylation site specific mutants and wild type were seeded on fibronectin coated 6 well plate to get monolayer. The cells were serum starved for 24 h. A straight, uniform wound (approx. 400 μm in width) was made. Wound closure of cells in response to the immobilized fibronectin was measured for 25 h by time lapse imaging using a Carl Zeiss inverted microscope at 10x magnification.

Densitometry quantitation and statistical Analysis-Densitometric quantitation of scanned images was performed by ImageJ 1.43 software (National Institutes of Health). Band intensities were normalized to respective loading controls. Statistical analysis was performed using GraphPad Prism 5 software.

Results

Objective 1: To investigate which site/s of O-GlcNAcylation on K18 is/are responsible for maintaining filament organization and stability:

A. To investigate O-GlcNAcylation site of K18 is responsible for maintaining solubility-As both, O-GlcNAcylation and phosphorylation on keratin 18 are known to increase its solubility, we investigated whether perturbing keratin 18 O-GlcNAcylation alters its phosphorylation dependent solubility. To assess phosphorylation induced solubility, we treated K18 O-GlcNAcylation mutant stably expressing cell lines with Okadaic acid (OA). OA treatment led to a notable increase in solubility of K18-WT whereas the solubility of K18-TM (S29A/S30A/S48A) was unaltered. Similarly, HHL17 cells expressing K18-S29A and K18-S48A mutants exhibited increase in

solubility whereas mutation of S30 prevented OA induced solubility of K18 (K18-S30A), similar to triple O-GlcNAc mutant of K18. This suggests that phosphorylation induced solubility of K18 is dependent on its O-GlcNAcylation at S30.

B. O-GlcNAcylation at Serine 30 regulates the stability of K18-O-GlcNAcylation of keratin 18 reduces its stability by mediating ubiquitination dependent proteasomal degradation. As disassembly and sequestration of keratin 18 subunits into soluble pool could be a prerequisite for its degradation. The stability of WT and various K18 O-GlcNAcylation mutants was assessed after inhibiting protein synthesis with Cycloheximide. As compared to K18-WT, which showed significant reduction upon Cycloheximide (CHX) treatment, the level of K18-TM remained unchanged. It was interesting to note that among all the K18 O-GlcNAc mutants, those which harboured S30A were highly stable with unaltered protein level upon Cycloheximide treatment. Further we confirmed these results using PUGNAc: a potent O-GlcNAcase inhibitor. These results conclusively indicate that O-GlcNAcylation specifically at S30 determines the stability of K18.

C. O-GlcNAcylation at serine 30 is essential for maintaining normal keratin 18 filament organization -Intensity quantification of K18-WT filaments across the cell reveal that filament density is highest around nucleus and gradually decreases towards cell periphery. This organization was severely affected in both, K18-TM and K18-S30A which displayed increased filament accumulation around nucleus with collapse of peripheral filaments; whereas, the filament organization of K18-S29A and K18-S48A mutant was similar to K18-WT . Moreover, rescue of O-GlcNAcylation at S30 in K18-S29A/S48A restored the abnormal filament organization of K18-TM implying a key role for gS30 in supporting filament organization of K18.

<u>Objective 2</u>: To investigate whether O-GlcNAcylation regulates phosphorylation on K18, and if yes then which site of glycosylation regulates which site/s of phosphorylation

A. **O-GlcNAcylation and phosphorylation exhibit both cooperative and antagonistic relationship at proximal sites on K18**-To address whether these two modifications on keratin 18 exhibit cross-talk or act independently to regulate K18 properties, we first investigated whether these two modifications coexist on K18. To achieve this, keratin 8 and keratin 18 were separated, purified and O-GlcNAcylated form of these proteins were purified by passing through WGA column followed by immunoprecipitation to enrich O-GlcNAcylated K8 and K18. Purified O-GlcNAcylated K8 and K18 showed notable levels of phosphorylation, suggesting co-existence of these two modifications on K8 and K18.

B. **O-GlcNAcylation at S30 regulates phosphorylation at S33**-We further aimed to investigate the relationship/cross-talk between these two modifications on K18 by assessing the levels of site specific phosphorylation (pS33 and pS52) on various O-GlcNAc mutants of K18. K18-TM showed significant reduction in basal levels of pS33 while the levels of pS52 were notably higher as compared to K18-WT suggesting that O-GlcNAcylation on K18 exhibits a cooperative relationship with pS33 and reciprocal relationship with pS52. To investigate the O-GlcNAc site/s on K18 which aid in phosphorylation of S33, we assessed the basal levels of pS33 in various single and double O-GlcNAc mutants of K18. Interestingly, all O-GlcNAc mutants of K18 harbouring S30A exhibited significantly lower levels of pS33 suggesting a dependency on O-GlcNAcylation of K18 at S30 for the occurrence of phosphorylation at S33. Further we confirmed dependency of S33 phosphorylation on S30 glycosylation by treatment of PUGNAc and Okadaic acid respectively to O-GlcNAcylation site specific mutants. To address this, we used stable cells where the K18 mutant can be O-GlcNAcylated only at serine 30 (K18-S29,48A). It was interesting to note that treating these cells with OA led to an increase in pS33 with no notable change in O-GlcNAcylation at Serine 30. This suggests that the

cooperative interplay between modifications at these two sites is one-sided, with gS30 acting as an upstream switch for phosphorylation at S33.

C. **O-GlcNAcylation mediated phosphorylation of keratin 18 at Serine 33 regulates its stability-**As O-GlcNAcylation at S30 induces solubility and subsequent degradation of K18 along with increased phosphorylation at Ser33, it is possible that pS33 mediates the effect of gS30 on solubility and stability of K18. Although phosphorylation at S33 has been previously shown to regulate solubility, its role in regulating the stability of keratin 18 is yet unclear. To investigate the role of site specific phosphorylation on K18 in regulating its stability, cells expressing K18-WT and phosphorylation mutants (K18-S33A and K18-S52A) were treated with Cycloheximide. While K18-WT and K18-S52A exhibited reduced K18 levels upon Cycloheximide treatment, the protein levels of K18-S33A remained unchanged suggesting a role for pS33 in regulating stability of K18. We validated S33A stability by treating these phospho-mutants with PUGNAc. Further, to confirm this, we assessed the stability of K18 after rescuing the phosphorylation at S33 by substituting with a phosphomimetic mutation (S33D). However, both K18-S33D and K18-S30A/S33D showed higher stability as assessed by Cycloheximide treatment, suggesting that S33D does not substitute for phosphorylation at S33 and therefore could not rescue the enhanced stability of K18 upon loss of phosphorylation at S33.

D. **Phosphorylation at S33 regulates keratin filament organization-** Since gS30 on K18 leads to phosphorylation at S33, we predicted that loss of pS33 could phenocopy the filament organization of K18-S30A and K18-TM. As expected, K18-S33A mutant exhibited perinuclear aggregation and loss of peripheral filaments, while the filament organization of K18-S52A mutant was similar to K18-WT. To confirm these results, we assessed the filament organization

in K18-S33D and K18-S30A/S33D phosphomimetic mutants. The collapsed filament organization was not rescued in either of these stable lines, suggesting that S33D is unable to compensate for phosphorylation at S33. These results imply that gS30 dependent phosphorylation of S33 is essential for maintaining the normal filament organization of keratin 18.

<u>Objective 3</u>: To investigate if O-GlcNAcylation regulates cellular processes such as cell adhesion, spreading and motility and which site of O-GlcNAcylation is critical for these functions.

We further went on to investigate the role of O-GlcNAcylation and phosphorylation in regulation of cellular processes mediated by cytokeratin 18. HHL17 cells stably expressing K18 O-GlcNAc and phosphorylation mutants and WT were assessed for extent of adhesion on fibronectin. There was no significant difference in cellular adhesion seen in O-GlcNAcylation and phosphorylation mutants compared to WT; suggesting that both O-GlcNAcylation and phosphorylation of K18 do not regulate cellular adhesion on fibronectin. Further we investigated cellular spreading and migration in O-GlcNAc and phosphorylation mutants of K18 as it is known that K8/18 regulates cellular spreading and migration on fibronectin (43,44). HHL17 cells expressing site specific O-GlcNAc single, triple and phosphorylation single mutants were cultured on fibronectin to study both cellular spreading and migration. Interestingly, significant reduction in cellular spreading was found in HHL17 cells expressing O-GlcNAc mutants S30A, triple mutant and phosphorylation mutant S33Awhen compared to the WT as seen by laser confocal microscopic images. The cytoplasmic to nuclear area was used to quantitate the relative spreading. Similarly, O-GlcNAcylation mutant S30A, triple mutant and phosphorylation mutant S33A showed reduced collective cell migration on fibronectin compare to that of WT and other mutants. These results

together suggest that, O-GlcNAcylation at S30 and phosphorylation at S33 regulates cellular spreading and migration mediated by keratin 18.

Summary and Conclusion

Summary:

1. The dynamic modifications: O-GlcNAcylation and phosphorylation coexist on K8/18 molecule.
2. O-GlcNAcylation of K18 regulates its phosphorylation in site dependent manner.
    i. Phosphorylation at Ser33 is regulated by O-GlcNAcylation at Ser30 site in positive manner.
    ii. The S30 O-GlcNAcylation acts as upstream switch for phosphorylation at S33.
    iii. Phosphorylation at Ser52 is regulated by O-GlcNAcylation at Ser48 site in reciprocal manner.
3. O-GlcNAcylation of K18 at Ser30 is critical for regulation of its solubility and subsequently degradation.
4. O-GlcNAcylation at Ser30 is critical for regulation of keratin filament organization process.
5. Phosphorylation at Ser33 has role in regulation of keratin solubility and filament organization. O-GlcNAcylation of K18 at Ser30 regulates its stability and filament organization by regulating phosphorylation at Ser33.
6. This positive correlation is important in regulation of cellular processes such as cellular spreading and migration on Fibronectin.

Conclusion: O-GlcNAcylation dependent phosphorylation is important for functional regulation of Keratin 18. This novel interplay between O-GlcNAcylation and phosphorylation is important in cellular processes mediated by K18.

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Publications in Refereed Journal

a) Published manuscripts –

 Kakade PS, Budnar S, Kalraiya RD, Vaidya MM, Functional implications of O-GlcNAcylation dependent phosphorylation at proximal site on keratin 18. J Biol Chem. 2016 Apr 8. pii: jbc.M116.728717

d) Other Publications / Conference Presentations:

- '*2015 cell biology annual meeting'* organized by American Society for Cell Biology (ASCB) at San Diego convention Centre, California, USA on 12th-16th December, 2015. **P.S. Kakade**, S. Budnar, R. Kalraiya, M. Vaidya; The crosstalk between O-linked β-n-acetylglucosamine (O-GlcNAcylation) and phosphorylation of keratin 18 regulate its functional properties and cellular processes. P1505 (poster), Abstract published in Mol Biol Cell. 2015 Dec 15; 26(25): 4523.doi: 10.1091/mbc.E15-09-0674
- "*Molecular Pathways to Therapeutics: Paradigms and Challenges in Oncology*", *Carcinogenesis 2015*, International conference organized by Carcinogenesis Foundation, USA and TMC-ACTREC, India on Feb-2015, TMC-ACTREC, Navi Mumbai. **P.S.**

Kakade, S. Budnar, R. Kalraiya, 'O-linked β-N-acetylglucosamine (O-GlcNAcylation) and phosphorylation crosstalk on keratin 18 regulates its stability and filament organization', (Oral presentation) abstract published in "Journal of Carcinogenesis 2015.

- National Conference on "*Glycobiology of Cancer; Lectins as tools and Targets*", Nov 2013, organised by Karnatak University, Dharwad, Karnataka, India, **P. S. Kakade**, B. Srikanth, R. Kalraiya, 'Understanding the role of O-GlcNAcylation of Keratin 18 in regulation of its critical properties and functions' (poster).
- *'31 Annual Convention Indian Association for Cancer Research 2012'* organized by TMC-ACTREC, India on Feb-2012, TMC-ACTREC, Navi Mumbai. **P. Kakade**, S. Budnar, R. Kalraiya, 'Role of O-GlcNAcylation in regulating keratin 8/18 properties' (poster).

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Chapter 1: Introduction

Intermediate filaments (IFs) are cytoskeletal proteins, form a super family, whose members are encoded by differentially expressed genes (45). Because of their properties and intracellular organization, IFs provide structural support to the cytoplasm and nucleus (45). Hence disturbed IFs cause cell and tissue fragility and lead to a large number of genetic diseases in humans (46,47). A number of nonmechanical roles have been recently unveiled for IF proteins, such as, regulation of key signalling pathways which control cell survival, cell growth, and vectorial processes including protein targeting in polarised cells (48). Emerging studies in the field have unfolded the rationale for large size of this family and context dependent regulation of IFs.

Keratins (K), form largest group of IF proteins (1). They are predominantly expressed in epithelial tissues (1). Keratins form ~10 nm thick filamentous network throughout cytoplasm of epithelial cells. They are non-covalent obligate heteropolymers composed of at least one member from each subfamily (type I and type II) (1,2). Their expression is tissue specific, differentiation dependent and developmentally regulated, suggesting a cell-type specific role for various keratins (3), e.g. basal epidermal keratinocytes express K5/14, suprabasal keratinocytes in the upper layer of skin express K1/10, wound healing epithelia express K6/16 and simple epithelial tissues predominantly express K8/18 (3),(49).

K8/18 pair is predominantly expressed in simple epithelial tissues such as, liver, lining of the alimentary canal, and pancreas as well as mixed epithelia such as lung and breast (2,3). Ectopic expression of K8/18 in squamous epithelia like lining of oral cavity is associated with malignant progression (4). Similar to other IF proteins, K8/18 maintain cellular integrity and are also central to various non-mechanical functions like protein biosynthesis, protection from apoptosis, regulation of cell cycle progression, motility, organelle transport (5-10). Their biological roles are majorly dependent on properties like solubility, filament organization and dynamics (11,12).

Interaction with associated proteins and post translational modifications (PTMs) are important determinants of keratin mediated cellular functions. For example, K8 and K18 interact with 14-3-3 family proteins in phosphorylation dependent manner and interact with AKT in O-GlcNAcylation dependent manner (8,50). They undergo several post translational modifications like phosphorylation, O-GlcNAcylation, acetylation, sumoylation and transamidation (13). Of these, phosphorylation is very well characterized for its role in regulating solubility and filament organization of both keratin 8 and 18 (5,9,14-16). Similarly, other modifications like O-GlcNAcylation, sumoylation of keratins 8/18 and acetylation of keratin 8 control their functional properties (17-19). However, the site specific roles of O-GlcNAcylation and its cross-talk with phosphorylation are yet to be uncovered.

O-GlcNAcylation was first identified by Hart et al. in 1984 (20). It is an addition of a single N-acetyl glucosamine (GlcNAc) to serine and threonine residues in nuclear and cytoplasmic proteins (20). Cycling of O-GlcNAc on proteins is catalysed by two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) (21,22). In contrast to, complex classical glycosylation which is static in nature (which decorates cellular surface); O-GlcNAcylation is a simple and dynamic modification, and is more similar to protein phosphorylation (23). O-GlcNAcylation is abundantly seen on diverse class of nuclear and cytoplasmic proteins viz., nuclear pore proteins, transcription factors, RNA polymerase II, kinases, phosphatases, cytoskeletal proteins and their associated proteins (21). O-GlcNAcylation regulates divers groups of proteins by modulating their essential properties such as phosphorylation, protein-protein/DNA-protein interactions (24), sub cellular localization (25) and proteasome mediated protein degradation (26,27); thereby controlling cellular processes like transcription, signal transduction, stress response and cell cycle progression (28,29). Aberrant O-GlcNAcylation is associated with various disorders such as, cancer, diabetes,

neurodegenerative and cardiovascular disorders (30-33). Hence it is considered as a sensor for cell homeostasis.

Albeit, O-GlcNAc modification does not have consensus sequence, it is often observed on the same or adjacent Ser/Thr residues that are used for phosphorylation, thereby negatively regulating phosphorylation(23). These two modifications can regulate each other by competitively occupying the same site (34), sterically hindering the addition at proximal site (26) or by influencing each other's modifying enzymes (35,36). The reciprocal interplay between O-GlcNAcylation and phosphorylation is exhibited on several proteins including intermediate filaments like neurofilaments (NF-M) and cytoskeleton associated proteins like Tau (37,38). With recent developments in mass spectrometry, more complex and extensive cross-talk between O-GlcNAcylation and phosphorylation has been discovered on many cellular proteins (36). This suggests that O-GlcNAcylation regulates protein properties in more diverse and complex manner.

Although, O-GlcNAcylation (gS29, gS30 and gS48) (39) and phosphorylation (pS33 and pS52) (5,40) modify K18 at proximal sites on keratin 18, their mutual interplay in regulating its crucial properties is still unexplored. O-GlcNAcylation on keratins 8/18 can regulate solubility, filament organization and stability (17). Here we aimed to understand the site specific role of K18 O-GlcNAcylation in regulating proximal phosphorylation and properties such as K18 solubility, stability and filament organization which are important for its function. We have used site specific single, double and triple O-GlcNAcylation and phosphorylation mutants and phosphomimetic mutants of K18.

1.1 Key Questions:

1.1.1. Do all sites of glycosylation on K18 regulate filament organization/stability or there are very specific sites which regulate these properties?

1.1.2. Does O-GlcNAcylation regulate phosphorylation on K18, if yes which residues are involved?

1.1.3. Do these sites of glycosylation regulate any of the properties essential for critical cellular processes like cellular adhesion, spreading and migration?

1.2 Objectives:

The following objectives were proposed to answer these questions:

1.2.1. To investigate which site/s of O-GlcNAcylation on K18 is/are responsible for maintaining filament organization and stability

1.2.2. To investigate whether O-GlcNAcylation regulates phosphorylation on K18, and if yes then which site of glycosylation regulates which site/s of phosphorylation

1.2.3. To investigate if O-GlcNAcylation regulates cellular processes such as cell adhesion, spreading and motility and which site of O-GlcNAcylation is critical for these functions.

Chapter 2: Review of Literature

2.1 Intermediate filaments:

Cytoskeleton is composed of three major classes of proteins, microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs) (51). Owing to diversity in protein composition, cell-specific presence and functions, IFs are the least characterized in comparison to other cytoskeletal systems. The MFs and MTs which are composed of actins and tubulins respectively, have few major subtypes (actin has α, β and γ subunits and tubulin has α, β, γ, δ, ε, ζ and η subunits) (47,51). There are ~70 different human cell type-specific IF proteins reported (45,46,52,53). MFs and MTs, utilize structural polarity to enable their cell motility and transport functions, whereas, IFs are apolar and flexible in nature, which offers them diverse roles such as protector of mechanical stress and regulator of multitude of cellular signalling events (45,54-56). There are six major IF protein subtypes (Table 1) (45,51). They belong to two distinct IF systems (either cytoplasmic or nuclear) (45,56).

IF name	Type	Size (kDa)	Cell and tissue distribution	Key feature and/or disease association
Cytoplasmic				
Keratins[a]	I (n = 28)	40–64	K9–K28 (epithelia); K31–K40 (hair/nail)	Types I and II keratins form obligate 1:1 heteropolymers. There are 54 functional keratin genes in the human genome. Mutated in >20 diseases.
Keratins[a]	II (n = 26)	52–68	K1–K8, K71–K74 (epithelia); K81–K86 (hair).	
Vimentin	III	55	Mesenchymal	Widely expressed in embryos.
Desmin	III	53	Muscle	Mutated in cardiomyopathies.
GFAP	III	52	Astrocytes/glia	Mutated in Alexander disease.
Peripherin	III	54	Peripheral neurons	Induced after neuronal injury.
Neurofilaments (L,M,H chains)	IV	61–110	CNS neurons	NF-L, M, and H form obligatorily heteropolymers with α-internexin.
α-Internexin	IV	66	CNS neurons	Neuronal IFs are key effectors of axonal radial growth.
Nestin	IV	177	Neuroepithelial	Markers of "early" progenitor (stem) cells in several tissues.
Syncoilin	IV	54	Muscle	Interacts with α-dystrobrevin.
Synemin	IV	182	Muscle	α and β isoforms; β form is also known as desmuslin; binds actin-associated proteins.
Nuclear				
Lamins B1, B2	V	66–68	Nuclear lamina	Enriched in progenitor cells.
Lamins A/C	V	62–78	Nuclear lamina	Subject to differential splicing; enriched in differentiated cells. Mutated in a progeria condition, muscular dystrophy, and others.
Orphan				
Phakinin (CP49)	undefined	47	Lens	CP49 and filensin form beaded filaments in lens epithelial cells. CP49 mutations cause cataracts.
Filensin	undefined	83	Lens	

[a]The keratin nomenclature has recently been revised (see Schweizer et al. 2006).

Table 1. The IF protein family (45,56).

2.1.1 Structure of IF proteins:

All IF proteins share a common three-domain structure that includes an N-terminal ‘head’ domain, C-terminal ‘tail’ domain, and a central α-helical ‘rod’ domain (46,54). The rod domain is further divided into four subdomains (coil-1A, 1B, 2A and 2B which are connected by linker domains (L1, L12, and L2) respectively. Rod domain is a highly conserved region. On the contrary, head and tail domains are substantially variable with respect to their amino acid sequences (Illustration 1). IFs form either homo or heterodimers as a result of parallel interaction between their rod domains (57,58).

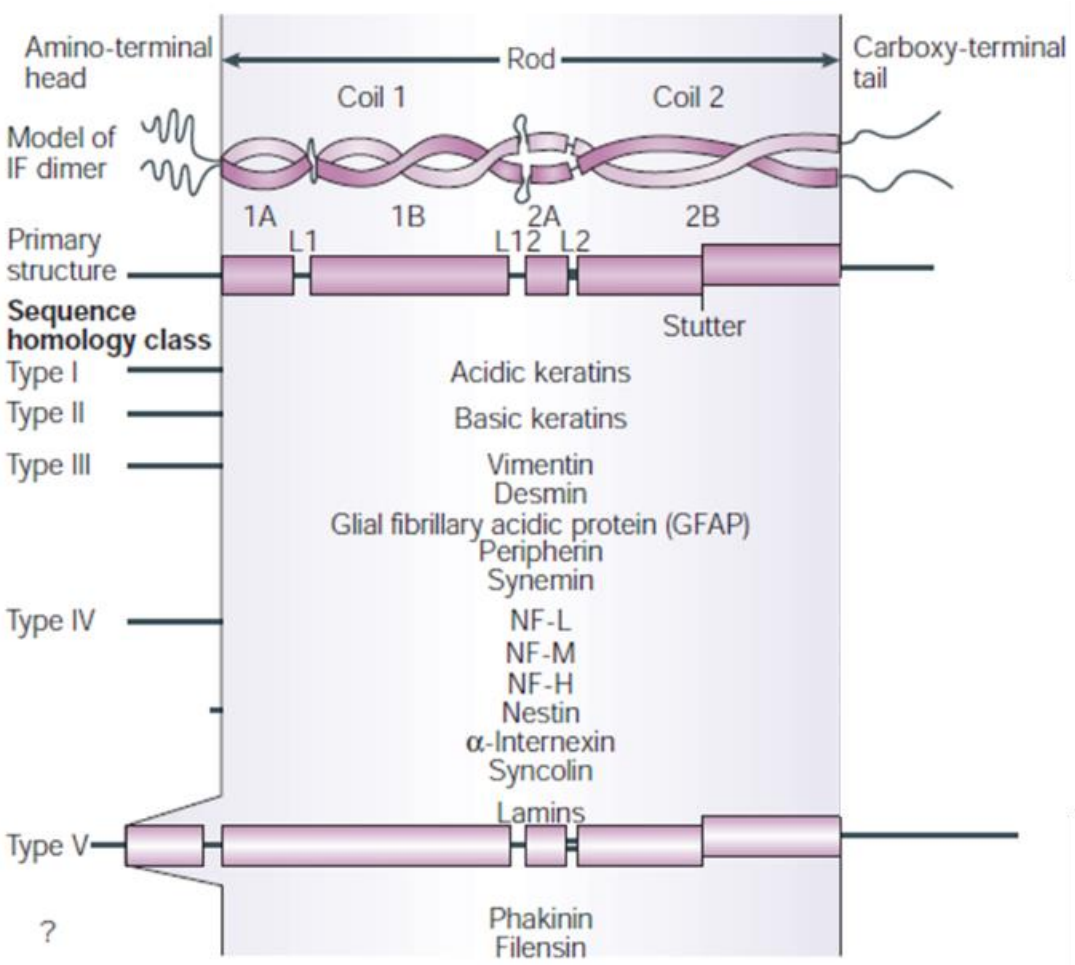


Illustration 1. Schematic diagram of the domain structure of IFs: All IF proteins are structurally divided in to three domains, a highly conserved α-helical central rod domain, which is flanked by non-helical head and tail domains. The rod domain consists of the heptad repeats, which are interrupted by three linker sequences (L1, L12 and L2). Hence rod domain is divided in to four segments: 1A and 1B contained 'coil 1', and 2A and 2B contained 'coil 2'. Six classes of IFs are illustrated, type-I and -II groups are the keratins. Vimentin, glial fibrillary acidic protein (GFAP), desmin, peripherin and synemin are categorised under type-III IFs. Neurofilaments (NFs) i.e. NF-L,NF-M and NF-H, α-internexin, syncolin and nestin comprise the type-IV IFs. Nuclear lamins are type-V IFs (59).

2.1.2 Functions of IFs:

The functions of IF are broadly divided in to two types, mechanical or non-mechanical (45,46,55,60). Maintenance of structural integrity of cell is a canonical function of IFs under mechanical category. It is best exhibited by the skin blistering disease caused by keratin 5 and 14 (K5/14) mutation (61,62), as K5/14 filament network can absorb significant mechanical stress. The non-mechanical IF functions include regulation of the cellular architecture, cellular growth, cell migration, metabolism and aging (48,55,56,63). Crystallization of full length IF proteins has not yet been possible due to, their polymerization-prone character, the absence of suitable inhibitors to trap intermediates and their structural polymorphism. However, by using 'divide-and-conquer' approach subdomain structure of some of the IFs has been elucidated (64,65). For example crystal structure of 2B subdomain of vimentin, lamin A, nonhelical tail domain of lamin A/C and 1A subdomain and partial coil2 of vimentin (65-71). However, there is still no structural information available for any heteropolymeric IFs, keratins.

2.1.3 Post translational modifications of IFs:

Several Post translational modifications (PTMs) are reported on IFs (table 2), which modify them at multiple sites.

PTM	IFs	PTM enzyme (on)	PTM enzyme (off)
Extensively studied			
Phosphorylation	most IFs*	SAPKs, AKT1, PKC, Cdk1, Cdk5, other	PP1, PP2A
Farnesylation	lamins	farnesyl transferase	Zmpste24 (via proteolysis)
Ubiquitination	most IFs**	CHIP/STUB, Ubc3, UbcH5, Siah1, Trim32	not known
Accumulating data			
Sumoylation	lamins keratins vimentin	Ubc9	not known
Glycosylation	keratins neurofilaments vimentin	OGT	O-GlcNAcase
Acetylation	keratins	not known	SIRT2
Limited data			
Transamidation	keratins	transglutaminases	none
ADP ribosylation	desmin vimentin	ADP-ribosyl transferase SpyA	not known

** phosphorylation of filensin, phakinin, and syncoilin is based on proteomic evidence*

*** putative ubiquitination sites for most IFs have been identified by large scale proteomic studies*

Table 2. IF protein PTMs (13)

The overview of IF functions in cellular processes suggest that many, if not most, of these functions are governed by various PTMs. Phosphorylation is an extensively studied modification among them, which regulates several properties and functions of IFs (Table 3) (72-74).

IF property and function	Comments and examples
Properties	
Spatiotemporal compartmentalization:	
Subcellular space	Soluble pool, membrane-associated, filaments, oligomers, inclusions
Polarized cells	Keratins in simple epithelia and NF in neurons
Tissues	K20 pS13 in goblet cells but not other enterocytes
Interaction with IFAP	Keratins and vimentin with 14-3-3; NF with kinesin
IF turnover	Phosphorylation protects against degradation via the proteasome or caspases
IF transport	Axonal NF transport; lamin nuclear import
Functions	
Response to stress:	
Protection from injury	Increased damage in mice expressing phosphorylation mutant IFs
Protection from apoptosis	Phosphorylation increases during apoptosis; K8 S73A overexpressing mice are more susceptible to apoptosis
Stress sensor	Overall increase in IF phosphorylation during stress
Cell signaling:	
Scaffolding of signaling molecules	14-3-3; kinases
Serving as phosphate sponge	K8 pS73 serves as a major SAPK substrate; NF
Cell-cycle regulation	Strong evidence in cell culture, some in animal studies
Regulation of protein synthesis and cell size	K17 binding with 14-3-3 during wound healing
Mechanical support	An established IF function but association with phosphorylation is correlative
Organelle function; protein targeting	Phosphorylated NF binds to isolated mitochondria; vimentin in integrin traffic
Cell migration	Vimentin in lymphocyte migration
Tissue-specific axonal function	NF-M tail phosphorylation in axonal calibre; nerve conduction

Table 3. Phosphorylation regulates a broad range of IF properties and functions (49)

2.2 Keratins:

Keratins (Ks) or cytokeratins (ck) are the major IFs of epithelia. The keratin family comprises of 54 proteins which are divided in to two types (types I, acidic, (K9-28 and 31-40) and type II, neutral or basic (K1-8 and K71-86) (1)(Table 1). They form obligate noncovalent heterodimers composed of type I and type II keratins.

Keratin proteins can be further divided into three functional groups: ‘simple’ keratins, expressed in single-layered or simple epithelia, e.g. liver, pancreas, and glandular epithelia; ‘barrier’ keratins, expressed in stratified squamous epithelia, e.g. epithelia of epidermis; and ‘structural’ keratins, expressed in hard tissues, e.g. hair and nail (75). Owing to their tissue specific expression, keratins are used as markers to distinguish different epithelial cell types. For example, basal epidermal keratinocytes express K5/14, suprabasal keratinocytes in the upper layer of skin express K1/10, wound healing epithelia express K6/16 and simple epithelial tissues predominantly express K8/18 (3).

2.2.1 Cellular functions of keratins:

Keratins are involved in various cellular processes and regulate critical functions. They are implicated in maintenance of cellular integrity, protein biosynthesis, organelle transport, cell cycle progression, protection from apoptosis, response to stress etc. (76).

Besides being a multimember family, particular keratin is required for respective tissue specific functions, e.g. mice ectopically expressing K1 in β cells of pancreas develop of diabetes because of reduction of insulin secretory vesicle, indicating their functional non redundancy (2). Keratins are also involved in multiple cellular processes (77). Hence deletion of particular keratin leads to defect in diverse cellular processes, e.g. deletion of K17 in mice model showed defects in hair follicle recycling, wound repair, protein synthesis, inflammatory cyclins profile alteration and reduction in cell size (78,79). Further, expression of appropriate pair of keratins is highly important for normal tissue function. For example, K14 knock out mice in which over expression of K16 and K18 leads to partial rescuing of normal phenotype (76,80).

2.2.2 Post translational modifications of keratins:

Keratins undergo several PTMs e.g. phosphorylation, glycosylation, acetylation, transamidation, proteolysis, sumoylation, caspase cleavage, etc (13). Out of these, phosphorylation is extensively studied modification which regulates several functions of keratins e.g. filament organization, cellular migration, organelle transfer, protection from stress, protein targeting, regulation of cellular signalling, regulation of apoptosis etc (13). The importance of PTMs in the keratin filament organization and interaction with its binding partners make them crucial in various cellular processes and specific functions mediated by keratins. Many human diseases are linked with alterations in keratin PTMs (49). These alterations could be result of either mutation in keratin genes or related to pathogenesis of disease. The alterations in PTMs of keratins might occur for

protecting cells from disease or which is required for progression of the disease. Hence targeting PTMs could be beneficial for therapeutic purposes.

2.3 Keratin 8 and 18 (K8/18):

K8 (type II keratin) dimerizes with K18 (type I keratin) to form intermediate filaments in single layered or simple epithelia, e. g. liver, linings of intestine, pancreas etc. and in mixed epithelia e.g. breast and lung epithelia (2) . K8/18 pair is distinct from rest of the type I and II keratins. K8/18 is the first pair expressed during early developmental stages of embryo, e.g. in trophectodermal layer of blastocyst (81), suggesting that K8/18 filaments are required for development. Secondly, type I and type II keratin genes are located on different chromosomes. On the contrary, K8 and K18 genes are adjacent to each other on same chromosome (chromosome12) (2). It suggests that their expression is regulated at the gene level. Another distinctive character of K8 and 18 is that they are persistently expressed in various carcinomas, which is in contrast to the other keratins, (82) their expression is either sporadic or lost during cancer progression. Hence K8, 18 and 19 are considered as carcinoma markers. In addition, ectopic expression of K8/18 in stratified epithelia is linked with malignant progression.

2.3.1 Structure of K8 /18:

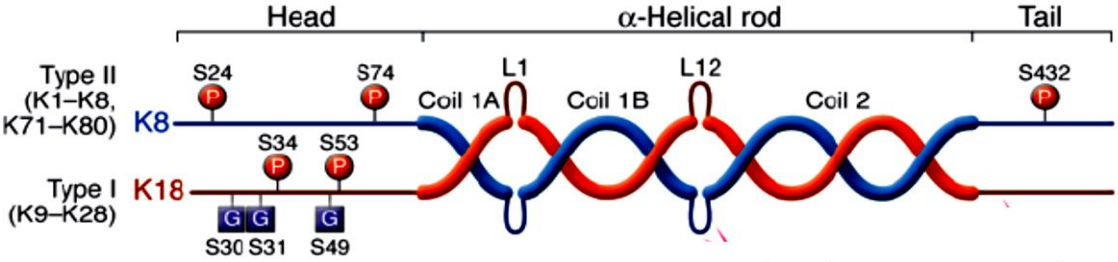


Illustration 2. Structure of K8/18: Dimer of K8 and K18 consists of a central coiled-coil α-helical rod domain which is flanked by highly variable non-helical head and tail domains. The

head and tail domains of K8/18 are dynamically modified with phosphorylation (P) and glycosylation (G). The rod domain is further divided into (coils 1A, 1B, and 2), 3 subdomains due to presence of 2 linkers (L1 and L12) (82).

Like all intermediate filaments, K8 and K18 proteins have three distinct domains: a central α-helical rod domain, flanked by nonhelical head and tail regions (Illustration 2) (82). The rod domains of both K8 and 18 are aligned in parallel manner. Further these dimers form antiparallel tetramers by overlapping the N- Tetramers which then assemble into 'unit length filaments' that are 60 nm length. The rapid assembly of unit length filaments in an end-to end manner leads to formation of 10 nm thick keratin filament, which covers entire cytoplasm (54). The antiparallel arrangement of keratin precursors (tetramers) offers apolar nature to K8/18 filaments. It is distinguishing character which separates them from other polar cytoskeletons such as MFs and MTs.

2.3.2 Functions of K8/18:

2.3.2.1 Cell polarity:

Maintenance of cellular polarity is an important function in simple epithelia (77). Cellular polarity offers directional secretory and absorptive functions to epithelia, and can be obtained by subcellular distribution of key molecules. Simple epithelial secretion is directly dependent on flexibility of K8/18 filaments. On the contrary, highly stable K1/10 filament network hampers membrane trafficking (77). The interaction of keratins with desmosome forms trans-cytoplasmic meshwork across the cell boundaries, which gives three dimensional network in tissues. The asymmetric organization of simple epithelial keratins (that is higher organization at the apical surface) directly contributes to cellular polarity (83).

2.3.2.2 Apoptosis:

K8 and 18 are reportedly linked with the regulation and attenuation of proapoptotic stimuli, which is considered as novel function of simple epithelial keratins. K8 and K18 interact and inhibit two apoptotic signalling molecules i.e. TNFα and Fas ligand (84,85). In addition, keratins also bind to their downstream molecules (e.g., TRADD) (86). Fas mediated apoptosis is induced by certain hepatotoxins. In response to Fas signalling, hepatocytes activate JNK signalling pathway and concomitantly, phosphorylation of K8 at S73 occurs (87). Phosphorylated K8 inhibits JNK and hence cannot activate c-jun (87). Thus, presence or absence of K8/18 interfere with and control signalling in apoptosis. Further, simple epithelial cells die if they do not form proper keratin filaments, owing to detachment of these cells from extra-cellular matrix (ECM) (88,89). The type I keratins in simple epithelia (K18 and 19) can be targeted in apoptosis, where they get hyper phosphorylated and cleaved by caspases (89,90).

2.3.2.3 Protection against stress:

Keratins reorganise in order to maintain cellular integrity during stressful conditions (91). Although internal epithelia are not exposed to intense shear and tensile stress, simple epithelia need to with stand osmotic and shear stress (92). Mice which are deficient in simple epithelial keratins exhibit placental defects and colonic hyperplasia (93). Keratins in simple epithelia help in maintenance of tissue positioning. In case of hepatocytes toxin insults may also result in osmotic imbalance (77). Hence K8/18 pair acts as a chemo-protective skeleton in liver. K8/18 serve as stress markers by increasing their expression up to 3-fold, (15) akin to heat shock protein (hsp)-70 which they bind to, and by undergoing posttranslational modifications such as phosphorylation, caspase-mediated fragmentation, ubiquitination and cross-linking (49,94). Dynamic increased/decreased keratin site-specific phosphorylation serves as reliable marker for human liver

disease progression/regression, and for simple epithelial cell injury (95). The K8/18 related genetic animal models and *ex vivo* cell culture studies demonstrated the importance of K8/18 in protecting hepatocytes from oxidative and other hepatotoxic injuries that promote apoptosis, by acting as a phosphate sponge (93,96). In addition, the natural K8 variants Y53H and G61C interfere with keratin filament reorganization in response to oxidative and other stresses as noted in transfected cell culture models (97).

2.3.2.4 Regulation of organelle transportation:

Other important and emerging K8/18 functions that are revealed by mutational analysis of K8/18 are roles in protein-targeting and organelle localization (98). For example, K8-null liver shows increased basolateral distribution of the bile canaliculus Ecto-ATPase, an increased cell surface distribution of Fas receptor, and altered distribution of desmoplakin (85,99). Further, expression of K18 R89C mutant in cultured cells alters Golgi distribution into keratin aggregates (100). Proteomic analysis of K8-null livers demonstrates significant alterations in many mitochondrial proteins. In addition, it affects size of mitochondria (7). K8 null mice showed smaller mitochondria and altered distribution in cytoplasm when compared with control livers (92).

2.3.3 Solubility and filament organization of K8/18:

2.3.3.1 Solubility:

Filamentous keratins disassemble in to oligomeric precursors in response to cellular stimuli. This process is known as keratin solubility. Keratin solubility is dependent on cell type and cell cycle stage. Among keratin family, K8 and K18 proteins are relatively soluble in nature (~5% soluble) (12). The exchange of keratins between highly polymerised filamentous keratins and soluble keratins takes place within minutes. Thus, the equilibrium between filamentous/ soluble keratins

is a dynamic process. In order to maintain epithelial homeostasis, continuous adaptive changes of tissue and cell architecture are needed to compensate for migration, proliferation and apoptosis of cells within the simple epithelium (101). These changes are required for modulation of cell shape, positioning, organelle transport, rearrangement of compartments and acquisition of specialized differentiation features which depend on flexibility and restructuring of the keratin cytoskeleton (101). Recent experiments further revealed that the protective effect of the K8/18 extends beyond just mechanical function by contributing to various stress responses and acting as a sink for regulatory factors that determine cell fate (92,102).

2.3.3.2 Filament organization:

K8/18 filaments are highly dynamic in nature. Keratin filaments formation is a spatially defined, step wise process. It begins at the cellular periphery, where keratin precursor molecules oligomerize (103,104), further elongate in to short length filaments, integrate into peripheral keratin network (104,105), and finally mature keratin filament bundles are formed, which encase the nucleus (also known as tonofibrils) (106,107). Alternatively, these keratin filaments get disassembled in to soluble oligomers, which are utilised for new filament formation (Illustration 3) (65,108). K8/18 filaments disassemble in to reusable non-filamentous subunits or can be targeted for degradation (89,102). Keratin filament assembly is independent of protein biosynthesis and does not require microtubules (109). Keratin filament turnover cycle is much faster (~25% hourly turnover rate) than biosynthesis of keratin (less than 5% per hour) (110). Soluble keratin precursors continuously diffuse in cytoplasm which mainly consists of detergent soluble, primarily tetramers and also higher order oligomers of K8/18 (111,112). The active transport machinery is not required to deliver disassembled keratins to the cell periphery (105,113).

These soluble precursors are also incorporated in to pre-existing K8/18 filaments throughout the cytoplasm by lateral exchange into existing filaments (110).

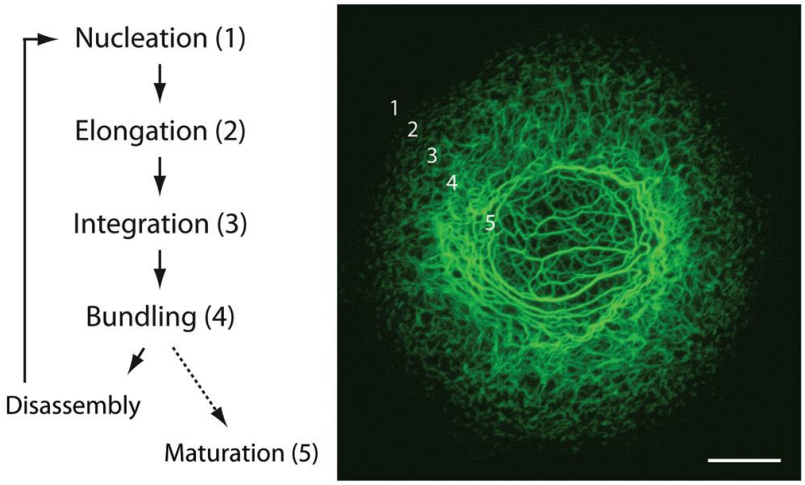


Illustration 3. Keratin filament organization: Steps of keratin filament organization are listed on the left side of the figure. Keratin filament organization takes place at topologically defined regions inside the cell. Keratin filament assembly (nucleation) begins in cell periphery at focal adhesions. Further, keratin particle elongation and integration into the peripheral keratin filament network takes place. Next, keratin filaments bundle formation takes place. Simultaneously disassembly into soluble subunits also takes place during inward-movement and bundling of keratin filaments. These soluble subunits are further re-utilized for another round of filament assembly. Next, the mature filaments encase the nucleus. Bar represents 10 µm (114).

2.3.4 Post translational modifications of K8/18:

Many of K8/18 functions are regulated by their post translational modifications. These PTMs regulate functional properties of keratins, such as their solubility, filament organization and turn over. K8/18 are modified at multiple sites with phosphorylation, glycosylation, ubiquitination and recently characterised acetylation and sumoylation.

2.3.4.1 Phosphorylation:

Phosphorylation of K8/18 is an extensively studied PTM, which regulates different cellular processes mediated by K8/18, in a site specific manner. Phosphorylation of K8/18 protects cell from apoptosis by serving as 'phosphate sponge' (49), where K8 S74 and K18 S54 are the major phosphorylation sites. The phosphorylation of K8/18 protects several pro-apoptotic molecules from getting phosphorylated (93,115). Mutation of S74 leads to apoptosis, and liver injury in mice which over express K8S74A has been reported (93).

Further, phosphorylation of K8/18 mediates their interaction with their associate binding partners, e.g. interaction of 14-3-3 protein with K18 during cell cycle progression and in tumorigenesis (116). K18 was the first identified binding partner of 14-3-3 among IFs (50). K18 S33 phosphorylation site is involved in binding with 14-3-3 and this interaction sequesters keratins in soluble form(50). Further, phosphorylation of K8/18 regulates cellular migration in context dependent manner (9,117). Whereas phosphorylation at K8 S432 increases migration of pancreatic and gastric epithelial cells, phosphorylation at the same site reduces migration in oral squamous carcinoma cells (9,117).

2.3.4.2 Sumoylation:

K8/18 are extensively modified with small ubiquitin-like modifier (SUMO). SUMO-2/3 type of sumoylation is preferred in comparison to SUMO-1 on K8/18 (18). K18 SUMO-2/3 takes place at Lys207 (18). Generally, keratin sumoylation is not detectable under basal conditions. It is upregulated during hyper phosphorylation, oxidative and apoptotic stress conditions, which induces keratin filament reorganization (18).

2.3.4.3 Lysine acetylation:

K8 rod domains are modified with lysine acetylation at Lys207 (19,118,119) which promotes dense K8 filament organization preferentially towards nucleus. During hyperglycemia and inhibition of NAD-dependent deacetylase, SIRT2 leads to increased K8 acetylation, which further results in perinuclear accumulation of K8 (19).

2.3.4.4 O-GlcNAcylation:

O-linked glycosylation is enzymatic addition of N-acetylglucosamine (GlcNAc) moiety to Ser/Thr residues of nuclear and cytoplasmic proteins. Among IFs K8, K18, K13, vimentin and neurofilament proteins are reported to get dynamically modified with O-GlcNAcylation (120-123). Among these IFs, K18 and neurofilaments are best characterised for role of O-GlcNAcylation in regulation of their functions. K18 has three O-GlcNAcylation sites *Viz*, Ser29, Ser30 and Ser48 in its head domain (39). Previous report from our lab has shown that, O-GlcNAcylation of K8/18 regulates its crucial properties such as solubility, stability and filament organization (17). O-GlcNAcylation of K18 is also involved in protection of simple epithelial tissues during stress and injury (8). The underlined mechanism involves inverse regulation of phosphorylation of AKT and PKCθ (at sites that are critical for their kinase activities, which are critical for its cell survival function) by K18 O-GlcNAcylation (8). K18 harbours multiple sites of O-GlcNAcylation which are proximal to phosphorylation sites (5,40). The interplay between these two dynamic modifications in regulating K18 functional properties is largely unexplored.

2.4 Glycosylation:

2.4.1 Introduction:

The process of covalent attachment of a sugar/glycan (oligosaccharide/monosaccharide) moiety to macromolecules such as proteins and lipids is known as glycosylation(124). Previously, the only

known function of carbohydrates was as energy source. Later on it was discovered that, they also play an important biological role in facilitating interactions between two cells and the surrounding matrix; hence they are important in assembly of complex multicellular organisms (124). The study of glycans lagged behind due to their structural complexity, difficulty in sequencing and the fact that structure of glycans could not be predicted by the DNA template (124). Classical glycosylation is an array of multiple monosaccharides, which are linked by either α, β or γ linkages, and at several positions, of the protein backbone (125). Glycosylation is broadly classified based on the linkages to the protein, i.e. N-linked or O-linked. The N-linked glycans (commonly begin with N-acetylglucosamine: GlcNAc moiety) are covalently attached to the amide group of the amino acid asparagine (126). Not all asparagine residues can accept glycosylation. Amino acid sequence beginning with Asn-X-Ser/Thr where X can be any amino acid except proline gets glycosylated. N-linked glycosylation is found abundantly in glycoproteins of serum e.g. immunoglobulins. O-linked glycans (which frequently begin with N-acetylgalactosamine: GalNAc moiety) are covalently attached to the hydroxyl group of amino acids serine and threonine (127).

2.4.2 The synthesis and degradation of classical glycans:

There are only few genes that code for enzymes involved in glycosylation, which are responsible for biosynthesis and assembly of various polysaccharides (128). During co-translation of proteins in rough endoplasmic reticulum (RER), concomitantly, multi-step glycosylation is also initiated (128). Differences in structures of N and O-linked oligosaccharides reflect their differential biosynthesis process (129). O-linked sugars are added one at a time, and by different glycosyltransferase enzymes (130). In contrast, N-linked oligosaccharides synthesis begins with attachment of a large preformed oligosaccharide, containing 14 sugar residues, which are subsequently further modified in a sequential manner by different enzymes (126,131). Well-

studied examples of different glycoproteins are glycophorin in erythrocyte plasma membrane Na^+/K^+ ATPase, viral glycoproteins destined for the plasma membranes of infected cells and enzymes in plant plasma membranes that synthesize cell-wall components such as cellulose.

Secretory pathway, that comprises organelles such as, endoplasmic reticulum (ER) and Golgi apparatus (Golgi), harbour a sequential competition between glycosyltransferases and glycosidases, active in specific compartments (128). For example, biosynthesis of O-linked oligosaccharide of glycophorin and similar glycoproteins begins with transfer of *N*-acetylgalactosamine (GalNAc) catalysed by a GalNAc transferase that is localized to the rough ER or the *cis*-Golgi network. Further, glycophorin moves to trans-Golgi vesicle, where, a galactose residue is added to the *N*-acetylgalactosamine by a specific *trans*-Golgi galactosyltransferase. In summary, secretory pathways decide the ultimate combination and complexity of polysaccharide on a protein. Hence the complete understanding of glycan structure is highly difficult, even though the complete gene expression profile of a given cell type is already elucidated (129).

Deglycosylation of these glycans is carried out by multiple glycosidases in lysosomes, concomitant with protein degradation. Owing to this distinct compartmentalization of the glycosylation and deglycosylation processes, classical glycosylation is mostly static in nature (132).

2.4.3 Functions of glycosylation:

Due to their ubiquitous and complex nature, glycosylated proteins have multiple and diverse cellular functions (133). They span the spectrum from subtle to vital functions involved in cellular activation, differentiation, growth, embryonic development, organogenesis, and survival of organism (134). Some genetic defects in glycosylation, which completely eliminate major glycans

from proteins, are embryonic lethal in nature, e.g. mutation in ALG1gene which encodes for mannosyltransferase I GDP-Man: $GlcNAc_2$- PP-Dol mannosyl- transferase which is one of the enzymes in N-linked glycosylation, causes severe psychomotor retardation, hypotonia, acquired microcephaly, intractable seizures, fever, coagulopathy, nephrotic syndrome and early death (135,136). Whereas, those genetic alterations which modify the composition of outer chain of polysaccharides, show more specific defects, e.g. 'Paroxysmal nocturnal hemoglobinuria' disorder caused due to a mutation in 'PIGA' gene, gives defective GlcNAcT enzyme. It leads to complement-mediated hemolysis (135,136).

2.4.4 Nucleocytoplasmic glycosylation:

A notion in general biochemistry and cell biology is that, glycosylation is restricted to those proteins which are present on a cell surface or lumens of organelles. However, large number of reports indicate that nuclear and cytoplasmic proteins are also extensively glycosylated, which are of both types: simple and complex (137). Potential mechanisms for complex type of glycosylation on nuclear and cytoplasmic proteins are as follows,

1. Glycosyltransferases are also present in the cytoplasm which glycosylate proteins in the cytoplasm e.g. glycosylation of Skp1 in *Dictyostelium* (138).

2. Translocation of the glycoproteins from secretory pathway to the cytoplasm, Sec61/63 complex is found to mediate such retrotranslocation from rough endoplasmic reticulum to the cytoplasm (139).

3. Several classes of glycans biosynthesis is initiated by membrane associated glycosyl transferases, whose catalytic domains are face the cytoplasmic surface. Such glycosylation

is observed on glycosylceramides and GPI anchors. These proteins ultimately, flip to the outer side of membrane (140).

Both O-linked and N-linked type of classical glycosylations are reported in nuclear and cytoplasmic compartments. High-mobility-group proteins (HMGs) 14 and 17, which are important structural components of chromatin, are classical type glycoproteins which were found to be N-linked glycosylated with *N*-acetylglucosamine, mannose, galactose, glucose, fucose, and possibly xylose residues. Complex O-linked glycosylation is also observed on cytoplasmic proteins, e.g. Glycogenin (glycogen binding protein) present in liver cells (141).

2.5 O-GlcNAcylation:

2.5.1 Introduction:

Unlike classical complex glycosylation decorating the cellular surface, O-GlcNAcylation is single sugar attachment (N acetyl-D-Glucosamine) through a hydroxyl linkage to Ser/ Thr residues of nuclear and cytoplasmic proteins (20). The attachment of bulky, uncharged, acetylated hexosamine sugar (GlcNAc moiety) is part of a rapidly emerging signalling mechanism, which competes with phosphorylation. The addition and removal of O-GlcNAc moiety is catalysed by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), respectively. These O-GlcNAcylation cyclic enzymes are distinct from glycosyl transferases and glycosidases present in the secretory pathway. On the contrary, these enzymes are more similar to kinases and phosphatases as both (O-GlcNAcylation and phosphorylation) are dynamic processes and both modify Ser/Thr residues (142,143). Further, OGT and OGA get phosphorylated similar to kinases and phosphatases, and many proteins get O-GlcNAcylated or phosphorylated at exposed regions of target protein,

suggesting that both the modifications have same recognition domain. However, these two modifications are mechanistically distinct. There are thousands of kinases and phosphatases responsible for protein phosphorylation, whereas cycling of O-GlcNAc is catalysed by a single pair of enzymes i.e. OGT and OGA. Secondly, the site of phosphorylation can be identified by primary sequence of amino acids, whereas, O-GlcNAc modification has no consensus sequence. Some similarities and differences of the two post-translational modifications are listed below (Table 4).

Sr. no.	**Phosphorylation**	**O-GlcNAcylation**
1	Single residue modification	Single residue modification
2	Dynamic	Dynamic
3	Modifies serine and threonine	Modifies serine and threonine
4	Catalysed by several kinases and phosphatases	Catalysed by OGT and OGA
5	Nucleo-cytoplasmic modification	Nucleo-cytoplasmic modification
6	Negatively charged	Non polar
7	Directly activates/inactivates signalling molecules	Activates signalling pathway by activating phosphorylation of signalling molecules
8	Nutrition level dependent modification, high energy donor substrate: ATP	Nutrition level dependent modification, high energy donor substrate: UDP-GlcNAc

Table 4. Similarities and differences in O-GlcNAcylation and phosphorylation (144,145)

2.5.2 O-GlcNAc addition and removal:

2.5.2.1 O-GlcNAc transferase:

OGT is an enzyme which catalyses the addition of a GlcNAc moiety to nucleo-cytoplasmic proteins (146,147). OGT is a soluble, ubiquitous and highly conserved enzyme. A single copy of OGT gene is present on X chromosome (Xq13.1) in mammals (148,149). Deletion of OGT gene is embryonic lethal, indicating that OGT is required for the viability of organism. Further, the tissue specific mutation of OGT leads to defective somatic cell functions. Interestingly, this single enzyme can transfer GlcNAc moiety to ~4000 different targets through diverse mechanisms (147), which implies diverse substrate specificity of O-GlcNAc transferase. The mechanisms such as splice variants, substrate interaction via multiple domains, tissue- and organelle-specific expression, post-translational modifications (e.g. phosphorylation), the substrates' post-translational modifications, and nutrient flux may contribute to this diverse substrate specificity (150-156).

Structurally, OGT contains a tetratricopeptide repeat (TPR) domain at the N terminal and a catalytic domain at the C terminal; both the domains are connected by a least conserved linker region. The single gene of OGT has three splice variants with different lengths of TPR (Illustration 4 A).

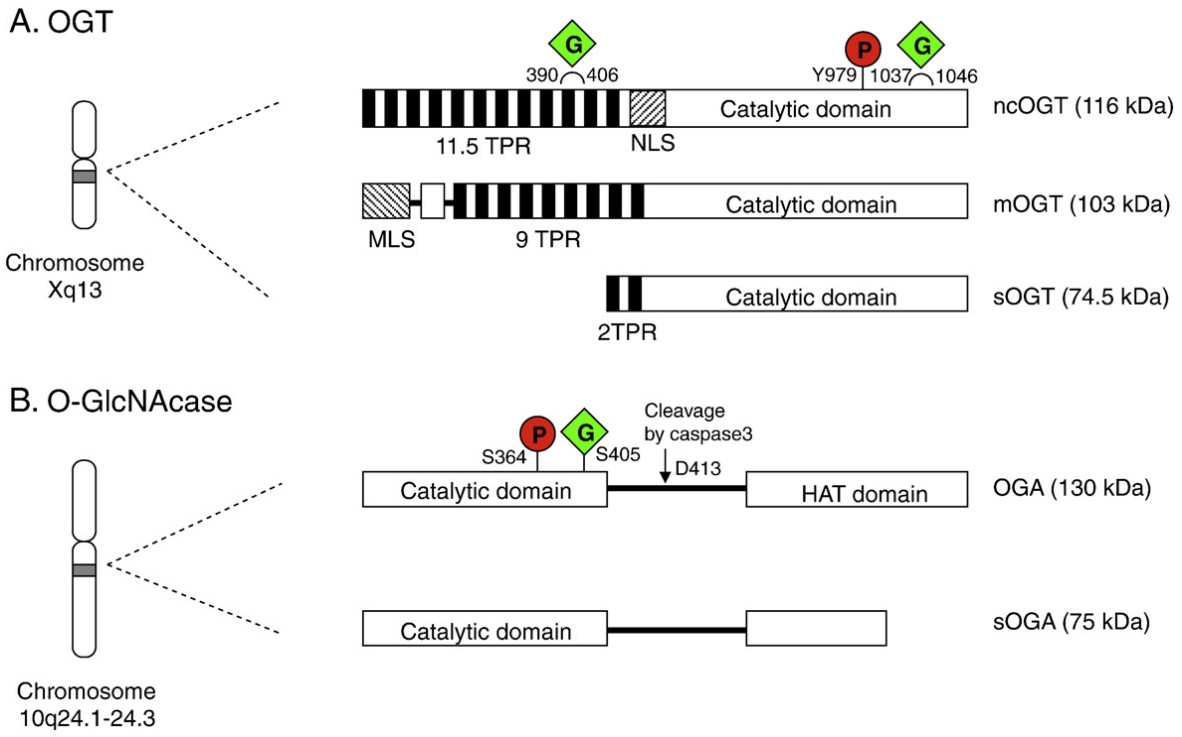


Illustration 4. Structure of OGT and OGA: A. OGT gene is located on X chromosome (Xq13) which gives three different splice variants (isoforms). All of the isoforms have identical C-terminal catalytic domains whereas, at their N-terminal different lengths of tetratricopeptide repeats (TPRs) are present. ncOGT has 11.5 TPRs and nuclear localization signal (NSL); mOGT has 9 TPRs and mitochondrial localization signal (MLS); sOGT has 2 TPRs. Among three isoforms ncOGT is dynamically modified with tyrosine phosphorylation (P) and O-GlcNAcylation (G); nc: nucleocytoplasmic; m: mitochondrial; s: short. B. O-GlcNAcase (OGA) gene is situated at X10q24 chromosome. OGA gene gives two isoforms with identical N-terminal catalytic domains. Full-length OGA contains a histone acetyl transferase (HAT) domain at its C terminal region. This domain is dynamically modified with phosphorylation (P) and O-GlcNAcylation (G); s: short (142).

2.5.2.1.1 Isoforms of OGT:

The largest isoform is nucleocytoplasmic OGT (ncOGT), ncOGT has 13 TRP domains. ncOGT is involved in transcriptional repression, proteasomal inhibition and stress tolerance activities (153). There are three isoforms of OGT. Mitochondrial OGT (mOGT) has 9 TPR domains, which is a

result of unique start point from the fourth intron, and has mitochondrial target signal (MTS) at the N terminal region. The mOGT has pro-apoptotic activity (149,157,158). The shortest isoform, sOGT, contains 3 TPR domains and has been linked to apoptosis (Illustration 4A) (158). Variable lengths of TPR domain contribute to variation in the amphipathic groove, the region which modulates substrate specificity of OGT (159-161). The molecular weights of different isoforms of OGT range from 70 kDa -110 kDa (149). OGT recognises multiple substrate protein sites by using mechanisms such as cellular UDP-GlcNAc concentration; hence at physiological concentration of UDP-GlcNAc, some but not all proteins are constitutively glycosylated (155). Further, phosphorylation of substrate protein can also modulate interaction with OGT, e.g. phosphorylation of calcium/calmodulin-dependent protein kinase type IV (CaMKIV) modulates affinity of hOGT (155). The interaction of OGT with its substrate or interacting partners at multiple domains affects its activity, localization and interaction with other partners of OGT (162).

2.5.2.2 O-GlcNAcase:

OGA also known as GCA or MGEA5, is an evolutionarily conserved single enzyme responsible for removal of GlcNAc moiety from proteins (163-165). OGA solely catalyses removal of O-GlcNAc from a number of targets. OGA is a soluble, highly conserved and ubiquitously expressed enzyme. Structurally, OGA is 917 amino acids long (166). The N terminal domain is hexosaminidase domain and the C terminal is pseudo-histone acetyl transferase (HAT) domain (Illustration 4B) (167). OGA has alternatively spliced two isoforms, long and short (OGA-L and OGA-S). Both the isoforms have an identical catalytic activity, although OGA-S lack pseudo-HAT domain (168). Each of the isoforms has been shown to have OGT interacting region. OGA-L resides in nucleus and cytoplasm (169), whereas OGA-S is present in lipid droplets (170,171). The molecular weight of these isoforms range between ~72 kDa – 100 kDa. Interestingly, OGA has

'apoptosis induced caspase-3 cleavage' site which yields two truncated fragments upon cleavage, both of which are functional even when separated. Although OGA structure is yet to be resolved, based on indirect structural approaches, it has been revealed that GlcNAc moiety is more important than protein backbone for its de-glycosylating activity (155).

2.5.3 Biosynthesis of UDP-GlcNAc (Hexosamine biosynthetic pathway):

Hexosamine biosynthetic pathway (HBP) comprises of multiple steps that lead to synthesis of activated UDP-GlcNAc (Illustration 5) (172). The pathway utilizes multiple metabolites such as carbohydrates, amino acids, ATP, lipids and nucleotides to yield UDP-GlcNAc as the end-product. Hence, HBP is a nutrient and metabolite sensitive and dependent pathway. Around 2-5% glucose entering into the cells is directed to the HBP. Cellular glucose is converted into fructose-6-phosphate (Fructose-6-P) as a result of phosphorylation and isomerisation in two enzymatic steps. Further, it is converted in to glucosamine-6-phosphate (Glucosamine-6-P) by the rate limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). In the next step, acetyl group is added to yield GlcNAc-1-phosphate (GlcNAc-1-P) catalysed by Glucosamine-6-phosphate N-acetyltransferase (EMeg32) (173,174). After second isomerization in to N-acetylglucosamine-1-P, the end product of HBP pathway i.e. activated UDP-GlcNAc is produced (Illustration 5). HBP pathway is tightly controlled at different levels. The activity of rate limiting enzyme GFAT is regulated by the pathway end product i.e. UDP-GlcNAc by feedback mechanism. The second important enzyme in the pathway is GlcNAc-6-phosphate acetyltransferase (EMeg32), whose deficiency in mouse embryonic fibroblasts is linked with defects in proliferation and adhesion. Mice with homozygous null embryos lacking EMeg32 die within 7-8 days. HBP pathway is solely responsible for entrance of cellular glucose in to the biosynthesis of UDP-GlcNAc (Illustration 5).

Hence, factors which inhibit HBP pathway have direct effects on O-GlcNAcylation on cellular proteins.

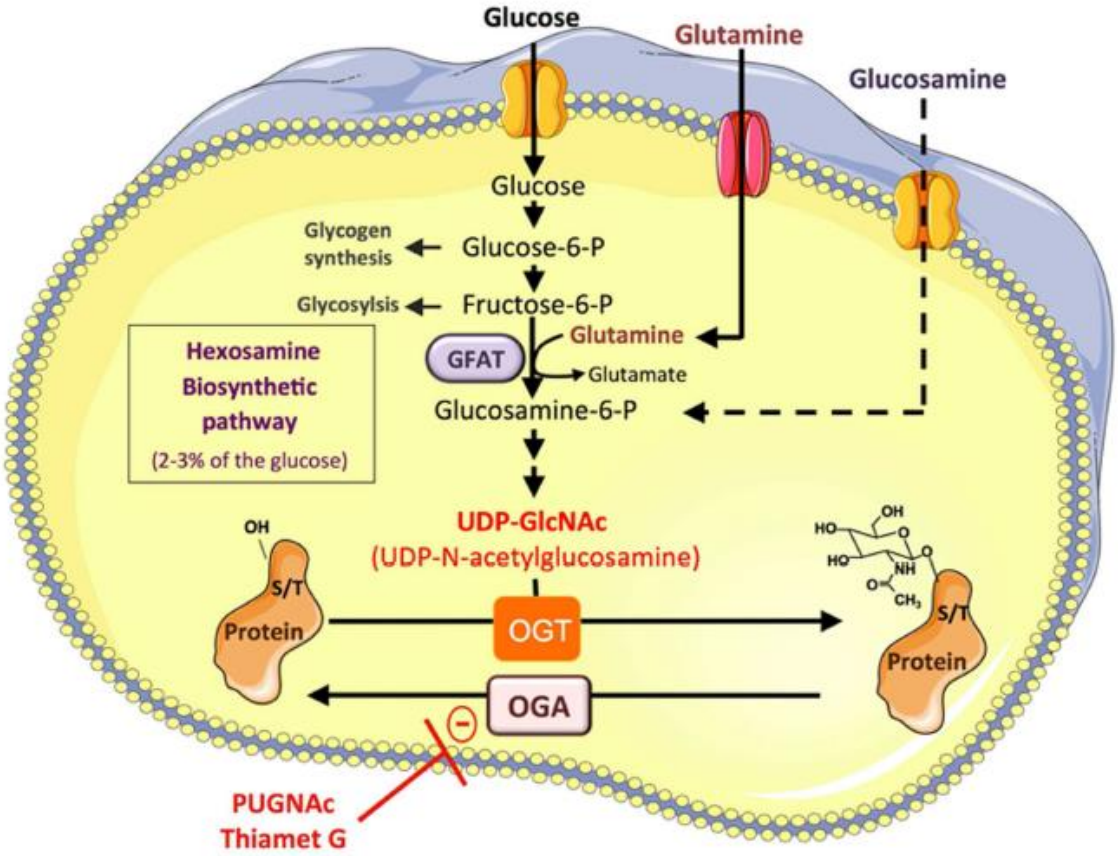


Illustration 5. Hexosamine biosynthetic pathway: Around 2-3% of total internalised cellular glucose enters in to HBP pathway. The end product of HBP is UDP-GlcNAc, a substrate of *O*-GlcNAc-transferase (OGT). Glucose is converted in to fructose-6-phosphate, which further enters in the HBP pathway. Next, glutamine:fructose-6-phosphate amidotransferase (GFAT), a rate limiting enzyme converts fructose-6-phosphate in to glucosamine-6-phosphate. After a subset of reactions, UDP-*N*-acetylglucosamine (UDP-GlcNAc) is synthesized which is used for O-GlcNAcylation of serine or threonine residues of target proteins by OGT. The removal of *O*-GlcNAc moiety from glycosylated proteins is carried out by the *O*-GlcNAcase (OGA). O-[2-acetamido-2-deoxy-D-glucopyranosylidene] amino-*N*-phenylcarbamate (PUGNAc) and O-(2-Acetamido-2-deoxy-D-glucopyranosylidenamino) N-phenylcarbamate (Thiamet G) are the OGA inhibitors which enrich O-GlcNAcylation on cellular proteins (175).

2.5.4 Protein properties regulated by O-GlcNAcylation:

2.5.4.1 Phosphorylation:

O-GlcNAcylation and phosphorylation both occur on Ser/Thr side chain on proteins. Virtually every O-GlcNAcylated protein is a phosphoprotein. Mapping of O-GlcNAcylation sites revealed that, several of them were either phosphorylation sites, or sites adjacent to the phosphorylation site. Hence, a "Yin-Yang" model was proposed to define relationship between O-GlcNAcylation and phosphorylation (176). This model was initially defined to illustrate mutual occupancy of O-GlcNAcylation and phosphorylation at the same site. However, continuously increasing list of O-GlcNAcylating proteins suggests that, "Yin-Yang" is an over simplified illustration of crosstalk between O-GlcNAcylation and phosphorylation. These observations underline the fact that the interplay is both complex and extensive.

Several mass spectrometry studies have reported global changes in O-GlcNAcylation levels after alteration in cellular phosphorylation. Reciprocal relationship between O-GlcNAcylation and phosphorylation has indeed been found in several global analysis, such as treatment of various kinases activators. For instance, activation of protein kinase A (PKA) and protein kinase C (PKC) reduces global O-GlcNAcylation. In addition, inhibition of these kinases increases global O-GlcNAc level (177).

In contrast to these findings, several mass spectrometry studies have suggested that, relation between O-GlcNAcylation and phosphorylation may not be always reciprocal. Effect of inhibition of glycogen synthase kinase (GSK-3) was investigated on 45 known O-GlcNAcylating proteins. Out of these, 10 proteins (which were mostly cytoplasmic) showed increased O-GlcNAcylation whereas, 19 other proteins (mostly localised in nucleus) had reduced O-GlcNAcylation. In another study, increased O-GlcNAcylation by inhibiting OGA was investigated on over 700

phosphorylation sites in non-stimulated NIH3T3 cells. Around 48% phosphorylation sites were unaltered, indicating that these were non-actively cycling phosphorylation sites, while 280 phosphorylation sites showed reduction of phosphorylation in response to increased global O-GlcNAcylation. Surprisingly, 149 sites showed increased phosphorylation in that condition (36). These studies indicate that O-GlcNAcylation and phosphorylation are most abundant modifications which showed extensive crosstalk on many cellular proteins. It is also reported that even OGT is regulated by phosphorylation and O-GlcNAcylation regulates variety of kinases (35,178,179).

Broadly, four types of crosstalk between O-GlcNAcylation and phosphorylation can occur (figure. 7) which are as follows:

2.5.4.1.1 Reciprocal crosstalk of O-GlcNAcylation and phosphorylation at same site:

Proteins like, c-Myc (Thr58), Estrogen receptor-β (ER-β) (Ser16) and IKKb (Ser733), in which O-GlcNAc and phosphorylation compete for same hydroxyl group, as postulated in "Yin-Yang" model (Illustration 6 A) (180,181). Each of the modifications has specific function for a given site. In case of ER-β, Ser16 is present in 'Pro, Glu, Ser and Thr', 'PEST' sequence, which is related to rapid protein degradation. O-GlcNAcylated ER-β is stable as it interferes with phosphorylation at the same site, which is required for degradation and transcriptional activation of ER-β (181,182). In case of IKKb, its reciprocal interaction between O-GlcNAcylation and phosphorylation at Ser733 site is responsible for regulating its catalytic activity (179).

2.5.4.1.2 Crosstalk of O-GlcNAcylation and phosphorylation at proximal sites:

Perhaps more common than competition for same site between O-GlcNAcylation and phosphorylation, is the competition for adjacent sites (Illustration 6 B). O-GlcNAc is a bulky modification (Stoke's radius of O-GlcNAc is much larger than that of phosphorylation) (145) and

hence it creates steric hindrance for phosphorylation at the adjacent site, e.g. O-GlcNAcylation at Ser149 and phosphorylation at Thr155 of p53 is responsible for its ubiquitin proteasome mediated degradation (26). A similar dynamic interplay between O-GlcNAcylation and phosphorylation occurs on RNAPII at the C-terminal domain (CTD) (183). Thr1618 is O-GlcNAcylation and Thr1616 and Thr1619 are phosphorylation sites. Both the phosphorylation sites reciprocally regulate their adjacent O-GlcNAcylation (184).

2.5.4.1.3 Crosstalk between O-GlcNAcylation and phosphorylation at distant sites:

O-GlcNAcylation and phosphorylation could also regulate each other at distance sites (Illustration 6 C). For example, in Insulin receptor substrate 1 (IRS1), phosphorylation sites are mostly located in the N-terminus, (Ser307, Ser612, Ser632/635). Thes negatively regulate insulin signalling, while Ser302, Ser629 have positive regulatory effects on insulin signalling (185). O-GlcNAcylation sites of IRS1 exist mainly in the C-terminus, such as Ser914, Ser1009, Ser1036, Ser1041. Increased O-GlcNAcylation on IRS1 negatively regulates insulin signalling, but exact nature of the crosstalk between specific O-GlcNAcylation and specific phosphorylation among these sites of IRS1 is still unknown (151,186).

2.5.4.1.4 Crosstalk between the cycling enzymes regulating O-GlcNAcylation and phosphorylation:

Competition between O-GlcNAcylation and phosphorylation leads to extensive crosstalk at the same or proximal sites on proteins. However, these two modifications also control each other by influencing the activities and localization of the other's cycling enzymes (Illustration 6 D). For example,

1. OGT activity is regulated by its tyrosine phosphorylation and further O-GlcNAcylation (187).

2. Calcium calmodulin-dependent protein kinase IV (CaMKIV) is repressed by O-GlcNAcylation (35).
3. Insulin stimulation recruits OGT to the plasma membrane where it interacts with and gets phosphorylated by insulin receptor, which is necessary for its activity. It is reported that, in rat brain, OGT forms a stable and active complex with Ser /Thr protein phosphatase i.e. PP1b and PP1c (186).

   OGT, OGA, Aurora B kinase and protein phosphatase 1 form a supra-complex during mitosis. The mitotic kinase Aurora B forms a complex with OGT and OGA during M phase of the cell cycle (121). This transient complex formed during cytokinesis regulates both the O-GlcNAcylation and phosphorylation of several proteins like vimentin which are involved in cell division.

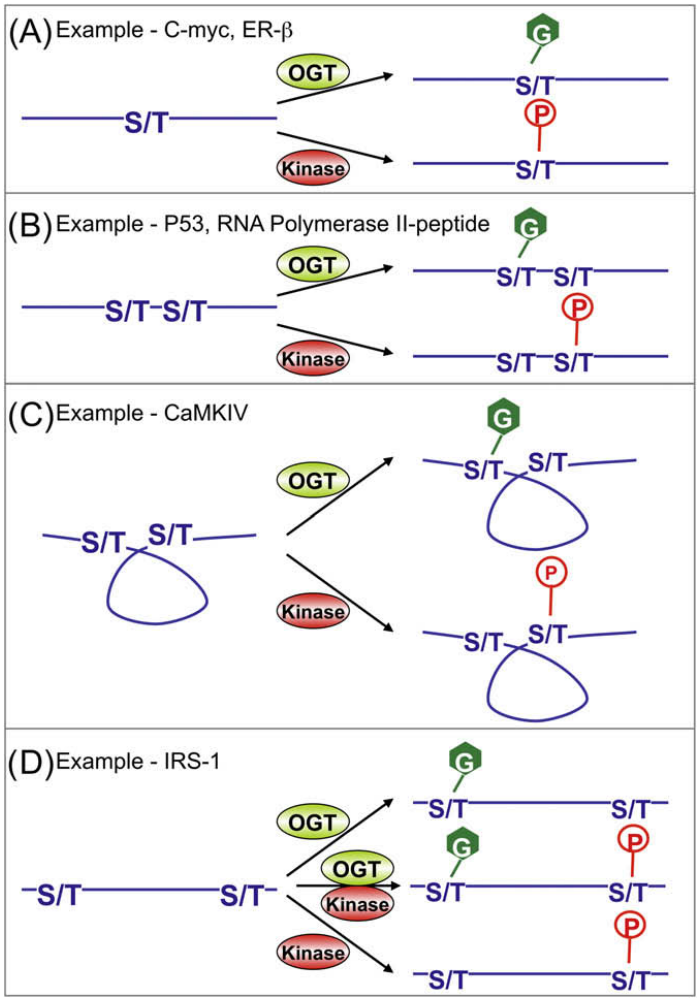


Illustration 6. Crosstalk between O-GlcNAcylation and phosphorylation: (A) Reciprocal interplay between O-GlcNAcylated and phosphorylated at same serine or threonine residue of proteins. (B) Reciprocal interplay at proximal sites (within 10 amino acids distance). (C) Distantly situated serine/ threonine residues in primary sequence, which are brought together due to protein folding can have reciprocal interplay. (D) O-GlcNAcylation and phosphorylation can have either positive or reciprocal interplay at a distant sites due to regulation of their cyclic enzymes. Serine (S), threonine (T) G: O-GlcNAcylation, and P: O-phosphorylation (145).

2.5.4.2 Interplay with other PTMs:

Since its discovery, crosstalk between O-GlcNAcylation and phosphorylation has been extensively studied. Interplay between O-GlcNAcylation and other PTMs such as methylation, acetylation, ubiquitination etc., is also under investigation and their role in cellular processes is emerging.

Increased O-GlcNAcylation level through over expression of OGT, alters acetylation and methylation of histones and coactivator associated arginine methyltransferase 1 (CARM1) (28). NFκB family member, RelA O-GlcNAcylation at T305 is required for its acetylation mediated by p300 at K310 (188). Further, O-GlcNAcylation of histone lysine methyltransferase (MLL5) promotes methylation of H3K4 by enabling retinoic acid induced granulopoiesis (189). Ten-eleven translocation (TET) enzymes, i.e. TET2 and TET3 proteins form a complex with OGT which maintain methylation of H3K4 (190). O-GlcNAcylation is also implicated in regulation of mono and poly ubiquitination of various cellular proteins. O-GlcNAcylation reciprocally regulates phosphorylation of proteins (191) and phosphorylation inturn can modulate ubiquitination of proteins (192). Complex cross talk between O-GlcNAcylation, phosphorylation and ubiquitination leads to protein degradation.

2.5.4.3 Protein degradation:

Ubiquitin proteasome mediated protein degradation is an important mechanism of protein turn over, which maintains steady state concentration of proteins in cell. O-GlcNAcylation reciprocally regulates phosphorylation, and phosphorylation facilitates ubiquitination (191,192). Hence, it can be concluded that O-GlcNAcylation regulates ubiquitin proteasome mediated degradation of cellular proteins (193). For instance, O-GlcNAcylation provides stability to p53, by reducing its interaction with MDM2 (E3 ubiquitin-protein ligase). In addition, O-GlcNAcylation at S149 of p53 reciprocally regulates its phosphorylation at T155, which is required for ubiquitination and degradation of p53 (26). This suggests that O-GlcNAc protects p53 from degradation by inhibiting its phosphorylation. Similarly, O-GlcNAcylation of Δ-Lactoferrin, Snail1, ERβ, etc. protects them from degradation by reciprocal regulation of their phosphorylation (193,194). However, O-GlcNAcylation of Casein kinase 2 alpha (CK2α), a serine/threonine protein kinase at S347 reduces

its stability by reciprocal regulation of its phosphorylation at T344 (194). Proteomics study performed by Ruan et al. demonstrated that many proteins of ubiquitination pathway including E3 ubiquitin ligases and deubiquitinases (DUBs) are OGT binding partners (195). This suggests that O-GlcNAcylation modulates ubiquitin system directly. For instance, O-GlcNAcylation of PGC-1α at S333 facilitates recruitment of Bap1 (DUB), which further deubiquitinates and stabilizes PGC-1α (195-197). Large number of ubiquitin processing enzymes are known to be modified with O-GlcNAc e.g. ubiquitin precursors, E1s, E2s, E3s and DUBs (195,198). Further, O-GlcNAcylation regulates activity of proteasome (27). Several proteins in 20S catalytic subunits and the 19S regulatory subunits of the proteasome are found to be O-GlcNAcylated. In case of 19S, O-GlcNAcylation inhibits its proteasomal activity (199).

2.5.4.4 Protein localization:

O-GlcNAcylation has been observed predominantly on nuclear proteins (200). Studies in Aplasia neurons have shown that proteins modified with O-GlcNAc rapidly shuttle from cytoplasm to nucleus (201). Therefore, O-GlcNAcylation is considered as similar to nuclear localization signal (NLS). Chemically synthesised glycoconjugated bovine serum albumin (BSA), when injected in cells' cytoplasm, showed its nuclear localization. The attached sugars glucose, mannose, fucose, or N-acetylchitobiose were shown to be facilitate nuclear uptake of BSA. This nuclear transportation was energy dependent and did not use typical translocation factors for their nuclear localization (202-204). Many proteins exhibit ability to bind O-GlcNAc, e.g. galectin 3, CBP)-35, CBP-67 and HSP70. Such lectins are known to shuttle between cytoplasm and nucleus (205). Proteins such as c-Myc, Tau, Stat5a, Pax-6, ELF-1, Sp1, and the mTOR α4 phosphoprotein, etc. are relatively more O-GlcNAcylated when they are in nucleus than their cytoplasmic forms (206-211). Hence, it is considered that O-GlcNAc acts as nuclear retention signal rather than NLS.

Similarly, increased phosphorylation of Tau proteins concomitantly reduces its O-GlcNAcylation because of which phosphorylated form of Tau shows reduced nuclear localization (207). Transfection of siRNA of OGT in Lymphoma cells resulted in increased phosphorylation of Sp1 and mTOR α4 as well as their relocalization to the cytoplasm (211). O-GlcNAcylation of PKC regulates its activity as well as its translocation to membrane (212,213). Astroglial cells treated with PUGNAc or streptozotocin (STZ) decrease levels of membrane bound PKC-α and PKC-ε and increase levels of PKC-βII. But exact mechanism of how O-GlcNAcylation regulates translocation of PKC is not yet known (213).

2.5.4.5 Protein-protein interactions:

O-GlcNAcylation has been shown to regulate protein-protein interactions. Roos et.al have identified O-GlcNAcylation site on SP1 at its SpE domain, which is involved in self-association of SP1 and binding to the TATA-binding protein associated factor 110 (TAF110) (214,215). Mutation at this site leads to less efficient binding of SP1 to TAF110. Other transcription factors such as YY1 and ELF-2 interact with RB. RB bound form of these proteins is relatively less glycosylated compared to unbound form (216). This suggests that, O-GlcNAcylation of YY1 and ELF-2 inversely regulates their interactions with RB (216). Stat5a is involved in cytosolic signalling and in mediating the expression of specific genes. In response to prolactin, Stat5a is phosphorylated, and O-GlcNAcylated (208). It is possible that O-GlcNAcylation site T92 when mutated to T92A, leads to interference in induced gene transcription by prolactin stimulation. In addition, the glycosylated form of Stat5a preferentially binds to the transcriptional co-activator CREB-binding protein. HSP70 is an O-GlcNAc binding lectin, as many of the interactions of this protein are regulated by O-GlcNAcylation (217,218). Its protein folding ability during stress is linked with O-GlcNAcylation of its target proteins (218).

2.5.5 Cellular functions of O-GlcNAcylation:

2.5.5.1 Transcription:

Regulation of gene transcription is one of the important functions of O-GlcNAcylation. Around 25% of O-GlcNAc modified proteins are involved in transcriptional regulation (219). O-GlcNAcylation of these proteins can modulate their functions in many ways (220). O-GlcNAcylation is often accumulated in transcriptionally inactive regions of chromosome of drosophila and in mammalian cells (221,222). Therefore, O-GlcNAcylation is considered as a transcription silencer. However, investigation of several O-GlcNAcylating transcription factors such as, p53, C-Myc, Sp1, c-Jun, c-Fos, CREB binding protein (CBP) and early growth response-1 (EGR-1) (183,223,224) indicates that role of O-GlcNAcylation in transcription is much complex. O-GlcNAcylation regulates phosphorylation, stability, localization of transcription factors as also their transcription activity and interactions with DNA as well as binding partners. For example, O-GlcNAcylation of p53 at S149 regulates its stability and activity by modulating its phosphorylation at T155 (26). In contrast, O-GlcNAcylation of β-catenin acts as inactivation signal, as O-GlcNAcylation retains β-catenin in cytoplasm leading to reduction of its nuclear localization and transcription activity (25,225). On the contrary, O-GlcNAcylation of transcription factor, SP1, which is involved in insulin signalling, acts as a nuclear localization signal (226,227). Inhibition of HBP pathway by targeting GFAT enzymatic activity by DON (6-diazo-5-oxo-l-norleucine) leads to reduction in SP1 O-GlcNAcylation. Sequestration of non-glycosylated SP1 in cytoplasm, leads to reduction of its transcription activity and increased degradation (228).

O-GlcNAcylation regulates interactions between transcription factors and their binding partners. For instance, in hyperglycemia, O-GlcNAcylation of p65 domain of NFκB at Thr322 and Thr352 is increased, which reduces interactions between NFκB and IκB. As a result, IκB is unable to mask

the nuclear localization signal of NFκB. Hence nuclear translocation and transcription activity of NFκB is increased (229-231).

2.5.5.2 Cell cycle:

Similar to other PTMs, O-GlcNAcylation regulates the progression of cell cycle. Dynamic O-GlcNAcylation of cellular proteins is reportedly altered in response to several mitogenic stimuli (232). Colcimid or nocodazole cause cell cycle arrest at G2/M transition phase, which concomitantly enhances phosphorylation and O-GlcNAcylation of K8/18 (233). Further, removal of the Nocodazole resulted in baseline O-GlcNAcylation level of K8/18 (233). In addition, pharmacological drugs induce altered O-GlcNAcylation which leads to defective cell cycle progression, e.g. treatment of PUGNAc (a hexosaminidase inhibitor), which blocks cycling of O-GlcNAc on cellular proteins leads to abrogation of the cell cycle (234).

Recently, two independent groups have demonstrated that O-GlcNAcylation of cellular proteins is significanly increased when quiescent cells enter in to the G1 phase (235,236). Furthermore, blocking the activity of OGT leads to delayed synthesis of cyclin D1 (a key regulator of G1 phase) and cell proliferation. In addition, inhibition of the HBP pathway reduces cell cycle progression (237). On the contrary, inhibition of OGA accelerates cell cycle progression. Interestingly, silencing of the OGT expression inhibits PI3K and MAPK pathways, the signaling cascade which is responsible for cellular entry into G1 phase (237). These reports indicate that O-GlcNAcylation and OGT are indispensable for G0/G1 transition. During G1/S transition, levels of OGA are increased, which result in reduction in global O-GlcNAcylation levels (235). In agreement with this, HBP pathway inhibition accelerates the S phase (234). In S phase, O-GlcNAcylation of histones is relatively lesser, which may help the pre-replicative complex to reach chromatin. During the G2/M transition phase, when a cell is prepared for mitotic division, the levels of O-

GlcNAcylation are elevated (238). Xenopus oocytes, microinjected with bovin galactosyl transferases, an enzyme which catalyses the capping of terminal GlcNAc molecules, inhibits the entry of these cells in M phase (239). The perturbation of O-GlcNAcylation level in Xenopus oocytes by either PUGNAc or Glucosamine treatment disturbs the maturation kinetics (240). When Xenopus oocytes blocked at the G2 phase are activated by hormonal stimulus, concomitant increase in the O-GlcNAcylation of its cellular proteins was observed (241-244). Slawson *et. al* demonstrated that during mitosis, OGT localizes at mitotic spindle body (234). OGT forms a supramolecular complex along with OGA, Aurora B kinase and phosphatase 1, and this complex targets several proteins such as vimentin (121). The proper balance of O-GlcNAc cycling enzymes is required in M phase i.e. OGT and OGA knock-down shows reduced expression of cyclin B1 (M phase check point) (236,237).

2.5.5.3 Stress response:

O-GlcNAcylation has protective role during stress and in cellular death. During stress such as treatment with ethanol, UV light, sodium chloride, arsenite, hydrogen peroxide, or heat shock, O-GlcNAcylation of cellular proteins and OGT levels are increased (245). Concomitantly, during heat shock treatment, heat shock proteins (HSP 70) levels are also increased. Some HSP70 family proteins, such as p70, p65 and p55 have specific lectin activity, which is increased during stress (217,218,245). This suggests that HSP proteins act as chaperones which refold misfolded O-GlcNAcylated proteins during stress.

Major component proteins of stress granules, such as, glyceraldehyde-3- phosphate dehydrogenase, prohibitin-2, receptor for activated C kinase 1 (RACK1), are modified with O-GlcNAc in response to stress (246). Large number of proteins, which regulate translation and degradation of mRNA, e.g. ribosomal proteins, are modified with O-GlcNAc in response to stress.

It is considered that, O-GlcNAcylation is required for assembly of stress granule proteins. In support of this, several stress protective roles of O-GlcNAcylation are reported, such as, treatment of Glucosamine raises O-GlcNAcylation levels and protects from apoptosis by increasing anti-apoptotic proteins like Bcl2 in Neonatal Sprague-Dawley rat ventricular myocyte primary cultures treated for ischemia (247,248). Further, elevated O-GlcNAcylation by treatment of PUGNAc can reduce infarct size of damaged heart (248). During recovery of trauma-haemorrhage in rat model, Glucosamine treatment improves cardiac function, and inhibited circulating inflammatory cytokines by suppressing NFκB pathway (249). Over expression of OGT had similar results like glucosamine treatment. OGT affects nuclear localization and DNA binding activity of NFκB. Further, phosphorylation of IκB is also dependent on O-GlcNAcylation. O-GlcNAcylation regulates mRNA expression of IL6 and TNF-α, indicating that experimentally increased O-GlcNAcylation can protect cell from death.

2.5.5.4 O-GlcNAcylation in neuronal regulation:

O-GlcNAc and its cycling enzymes OGT and OGA are abundantly present in brain (165,250,251). Levels of mRNA coding for OGT and OGA were found to be highest in brain. In addition, activity of OGT has been found to be 10 fold higher in brain compared to other tissues (252). Some brain regions like cerebellar cortex and hippocampus showed high level of OGT expression (253,254). O-GlcNAcylation has important functions in neuron, which are revealed by deletion of OGT leading to neuronal apoptosis (224). In addition, recently it is shown that O-GlcNAc is also involved in mitochondrial transfer in neurons (255). Proteomics studies of human and mouse have demonstrated that over 1000 proteins are O-GlcNAcylated in brain, which range from abundant cytoskeleton associated proteins such as Tau to membrane associated proteins like Amyloid Precursor Protein (APP) (21). O-GlcNAcylation is involved in neuronal development, synaptic

transmission and synaptic plasticity (251,256,257). Study in primary chicken forebrain neurons indicates that during axon branching O-GlcNAcylation plays an important role (251). cAMP signalling stimulates axon branching which can be inversely regulated by O-GlcNAc levels, as over expression of OGA increases neurons exhibiting axon branching (251). Synaptosome proteins such as Bassoon and Piccolo which are involved in synaptic transmission are dynamically O-GlcNAcylated (256).

Increased O-GlcNAcylation by inhibiting OGA resulted in increased synaptic plasticity in hippocampus slice by activating calmodulin kinase II and Erk1/2. Both these kinases are essential for long-term potentiation (LTP) which is associated with ability to learn and for memorization (257). Increased O-GlcNAcylation of synapsin I/II concomitantly increases phosphorylation at S9, S62/67 and S603 residues (257). This promote synaptic vesicle formation during synaptic transmission.

2.5.6 O-GlcNAcylation and chronic diseases:

Aberrant O-GlcNAcylation is reported in plethora of human disorders including various chronic diseases such as cancer, diabetes, neuronal disorders, circulatory disorders etc.

2.5.6.1 Neurodegenerative disorders:

Alzheimer disease (AD) is the most common neurodegenerative disease (258). Although root cause of AD is yet to be known. Increased production of amyloid-β peptides (Aβ), which are proteolytic product of the amyloid precursor protein (APP) are involved in AD pathology (259). Similarly, another causative factor is aggregation of tau protein. Tau aggregation is driven by its pathological hyper phosphorylation (260). Stoichiometry of phosphorylation on Tau is increased by 6–8-folds which includes about 45 serine and threonine residues (260). A major consequence of hyper phosphorylation of Tau is seen in its disability to bind with microtubules thereby, leading

to acceleration of microtubule polymerization. Hyper phosphorylated tau gets accumulated in cytoplasm, which leads to increased toxicity similar to that seen in prion like aggregates (260). Regional glucose hypometabolism is a characteristic feature of Alzheimer's disease (AD) (259). Nutrient-responsive posttranslational modification i.e. *O*-GlcNAcylation, is an emerging link between glucose hypometabolism and progression of AD. Human as well as mouse brain tissues proteomics studies have revealed, presence of *O*-GlcNAc modification on over 1000 proteins (21). These proteins include highly abundant cytoskeletal associated Tau protein, membrane-associated APP and low-abundance proteins, including transcription factors (21). Gong et.al have demonstrated that, phosphorylation and *O*-GlcNAcylation on Tau exhibit reciprocal relationship at various sites (261). Interestingly, same group has also demonstrated that, the aggregates of Tau proteins from human AD patients do not have *O*-GlcNAc modification (262). This suggests that, increased brain *O*-GlcNAcylation protects against Tau and amyloid-peptide toxicity. Decreased *O*-GlcNAcylation occurs in AD, indicating that glucose hypometabolism can impair the protective roles of *O*-GlcNAcylation within neurons leading to neurodegeneration (245).

2.5.6.2 Cancer:

O-GlcNAcylation is shown to be significantly altered during progression of cancer through different mechanisms (144). Levels of O-GlcNAcylation and OGT are significantly elevated in breast, colon and prostate cancers (144,263-265). O-GlcNAcylation plays important role in cell division, metabolism and cytoskeletal regulation in tumor growth (144). Decreased O-GlcNAcylation is reported in breast tissue and thyroid cancer (263). In contrast, increased O-GlcNAcylation levels and its cycling enzymes are reported in cancers like lung, colon and chronic lymphocytic leukaemia (264,266). OGT knockdown suppresses tumor growth in mouse model. Major oncogenic factors, such as cMyc, NFκB, p53, SNAIL1, HCF1 and β-Catenin are O-

GlcNAcylated proteins (267). Thus perturbation of O-GlcNAcylation alters cell signalling, transcription, cell cycle, which inturn control tumorigenesis and metastasis (268-270). Mitogenic signals elicit activation of PI3K/AKT or MAPK pathway. In cancer cells activation of PI3K/AKT pathway is sensitive to nutritional conditions (271,272).

Some of the transcription factors which are O-GlcNAcylated are involved in cancer progression. For instance, NFκB signalling is activated by O-GlcNAcylation in two ways. First, O-GlcNAcylation of IKKB kinase catalyses activation of NFκB. Secondly, NFκB itself gets O-GlcNAcylated at T322 and T352 at a high glucose concentration. O-GlcNAcylation of p53, a tumor suppressor protein, is known to stabilize it (273).

O-GlcNAcylation also regulates various steps in cancer progression, such as cell adhesion, invasion and migration (268,269). Several reports have demonstrated that O-GlcNAcylation favours cancer progression by regulation of E-Cadherin/ β-Catenin. E-Cadherin/ β-Catenin is known to regulate cell-cell adhesion. Interaction between E-Cadherin and β-Catenin was markedly reduced in OGT depleted cells, suggesting O-GlcNAcylation is involved in reduction of cellular attachments with other cells, which is important for metastasis (268). Down regulation of OGT leads to surface localization of E-Cadherin and p120. Inhibition of E-cadherin restores invasiveness in 4T1 cells (268).

ECM remodelling enzymes which play crucial role in invasion and metastasis alter cell-cell and cell ECM interaction, migration and angiogenesis. Reqinato et.al have demonstrated that expression of MMP2 is inhibited upon down regulation of OGT by shRNA in breast cancer cells (269).

Thus the above review of literature clearly suggests that O-GlcNAcylation plays a vital role in regulating many cellular processes, such as metabolism, transcription, cell signalling, protein turnover and response to stress. Hence, O-GlcNAcylation is considered as a sensor of cell homeostasis. Therefore, it is not surprising that, alterations in O-GlcNAcylation are linked to various diseases, such as, cancer, diabetes, neurodegenerative disease, cardiovascular disease and so on. The developments in high efficient site mapping approaches have enable more rapid advances in the understanding of the consequences of O-GlcNAcylation on cellular proteins. Recent advances in the detection tools have increased our understanding of O-GlcNAcylation on complex cellular proteins having multiple sites of O-GlcNAcylation and phosphorylation, such as, Keratin 8/18. The present study was undertaken to understand role of multiple sites of O-GlcNAcylation and phosphorylation of keratin 8 and 18, in the regulation of keratin solubility, stability and filament organization, and in regulation of certain cellular processes such as cellular migration, adhesion and spreading.

Chapter 3: Material and Methods

3.1 Materials:

3.1.1 Cell lines:

HHL17 (Human Hepatocyte Line 17) cell line was obtained from Dr. A.H. Patel, Institute of Virology, Glasgow, UK (274). It is an adherent cell line routinely cultured in (Dulbecco's Modified Eagle Medium) DMEM containing 10% fetal bovine serum (FBS).

3.1.2 Reagents:

FBS, DMEM powder, Antibiotic-Antimycotic solution, L-Glutamine, were obtained from GIBCO, Invitrogen Corporation, USA. Cycloheximide, Okadaic acid, PUGNAc, fibronectin, Aprotinin, Leupeptin, Pepstatin, PMSF, Trypsin, Dextrose, Tween-20, Bovine Serum Albumin (BSA), TEMED (N,N,N',N'-Tetramethyl-ethylenediamine), β-mercaptoethanol, Glycine, Paraformaldehyde, Coomassie Brilliant Blue R-250, Ponceau-S, Lysolecithin, Phalloidin-TRITC (Tetramethylrhodamine), DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), Trypan Blue, PIPES, RNase A, Ethidium Bromide, Agarose, Polybrene, Puromycin, and primers for PCR were obtained from Sigma Chemical Co, USA. Ampicillin, Yeast Extract, Tryptone, Luria-Bertani broth and Agar powder were obtained from HIMEDIA, India. Tris, NP-40, Sodium Dodecyl Sulphate (SDS), Bisacrylamide, Neomycin (G418) and Triton X-100 were obtained from USB, USA. Taq DNA polymerase, Phusion high-fidelity DNA polymerase, T4 DNA ligase buffer, ATP, dNTP mixture, T4 DNA ligase enzyme and restriction enzymes were obtained from either New England Biolabs (NEB), USA or Fermentas, USA. Plasmid DNA extraction and DNA gel extraction kits were obtained from Qiagen, USA. Acrylamide, PVDF membrane and ECL plus kit were acquired from Amersham-Pharmacia Biotech Ltd., England. WesternBright ECL Western blotting detection kit was from Advansta, USA. WGA-sepharose beads were obtained from Amersham Biosciences, UK. Folin & Ciocalteu's phenol (FCP) Reagent and Ammonium persulphate (APS)

was obtained from SRL Pvt. Ltd. India. Vectashield with or without DAPI mounting medium were obtained from Vector Labs, USA. Protease Inhibitor cocktail and Phosphatase Inhibitor cocktail were procured from Calbiochem, USA. Protein-G Sepharose beads were procured from Pharmacia Fine Chemicals, Sweden AB. All other fine chemicals were obtained locally and were of Analytical (AR) or better grade. Plastic ware for tissue culture was obtained from Nunclon MA USA. Water used to prepare all reagents was of Milli-Q grade.

3.1.3 Antibodies:

Antibodies used in this study were mouse keratin 18 clone CY-90 (C 8541), mouse keratin 8 clone M20 (C5301), mouse HRPO (A4416) from Sigma Chemical Co, St. Louis, USA, mouse Anti-O-GlcNAc clone RL-2 (MA1-072 Affinity Bioreagents, USA), mouse Ser/Thr phosphorylation clone 22a (612549 BD bioscience, Clontech, USA), K18 phosphorylation specific antibodies, K18-pS33 (clone IB4) and Anti K18-pS52 (clone 3055), were a kind gift from Prof. Bishr Omary, Michigan Medical School, USA. Anti-GAPDH clone ABM22C5 (10-10011, Abgenex, India), Rabbit HRPO (sc-2004 Santa Cruz biotechnology, USA), mouse Alexa Fluor 568 (A11004 Molecular Probes, Invitrogen, USA) were also employed in the study.

3.2 Methods:

3.2.1 Cell culture:

HHL17 cell line was routinely cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 0.03% glutamine, 10 units/ml penicillin G-sodium, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B, and 10% FBS at 37°C and 5% CO_2.

3.2.2 Cell revival:

Cell lines which were preserved in FBS, containing 10% DMSO in liquid nitrogen, were revived by snap thawing at 37ºC, and resuspended in double volume of complete DMEM (DMEM+10% FBS). Further the cell pellet was obtained by centrifuging at 1200 rpm at room temperature (RT) for 5 minutes. Supernatant was discarded and cell pellet was gently resuspended in fresh complete DMEM medium. The suspension was dispensed in tissue culture dishes and cultured in humidified 5% CO2 incubator at 37ºC.

3.2.3 Sub-culture or passaging and preservation:

Subculture of cells was carried out by double washes with autoclaved, sterile PBS, pH 7.4 to remove serum. Adherent cells were incubated with trypsin solution (TPEG: 0.25% Trypsin, 0.02% EDTA, 0.05% Glucose in autoclaved sterile PBS, pH 7.4 filtered in 0.1 μ filter) for 60 seconds to break the contacts of these cells with surfaces of culture plates. After this, double the volume of complete DMEM was added to inhibit enzymatic action of trypsin. The cells were flushed, collected and centrifuged at 1200 rpm at RT for 10 minutes. The supernatant was discarded and cell pellet was gently resuspended in complete DMEM. The suspension was dispensed in tissue culture dishes and cultured at 37ºC, 5% CO_2 in the humidified incubator. Alternatively, these cells were used for experimental purpose or the cell pellet was resuspended in FBS containing 10% DMSO (cell density was adjusted approximately to 2.5×10^6/ml) and subjected for gradual cooling and finally stored or preserved in liquid nitrogen.

3.2.4 Treatment of cells with inhibitor drugs:

3.2.4.1 Okadaic acid treatment:

Cells in culture were treated with Okadaic acid at concentration of 400 nM/ml to inhibit type 1 and type 2A protein phosphatase which leads to enrichment of cellular phosphorylation.

3.2.4.2 Cycloheximide treatment:

Cells in culture were treated with Cycloheximide at concentration of 200 µM/ml for 24 H to inhibit protein synthesis and half-life of proteins was assessed.

3.2.4.3 PUGNAc treatment:

Cells in culture were treated with PUGNAc to increase O-GlcNAcylation on cellular proteins at concentration of 100 µM/ml for various time points.

3.2.5 Preparation of total cell lysates and differential extraction of soluble and insoluble keratin fractions:

HHL17 cells were harvested and washed with chilled PBS. To obtain total cell lysates (TCL), the cells were solubilised in lysis buffer containing 2% SDS (62.5 mM Tris and 2% SDS, pH 6.8), followed by three rounds of sonication on ice to shear the genomic DNA. The suspension was then heated at 100ºC for 5 minutes to denature the proteins and centrifuged at 16,000 g for 45 minutes. The supernatant was collected as total cell lysate and was stored at -20ºC until further use.

Differential extraction of soluble and filamentous keratins was performed as described in (17). In brief, soluble keratins were extracted by resuspending cells in Phosphate Buffered Saline containing 1% Nonidet P40, 5 mM EDTA, protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 10 µM pepstatin, and 25 µg/ml aprotinin) and phosphatase inhibitor cocktail. The suspension was incubated for 20 minutes at 4ºC followed by centrifugation (16,000×g for 1 hr.). The supernatant was collected as the soluble fraction and the resulting insoluble pellet was solubilized in 2% SDS cell lysis buffer (insoluble fraction).

3.2.6 Protein estimation using modified Peterson Lowry method:

Total protein contents were measured by modified Peterson's Lowry method. (275). Standard curve was obtained ranging between 5-30 µg by using 1 mg/ml BSA stock solution. In 1 ml

distilled water, appropriate volume of protein samples or BSA standard was added. Further, 1 ml CTC reagent [equal volumes of Solution A (0.1% Copper Sulphate, 0.2% Sodium Potassium Tartrate and 10% Sodium Carbonate), 10% SDS, 0.8 N NaOH, and distilled water] was added and incubated for 10 minutes. 0.5 ml of six times diluted Folin and Ciocalteau's reagent was then added to each tube and mixed by vortexing. After incubation for 30 minutes in dark, absorbance was measured at 750 nm using a UV Spectrophotometer.

3.2.7 Immunoprecipitation using K18 antibody:

For immunoprecipitation of K18,

- HHL17 cells were lysed in phosphate buffer (PBS (150 mM NaCl and 10 mM phosphate (pH 7.4)) containing 2% Empigen, phosphatase, and protease inhibitor mixture, followed by three rounds of sonication on ice.
- Pre-cleaning of cell lysates was carried out by incubating the protein-G sepharose beads (40 µl of 50% slurry in PBS pH 7.4) for 1 H at room temperature with gentle rocking.
- The suspension was centrifuged at 1500 g for 5 minutes at 4ºC and the precleared supernatant was used for the immunoprecipitation.
- Then 17.4 µg of K18 antibody was added in to 280 µg lysate and incubated for 1 H at room temperature with gentle rocking.
- Subsequently, the immune-complex was recovered by adding Protein-G sepharose beads (60 µl of 50% slurry in PBS pH 7.4) and incubating over night at 4ºC with gentle rocking.
- The immune complex was washed thrice with RIPA buffer (20mM HEPES pH 7.0, 0.4% NP40, 150mM NaCl, 5 mM EDTA) for 5 minutes at room temperature with gentle rocking followed by centrifugation at 1500 g for 5 minutes at 4ºC.

- Following this, the immune complex was eluted by adding 100 µl of lysis buffer (62.5 mM Tris and 2% SDS, pH 6.8) and incubated at 100ºc for 5 minutes. The suspension was centrifuged at 2000 g for 10 minutes at room temperature and the immunoprecipitated protein containing supernatant was collected and stored at -20ºC until further analysis.
- The amount of K18 in the Immunoprecipitate of samples to be compared was normalized so that the differences in the modifications could be easily evaluated.

3.2.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS-PAGE was performed to resolve the proteins based on their molecular size. 30% Acrylamide (29.2% acrylamide and 0.8% N,N'-methylene-bisacrylamide) was mixed with 1 M Tris base (pH 8.8) and 20% SDS, in order to obtain 30 ml solution of the desired resolving gel concentration (12%) containing 0.37 M Tris-Base and 0.1% SDS. One fifty micro litres ammonium persulphate (20% w/v) and 15 µl TEMED (Tetraethylmethyl ethylene diamine) 10% (v/v) were added and the solution was poured between sealed glass plates with 1.5 mm spacers. Distilled water 1 ml was carefully overlaid over the gel solution for efficient polymerization and preventing drying of the gel post polymerization. The set up was allowed to stand for 30 minutes at room temperature to allow the solution to polymerize in to gel. Post polymerization the distilled water was carefully drained .A stacking gel (4.5% Acrylamide, 0.0625 M Tris pH 6.8, 0.2% SDS), was overlaid on the polymerized resolving gel with 1.5 mm comb inserted between the plates. The set up was allowed to stand at room temperature for 30 minutes to allow polymerization of stacking gel. Post polymerization the comb was carefully removed. The protein samples were boiled for 5 minutes in sample buffer (0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol (w/v), 750 mM β-mercaptoethanol and 0.05% Bromo Phenol blue) and loaded into wells. Electrophoresis was carried out using

electrode buffer (0.025 M Tris.chloride, 0.2% SDS (w/v) and 0.192 M glycine) at 100 Volts (constant voltage).

3.2.9 Western Blotting:

Transfer of proteins from the gel to a PVDF membrane was performed as follows (276). Resolving gel was equilibrated in chilled transfer buffer (0.025 M Tris, 0.192 M Glycine, and 20% Methanol) for 15 minutes. PVDF membrane was pretreated with methanol for activation, super-imposed on the gel and the transfer apparatus was set as per manufacturer's instructions. Transfer of proteins to the membrane was carried out with chilled transfer buffer using a constant voltage of 90 Volts for 3 hours.

3.2.10 Ponceau–S staining:

Extent of protein transferred to the membrane was checked by soaking it in 0.2% (v/v) Ponceau–S stain for 2 minutes. The stain was later washed off with Tris Buffered Solution (TBS-20 mM Tris and 500 mM NaCl).

3.2.11 Probing of PVDF membrane with specific antibodies:

PVDF membranes blotted with proteins were blocked with either 3% BSA or 5% skimmed milk and probed with respective primary antibodies followed by secondary HRP conjugated antibody. Table 4 shows concentration of primary antibodies and their respective secondary antibody. The blots were developed using Enhanced Chemiluminescent (ECL plus) reagent.

Antibody	Blocking (1 H prepared in T-TBS)	Primary antibody in (1 H) prepared in T-TBS	Secondary antibody prepared in T-TBS (1 H)
Keratin 8	3%BSA	1.8 µg/ml (1:5000) in 1% BSA	1:2000 anti-Mouse HRP in 1% BSA
Keratin 18	3%BSA	1.8 µg/ml (1:55,000) in 1% BSA	1:10,000 anti-Mouse HRP in 1% BSA
O-GlcNAcylation	3%BSA	1:2000 1% in BSA	1:2000 anti-Mouse HRP in 1% BSA
Phospho serine/threonine	3%BSA	1:1000 in 1% BSA	1:2000 anti-Mouse HRP in 1% BSA
Keratin 18 phospho serine 33	5% Skimmed milk	1:4000 2.5% in Skimmed milk	1:4000 anti-Mouse HRP in 2.5% Skimmed milk
Keratin 18 phospho serine 52	3%BSA	1:4000 in 1% BSA	1:4000 anti-Rabbit HRP in 1% BSA
GAPDH	3%BSA	1:4000 1% BSA	1:4000 anti-Mouse HRP in 1% BSA

Table 5. List of Antibodies

3.2.12 Coomassie staining:

To ensure equal loading probed PVDF membrane was stained with Coomassie Brilliant Blue R-250, as follows**:**

After immunoblotting the membrane was washed once with TBS for 15 minutes and stained with 0.2% Coomassie Brilliant Blue (CBB) by soaking the membrane in for 3 to 5 minutes. The Coomassie stained blot was then washed with de-staining solution (50% methanol and 10% acetic acid) to fully de-stain the excess stain. The de-staining is performed with multiple changes of the de-staining solution till the background is completely free of stain and the protein bands are clearly

visible as sharp bands with even staining. After the de-staining, the blot is air dried and scanned immediately. The regions of the blot consisting of multiple, evenly stained, sharp bands were considered for Densitometric analysis.

3.2.13 Purification of the glycosylated form of K8 and 18:

Proteins from total cell lysate were resolved on 10% PAGE along with standard molecular weight markers. After the electrophoresis the resolved gel was incubated in 4 M sodium acetate solution for 2 H at -20ºC. The proteins in the gel can be visualised as clear transparent bands against the white opaque gel, due to precipitation of free SDS in the gel. The protein bands in the region of K8 (52 kDa) and keratin 18 (48 kDa) were collected separately in a microfuge tube by excising the gel with reference to standard molecular weight markers. The gel pieces were crushed and 500 µl of extraction buffer (0.5% SDS, 150 mM NaCl, 62.5 mM Tris pH 6.8) was added and the tubes were vortexed vigorously for 45 minutes at room temperature. Following this, tubes were centrifuged for 5 minutes at 10,000 g and a supernatant was collected in a fresh tube. SDS from the purified samples was removed by adding 150 mg of activated SM-2 Biobeads and incubated at room temperature for 2 hr with gentle rocking. The sample was centrifuged at 2000 g for 5 min at room temperature and the supernatant containing pure keratin 8 or 18 was collected and stored at -20ºC. Further, O-GlcNAcylated forms of K8 and K18 were precipitated by WGA-Sepharose beads as described in (17). Supernatant containing pure K8 and K18 were precleared by incubating with Sepharose beads (100 µl of 50% slurry in PBS pH 7.4) for 1 H at room temperature with gentle rocking. The suspension was centrifuged at 1500 g for 5 minutes at 4ºc and the precleared supernatant was used for enriching WGA binding proteins. To the precleared lysate, WGA-Sepharose beads (150 µl of 50% slurry in PBS pH 7.4) was added and incubated overnight at 4ºC with gentle rocking. Following this, the suspension was centrifuged at 2000 g for 5 minutes at 4ºC.

The supernatant was collected as unbound fraction. The WGA beads containing the bound proteins were washed thrice with RIPA buffer. (20 mM HEPES pH 7.0, 0.4% NP40, 150 mM NaCl, 5 mM EDTA) for 5 minutes each at room temperature with gentle rocking followed by centrifugation at 1500 g for 5 minutes at 4ºC. Following this, the WGA bound proteins were eluted by adding 100 µl of lysis buffer (62.5 mM Tris and 2% SDS, ph 6.8) and incubating at 100ºC for 5 minutes. The suspension was centrifuged at 2000 g for 10 minutes at room temperature and the WGA bound proteins containing supernatant was collected and stored at -20ºC until further analysis. The glycosylated forms of K8 and 18 were further immuno-precipitated using K8 and K18 specific monoclonal antibodies as described in immunoprecipitation method.

3.2.14 Methods in molecular biology:

3.2.14.1 Plasmids, site directed mutagenesis and cloning:

Human K18 WT EYFP and K18 *O*-GlcNAcylation triple mutant (TM) (S29A/S30A/S48A) EYFP, cloned in pEYFPN1 plasmids were used to generate site-specific (Ser-to-Ala) single and double *O*-GlcNAc mutants respectively of K18 using the QuikChange II site-directed mutagenesis kit according to the protocol of the manufacturer. The method involved high fidelity PCR based amplification of EYFP plasmid vector containing YFP tagged K18-WT cDNA using sense and antisense complementary primers carrying the desired mutation (Table 5). The PCR product was used to transform competent E.coli cells after digesting the methylated K18 WT parent template plasmid with DpnI restriction enzyme. The bacterial colony harbouring the plasmid containing the mutated K18 cDNA was grown in 10 ml LB medium overnight at 37ºC with shaking followed by extraction of the plasmid DNA. The plasmid DNA was resolved on 0.8% agarose gel and purified using Qiagen's QiaQuick gel extraction kit as per manufacture's instruction. The K18 gene was sequenced completely using forward and revers primers which anneal in the region of CMV

promoter and YFP sequence respectively. The sequence was aligned with K18-WT cDNA sequence to confirm mutation at specific sites.

Conditions for primer design were:

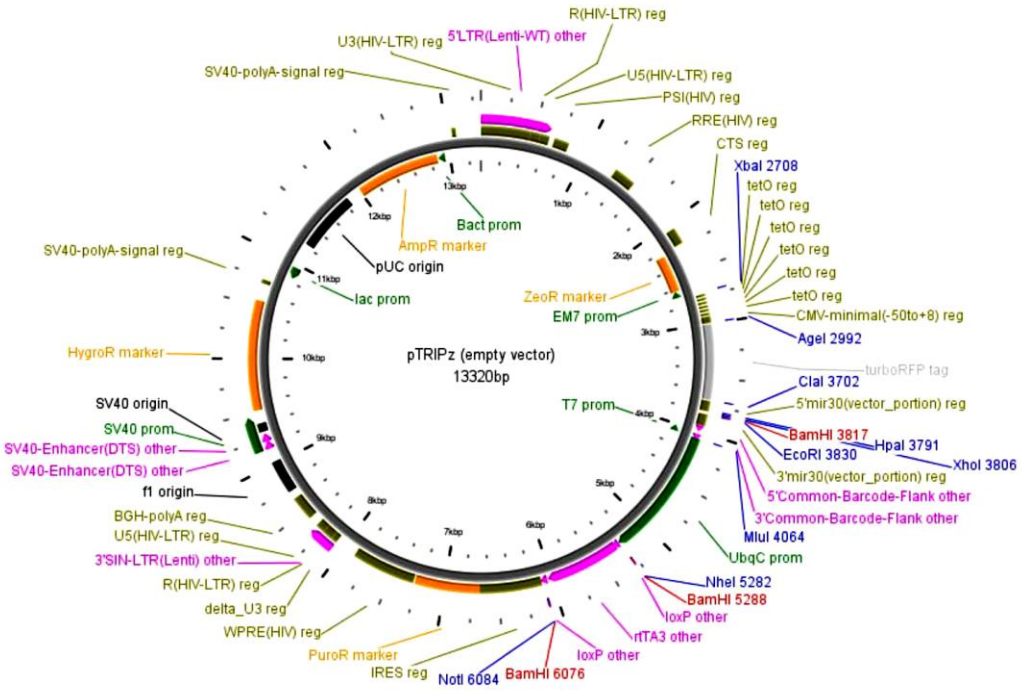
1. Both sense and antisense mutagenic primers contained the desired mutation and were complementary.
2. Primers were between 25 and 45 bases in length, with melting temperature (tm) of ≥78ºC.
3. The desired mutation (deletion/insertion) was in the middle of the primer with ~10-15 bases of correct sequence on both sides.
4. The primers optimally had a minimum GC content of 50% and terminated in one or more C or G bases.

Sr. No.	Mutation	Template	Primer	Sequence (5'-3')	PCR Product
1	S29A	K18 WT	Forward	GGCCGGTC**GC**CAGCGCGGCCAGCG	S29A
			Reverse	CGCTGGCCGCGCTG**GC**GACCGGCC	
2	S30A	K18 WT	Forward	CCGGCCGGTCAGC**GC**CGCGGCC	S30A
			Reverse	GGCCGCG**GC**GCTGACCGGCCGG	
3	S48A	K18 WT	Forward	CGGATCTCCGTG**G**CCCGCTCCACCA	S48A
			Reverse	TGGTGGAGCGGG**C**CACGGAGATCCG	
4	A29S	K18 TM	Forward	CCGGCCGGTC**AG**CGCCGCGGCC	S30A/S48A
			Reverse	GGCCGCG**GC**GCTGACCGGCCGG	
5	A30S	K18 TM	Forward	GGCCGGTCGCC**AG**CGCGGCCAGCG	S29A/S48A
			Reverse	CGCTGGCCGCGCTG**GC**GACCGGCC	
6	A48S	K18 TM	Forward	CGGATCTCCGTG**T**CCCGCTCCACCA	S29A/S30A
			Reverse	TGGTGGAGCGGG**A**CACGGAGATCCG	
7	S33A	K18 WT	Forward	CAGCAGCGCGGCC**GC**CGTCTATGCA GGC	S33A
			Reverse	GCCTGCATAGACG**GC**GGCCGCGCTG CTG	
8	S52A	K18 WT	Forward	GTCCCGCTCCACC**GC**CTTCAGGGGCG GC	S52A
			Reverse	GCCGCCCCTGAAG**GC**GGTGGAGCGG GAC	
9	S33D	K18 WT	Forward	CAGCAGCGCGGCC**GA**CGTCTATGCA GGC	S33D
			Reverse	GCCTGCATAGACG**GA**GGCCGCGCTG CTG	
10	S33D	K18 S30A	Forward	CAGCAGCGCGGCC**GA**CGTCTATGCA GGC	S30A/S33D
			Reverse	GCCTGCATAGACG**GA**GGCCGCGCTG CTG	

Table 6. Primer sequences used for site directed mutagenesis: Site directed mutagenesis primers 5'-3' forward and reverse primer sequences, template used for PCR reaction and mutant product after reaction. Nucleotide sequences in bold red letters indicate point mutations. WT= Wild Type, TM= Triple mutant, S= Serine, A= Alanine, D= Aspartate.

3.2.14.2 Cloning of K18 WT and O-GlcNAcylation and phosphorylation site specific mutants in to lentivirus plasmid:

K18 WT, O-GlcNAcylation and phosphorylation site specific mutants were further cloned in to infectious lentivirus plasmid vector, pTRIPz as described elsewhere (41). Briefly, CMV-K18-YFP expression cassette was PCR amplified from pEYFPN1 using forward and reverse primers with XbaI and NotI sites respectively such that they are in coding frame in pTRIPz. The PCR amplicon and pTRIPz vector were sequentially restriction digested with XbaI and NotI enzymes in their respective buffers and purified using DNA gel extraction kit (Qiagen) as per manufacturers' protocol. The linearized vector and digested CMV-K18-YFP amplicon were mixed in 1:3= Vector: insert molar ratio and ligated using T4 DNA ligase enzyme (NEB) overnight at 22ºC.The ligated mixture was used for transformation of ultra-competent DH5α E.coli cells. Recombinant clones were confirmed by restriction digestion of either XbaI/NotI restriction enzymes digestion or by EcoRI/NotI restriction enzymes digestion (Fig. 3.1).



pTRIPz lentivirus vector map

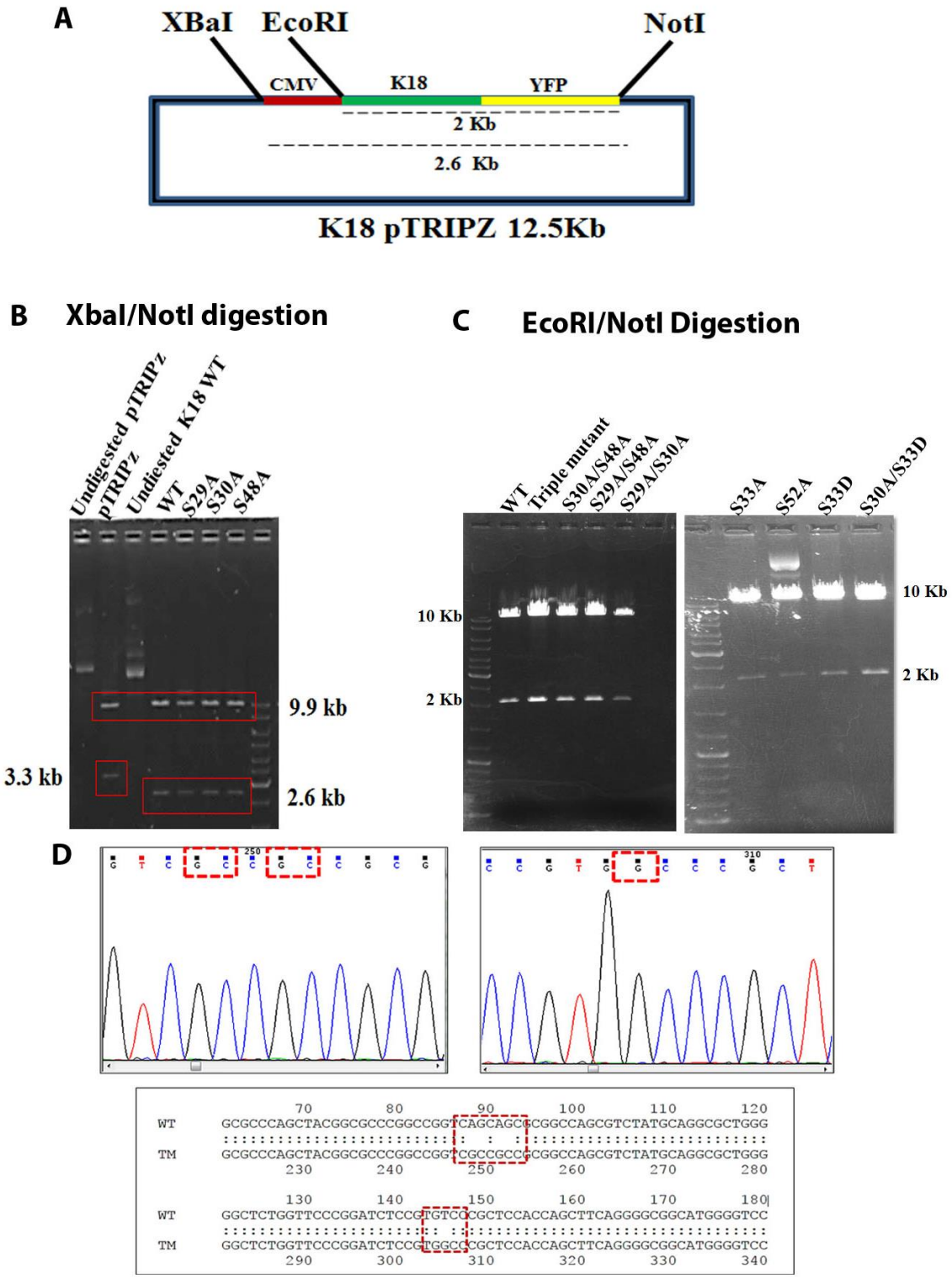


Figure 3.1. Cloning of CMV-K18-YFP expression cassette in to pTRIPz lentivirus vector. A Schematic representation of cloning of CMV-K18-YFP expression cassette in to pTRIPz lentivirus vector using XbaI and NotI restriction digestion strategy. B and C, Screening of recombinant

clones of O-GlcNAcylation and phosphorylation site specific mutants of K18 by using either, Xba1/Not1 (B) or EcoRI/NotI (C) restriction digestion. D, confirmation of desired point mutation in K18-cDNA by DNA sequencing method. Representation of K18 O-GlcNAc triple mutant (TM) point mutations mutations.

3.2.14.3 Preparation of ultra-competent *E. coli* DH5α cells:

Supper optimal broth (SOB) (300 ml) (Tryptone 6 g, Yeast extract 1.5 g, NaCl 0.15 g, KCl 5.6 g). The pH of the SOB medium was adjusted to pH 7 with 1 N NaOH, followed by the addition of 4 ml of 1 M glucose and 1 ml of 2 mM $MgCl_2$, just prior to inoculation of bacteria. Transformation Buffer (200 ml) (PIPES 0.6 g, $CaCl_2$ 0.4 g, KCl 3.7 g) was also prepared. The pH was adjusted to pH 6.7 with 10 N KOH and 2.18 g $MnCl_2$ and sterilized by filtering it through 0.22 μm filter.

Ultra-competent *E. coli* DH5α cells were prepared as per follows. A single colony of DH5α strain of *E. coli*, from an overnight grown LB agar plate was inoculated into 250 ml of SOB medium and incubated at 18°C with mild shaking till the OD at 600 nm reached about 0.3 to 0.5. The culture was incubated on ice for 10 min followed by centrifugation at 2500 g for 10 min at 4°C to pellet the bacterial culture. The culture supernatant was discarded and the bacterial cells pellet was gently resuspended in 80 ml of ice cold transformation buffer. The cell suspension was incubated on ice for 10 min followed by centrifugation at 2500 g for 10 min at 4°C. The supernatant was discarded and the bacterial cell pellet was resuspended in 20 ml of ice cold transformation buffer containing 7% DMSO. The suspension of cells was incubated on ice for 10 min and was subsequently aliquoted into nearly 200 μl aliquots in 1.5 ml micro centrifuge tubes, snap freezen into liquid nitrogen and immediately stored at -80°C until further use.

3.2.14.4 Transformation:

Ultra-competent cells were transformed with pTRIPz plasmid vector. After thawing of competent cells, ligated plasmid mixture was added in it and kept on ice for 30 minutes. The cells were placed

at 42 ºC (water bath) for exactly 90 seconds and subjected to cold shock on ice for 2-5 minutes. The cells were then mixed with 1 ml of sterile LB broth and incubated at 37ºC for 30 minutes in a shaker incubator. The cells were spun at 5000 rpm for 5 minutes, pellet was resuspended in 200 µl of LB broth and spread on to LB agar plate and incubated for 24 hours at 37ºC.

3.2.14.5 Plasmid extraction using alkaline lysis method (Mini-Prep):

Individual colony was picked up and inoculated into 5 ml LB broth containing 100 µg/ml ampicillin for plasmid extraction and allowed to grow overnight at 37°C. Plasmid isolation from the overnight grown culture was carried out by the alkaline lysis method. The overnight grown 1.5 ml bacterial cultures were centrifuged at 5000 rpm at 4°C for 5 min, the medium was removed and the pellets were dried. To the dried bacterial pellets, 100 µl of alkaline lysis solution I (GTE buffer: 50 mM Glucose, 25 mM Tris pH 8.0 and 10 mM EDTA) was added and vortexed till the pellets were completely dissolved. Then, 200 µl of alkaline lysis solution II (0.2 N NaOH and 1% SDS) was added, mixed gently by inverting and kept for 2 min at room temperature 150 µl of ice-cold alkaline lysis solution III (3 M potassium acetate pH 4.8 in glacial acetic acid) was then added to each tube and kept on ice for 10 min. The tubes were then centrifuged at 13000 rpm, 4°C for 15 min and the supernatants containing the renatured plasmids were transferred to fresh tubes. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to supernatant for removal of proteins and saccharides, mixed by vortexing and centrifuged as above for 5 min. The aqueous phase was transferred to a fresh microcentrifuge tube and double the volume of absolute alcohol was added and mixed well for precipitation of plasmid DNA. Tubes were kept on ice for 15 min and centrifuged at the above conditions for 20 min. The ethanol was removed and the pellets were washed with 70% chilled ethanol to remove salts, centrifuged as above for 5 min and all the traces of ethanol were removed. The pellets were completely dried at 37°C for 30 min. The dried pellets

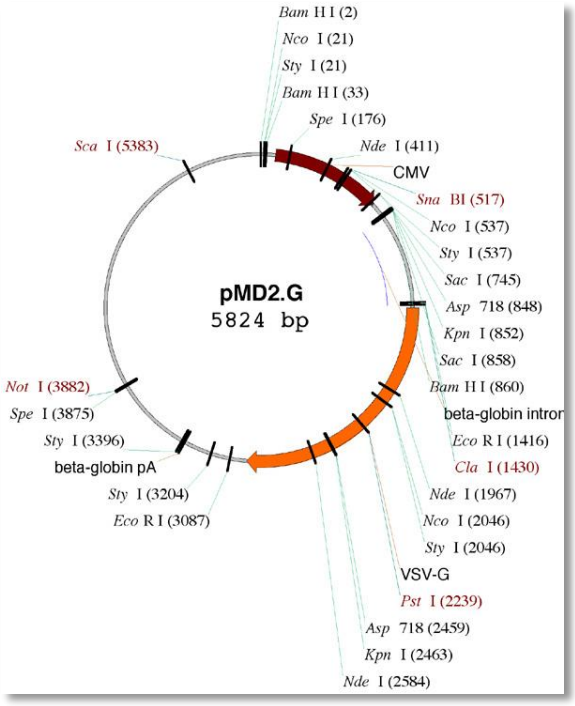
were reconstituted in 20 µl of autoclaved distilled water. 0.3 µl of RNase (1 µg/ml) was added to each tube and incubated at 37°C for 45 min to degrade RNA molecules.

3.2.14.6 Maxiprep for large scale plasmid DNA extraction:

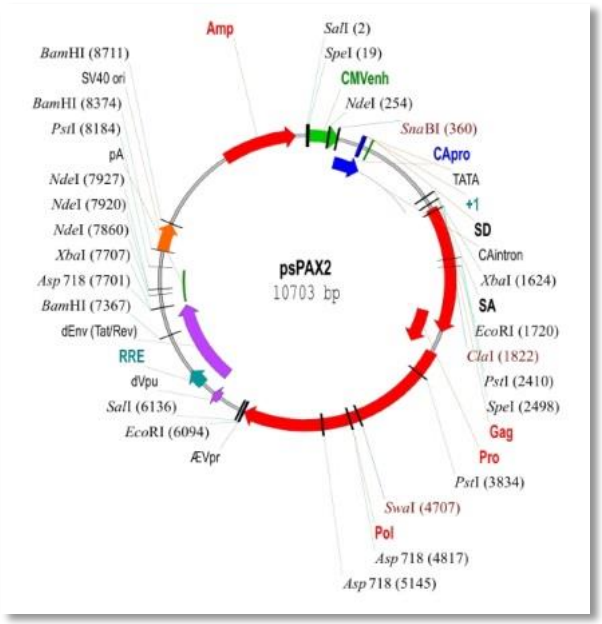
Maxiprep is different from that of Miniprep as the starting *E. coli* culture volume is 100-200 ml LB broth. Maxiprep was performed using kit based method from Sigma Aldrich. 200 ml of overnight grown culture was pelleted by centrifugation at 5000 g for 10 min. The pellet was resuspended in 12 ml resuspension solution and vortexed until the pellets were completely dissolved. Cells were lysed by adding 12 ml of lysis solution and mixed thoroughly by gently inverting the tubes 6-8 times. Then the tubes were kept at room temperature for 3 min. 12 ml of chilled neutralization solution was added to neutralize the lysed cells resulting in the formation of a white aggregate (cell debris, proteins, lipids, SDS and chromosomal DNA). Subsequently, 9 ml of binding solution was added to the pellet and poured into the barrel of the filter syringe and was incubated for 5 min at room temperature so that the white aggregate floated to the top. During incubation, the binding column was prepared by adding 12 ml of column preparation solution and was centrifuged at 5000 g for 5 min. By holding the filter syringe barrel over binding column, the plunger was gently inserted to expel the clear lysate into the column. The binding column was then spun in a swinging bucket rotor at 3000 g for 2 min and the eluent was discarded. The column was then washed with wash solution 1 and 2 and centrifuged at 5000 g for 2 min and 5 min respectively. The column was then transferred to a fresh 50 ml collection tube and 3 ml of elution solution was added and was kept at room temperature for 20 min and was then centrifuged at 5000 g for 5 min for the elution of DNA.

3.2.15 Generation of HHL17 stable cell line:

HEK293FT cells were cultured in DMEM complete medium containing G418 500 µg/ml in 90 mm culture plate to achieved 50 % confluency. Co-transfection of pTRIPz and packaging vectors i.e. psPAX2 and pMD2.G was performed, by calcium phosphate precipitation as described (Illustration 7). Desired amount of DNA (pTRIPz 12 µg, psPAX2 10 µg and pMD2.G 4 µg) was diluted to 260 µl of sterile distilled water in a sterile microcentrifuge tube. Equal volume of 0.5 M $CaCl_2$ was then added followed by addition of 520 µl of BES Buffered Saline (BBS). The mixture was incubated at room temperature for 30 minutes and then added to culture dishes dropwise with 50% confluent culture plate of HEK293FT, the plate was gently swirled and incubated for 16-24 hours. After the incubation, the medium was replaced with fresh complete DMEM. After 24 hours viral supernatant was collected in sterile 15 ml tube and centrifuged for 10 minutes at 2500 rpm to remove treces of HEK 293FT cells. Supernatant was collected and stored in -80ºC or used for transduction of HHL17 cells.



Vector map of pMD2.G plasmid



Vector map of psPAX2 plasmid

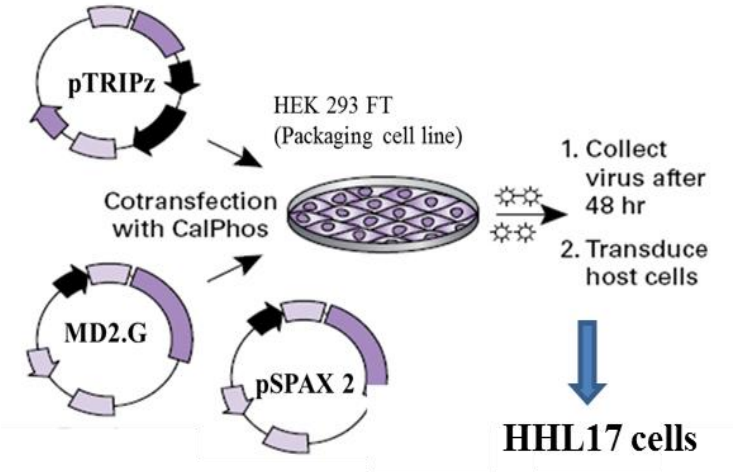


Illustration 7. Schematic representation of preparation of viral particles and transduction in HHL17 cells.

3.2.15.1 Lentivirus mediated transduction of HHL17 cell line:

The viral supernatant collected was added along with polybrene 8µg/ml (Polybrene is a polycation that neutralizes charge interactions to increase binding between the pseudoviral envelope and the cellular membrane) on 60% confluent HHL17 cells supplemented with complete DMEM. After

24 hours, the medium was changed. The HHL17 cells were grown in complete medium containing 0.8µg/ml puromycin for selection of cells.

3.2.16 Immunostaining and Imaging:

HHL17 cells stably expressing YFP-tagged K18-WT or O-GlcNAcylation or phosphorylation site specific mutants were fixed in chilled methanol (-20 °C for 5 min). Antibody staining for indirect immunofluorescence was carried out as follows. Cells were incubated with 0.4% Triton X-100 in PBS, pH 7.4, for 5 min followed by a 10-min incubation in PBS. The coverslips were overlaid with 5% BSA for 15 min, followed by incubation for 1 H with anti-K8 antibody (1: 300 diluted in 1% BSA containing PBS). The coverslips were subsequently incubated with PBS for 30 min with three changes. This was followed by incubation with anti-mouse Alexa Fluor 568 (1: 300 diluted in 1% BSA containing PBS) for 1 H with subsequent incubation with PBS for 30 min with three changes. Following this, the nucleus was stained with 25 µl of 5 µg/ml of 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) for 1 min, and the coverslips were incubated for 15 min in PBS with three changes. All the incubations were at room temperature. The coverslips were mounted using Vectashield mounting medium. Cells were examined under Zeiss LSM510 META confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Images were obtained using a 63X PlanApochromat phase contrast objective (numerical aperture: 1.4) and processed using LSM510 imaging software, version 4.2.

3.2.17 Adhesion assay:

HHL17 cells stably expressing O-GlcNAcylation and phosphorylation site specific mutants were harvested, washed with plain medium (DMEM without serum) to remove serum. Adhesion assay was performed in 96 well plates. The wells were coated with fibronectin (representative of ECM component) overnight at 4°C, each at a concentration of 10 µg/ml in 0.1 ml plain DMEM. 30,000

HHL17 cells, suspended in 100 µl of plain DMEM were added to each well in triplicate and incubated at 37°C for 1 H in a CO_2 incubator. Wells were gently washed three times with 100 µl of PBS to remove the unbound cells. A plate seeded with identical number of cells from each of the cell types without PBS washes, served to estimate the number of cells seeded. Fibronectin adherent HHL17cells were estimated by MTT assay as follows. 20 µl of MTT reagent (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (5mg/ml prepared in sterile PBS pH 7.4 filtered with 0.1 µ filter) was added in each of the well and incubated overnight at 37ºC in CO2 incubator. Next day, purple precipitation was observed. 100 µl of detergent solution (10% SDS, 0.01 N HCl solution) was added in to each of the well and incubated for 2 hours in dark to dissolve purple precipitation Absorbance was recorded at 570 nm.

3.2.18 Cell spreading assay:

Cell spreading assay was performed as per protocol described elsewhere (42). HHL17 cells stably expressing O-GlcNAcylation and phosphorylation site specific mutants and WT were harvested, serum was recovered by three times washing with plain medium (DMEM without FBS) to remove serum and seeded at a density of 0.5×10^6 cells/ml in plain medium on the coverslips coated overnight with 10 µg/ml fibronectin in serum free DMEM at 4°C. After 45 minutes of incubation in 5% CO_2 incubator at 37ºC. Unbound cells were removed by three time washes with warm PBS. Bound cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 5 min and stained with 2 µg/ml Phalloidin TRITC staining solution (containing 1µg/ml of Lysolecithin, 10% Methanol, 0.5% BSA in PBS) for 15 min at 37°C. Nucleus was stained with 5 µg/ml of 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 1-2 min. The stained cells were mounted using Vectashied (Vector lab) and images were acquired using a Carl Zeiss Laser confocal Microscope at 63x magnification.

3.2.19 Wound healing assay:

For wound healing assays, 6-well culture dishes were coated overnight with 10 µg/ml fibronectin in serum-free DMEM at 4 °C. HHL17 cells stably expressing *O*-GlcNAcylation and phosphorylation site-specific mutants were seeded at a density of 1 million cells/ml of complete medium and incubated at 37 °C for 24 H in a CO2 incubator. The cells were serum-starved for 24 H to inhibit cell proliferation. A straight, uniform wound (~400 µm in width) was made using a micropipette tip on the monolayer, and the cells were maintained in serum-free DMEM. The wound closure of cells in response to the immobilized fibronectin was measured for 25 H by time-lapse imaging of at least three different positions across the length of the wound using a Carl Zeiss inverted microscope at 10X magnification.

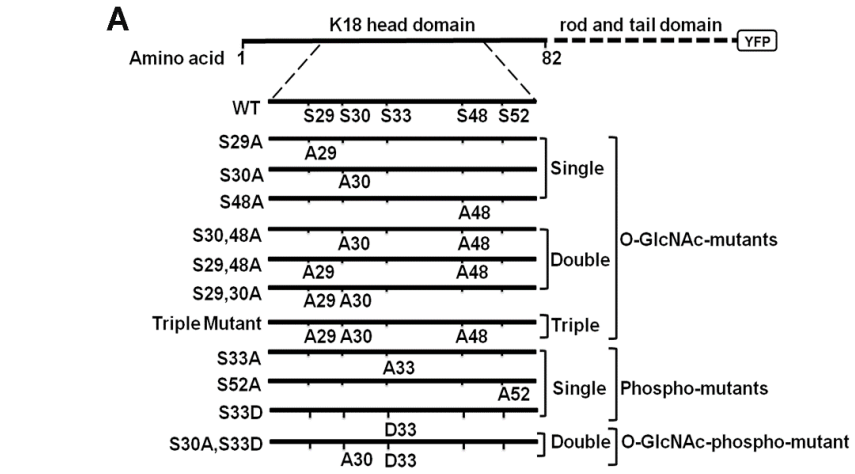
3.2.20 Densitometry quantitation and statistical Analysis:

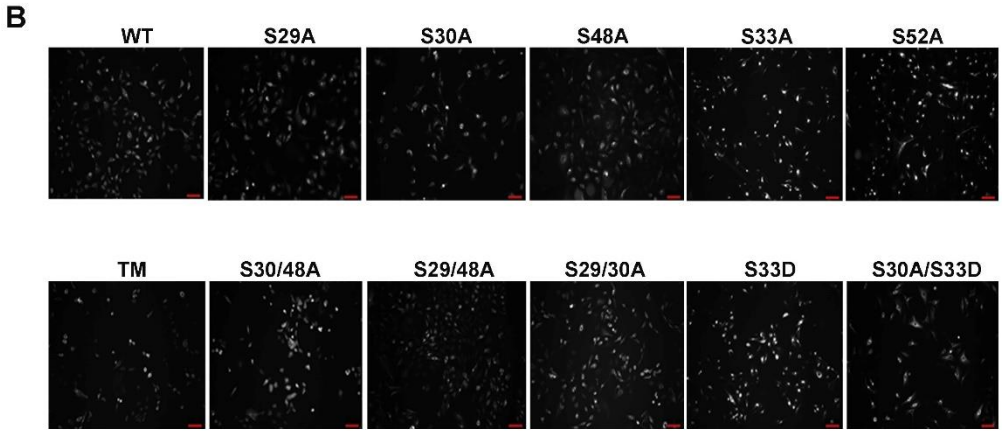
Densitometric quantitation of scanned images was performed by ImageJ 1.43 software (National Institutes of Health). Band intensities were normalized to respective loading controls. Statistical analysis was performed using GraphPad Prism 5 software. Significance was analysed by unpaired Student's *t* test for two samples and two-way analysis of variance for grouped data. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) values were considered significant. Photoshop (CS2; Adobe) was used for preparing figures.

Chapter 4: Results

4.1 Generation of stably expressing O-GlcNAcylation and phosphorylation site specific mutants of K18 HHL17 cell line:

In order to investigate which of the O-GlcNAcylation site of K18 regulates its solubility, stability, filament organization and phosphorylation, O-GlcNAcylation and phosphorylation site specific mutants were generated as depicted in Fig. 4.1, A. Further, we generated stable lines with HHL17 cells expressing O-GlcNAcylation and phosphorylation mutants of K18. The expression of the YFP-tagged K18 transgene in these stable lines was confirmed by immunofluorescence and immunoblotting, as shown in Fig. 4.1, B and C.


A
K18 head domain
rod and tail domain
YFP
Amino acid 1
82
WT
S29 S30 S33 S48 S52
S29A
A29
S30A
A30
S48A
A48
Single
S30,48A
A30 A48
S29,48A
A29 A48
S29,30A
A29 A30
Double
Triple Mutant
A29 A30 A48
Triple
O-GlcNAc-mutants
S33A
A33
S52A
A52
S33D
D33
Single
Phospho-mutants
S30A,S33D
A30 D33
Double
O-GlcNAc-phospho-mutant


B
WT
S29A
S30A
S48A
S33A
S52A
TM
S30/48A
S29/48A
S29/30A
S33D
S30A/S33D

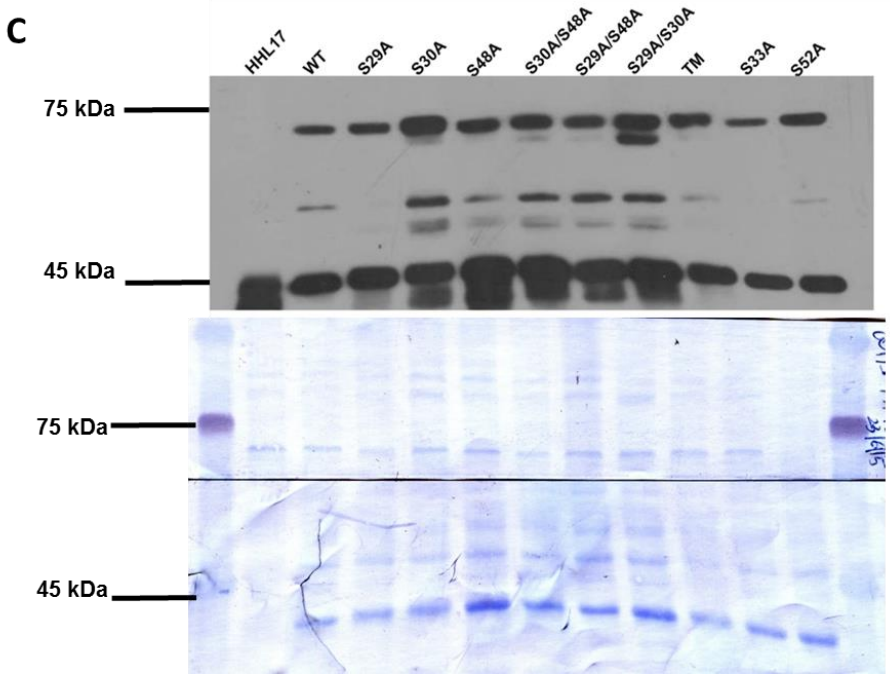

C
HHL17
WT
S29A
S30A
S48A
S30A/S48A
S29A/S48A
S29A/S30A
TM
S33A
S52A
75 kDa
45 kDa
75 kDa
45 kDa

Figure 4.1. Validation of HHL17 cells stably expressing K18-YFP O-GlcNAc and phosphorylation site specific mutants. A, Schematic outlining YFP-tagged K18, the WT, and various mutants of O-GlcNAcylation sites (gSer29, gSer30, and gSer48) and phosphorylation sites (Ser(P)33 and Ser(P)52) used to generate stable HHL17 lines. B, Fluorescence images of HHL17 cells stably expressing YFP tagged K18-WT, single (S29A, S30A and S48A), double (S30A/S48A, S29A/S48A and S29A/S30A), triple (S29, 30 and 48A) O-GlcNAc mutants and phospho mutants (S33A and S52A) of K18. Scale bars = 100 μm C, K18 immunoblots of total proteins from HHL17 cells stably expressing single, double, triple O-GlcNAc mutants and single phospho mutants. Endogenous K18 exhibits a molecular weight of 48 kDa whereas YFP tagged K18 mutants are at 72 kDa.

4.2 Objective :1

To investigate which site/s of O-GlcNAcylation on K18 is/are responsible for maintaining filament organization and stability.

4.2.1 Phosphorylation-induced solubility of keratin 18 is dependent on its O-GlcNAcylation at serine 30:

O-GlcNAcylation and phosphorylation on keratin 18 are known to increase its solubility (17). During stress or physiological conditions such as mitosis, increased phosphorylation is a key event to solubilize filamentous keratins in to soluble precursors for new filament formation (5). Hence, we investigated whether perturbing keratin 18 O-GlcNAcylation alters its phosphorylation-dependent solubility. For this purpose, we treated stable cell lines with OA, a broad-spectrum phosphatase inhibitor that increases phosphorylation and thereby the solubility of keratin 8/18 filaments. OA treatment led to a notable increase in solubility of K18-WT, whereas the solubility of K18-TM (S29A/S30A/S48A) was unaltered (Fig. 4.2, A and B). Similarly, HHL17 cells, stably expressing K18-S29A and K18-S48A mutants exhibited an increase in solubility, whereas mutation of Ser30 prevented OA-induced solubility of K18 (K18-S30A) (Fig. 4.2, C and D), similar to the triple O-GlcNAc mutant of K18 (Fig. 4.2, A and B). This suggests that phosphorylation-induced solubility of K18 is dependent on its O-GlcNAcylation at Ser30.

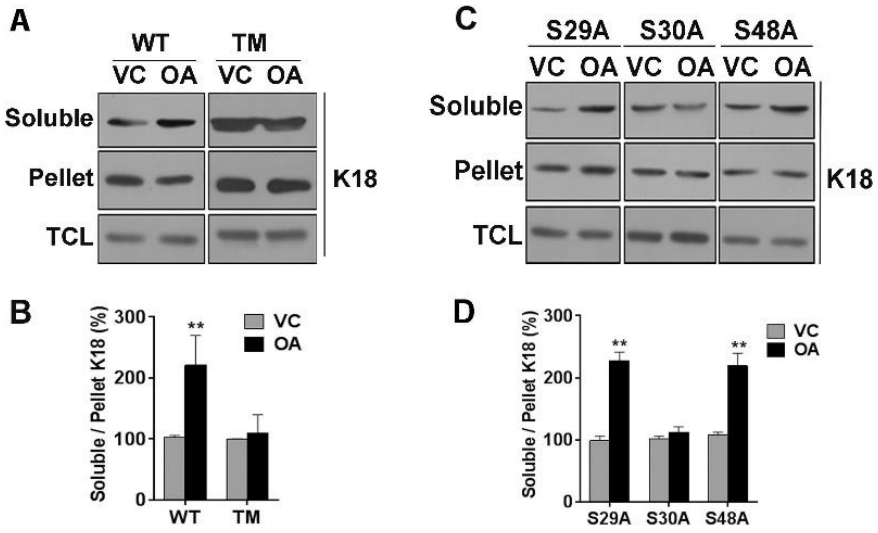


Figure 4.2. Phosphorylation-dependent solubility is impaired in Ser30 *O*-GlcNAc mutants of keratin 18. A and C, similar numbers of cultured cells were treated for 2 h either with DMSO vehicle control (VC) or OA to extract the soluble and pellet fraction or total cell lysate (TCL) in identical volumes. Three parts of the soluble fraction (45µl) and one part each of pellet and total cell lysate (15 µl) were immunoblotted for K18. A, Fractions of HHL17 cells expressing K18-WT and K18-TM and C, Fractions of HHL17 cells expressing single O-GlcNAc mutants of K18 (S29A, S30A, and S48A). B and D, Densitometric quantification (average of three independent experiments, including A and C) indicating the percent change in keratin 18 solubility, which is calculated as the ratio of K18 in soluble and pellet fractions. The ratios in *VC* were taken as 100%. **, $p<0.01$; unpaired Student's *t* test. Error bars represent mean ±S.E.

4.2.2 O-GlcNAcylation regulates turnover of K18:

O-GlcNAcylation is required for ubiquitination-dependent proteasome mediated degradation of K18 (17). Increased O-GlcNAcylation reduces stability of K18. Because disassembly and sequestration of keratin 18 subunits into a soluble pool could be a prerequisite for its degradation (43), we predicted that K18 O-GlcNAcylation at serine 30 could solubilise K18 and target it for degradation and reduce its stability. The stability of the WT and various K18 O-GlcNAcylation mutants was assessed after inhibiting protein synthesis with Cycloheximide. Compared with K18-

WT, which showed a significant reduction upon CHX treatment, the level of K18-TM remained unchanged (Fig. 4.3, A and B). It was interesting to note that, among all K18 O-GlcNAc mutants, those that harboured S30A (K18-S30A, S29A/S30A, S30A/S48A and TM) were highly stable with an unaltered protein level upon Cycloheximide treatment (Fig. 4.3, C–F).

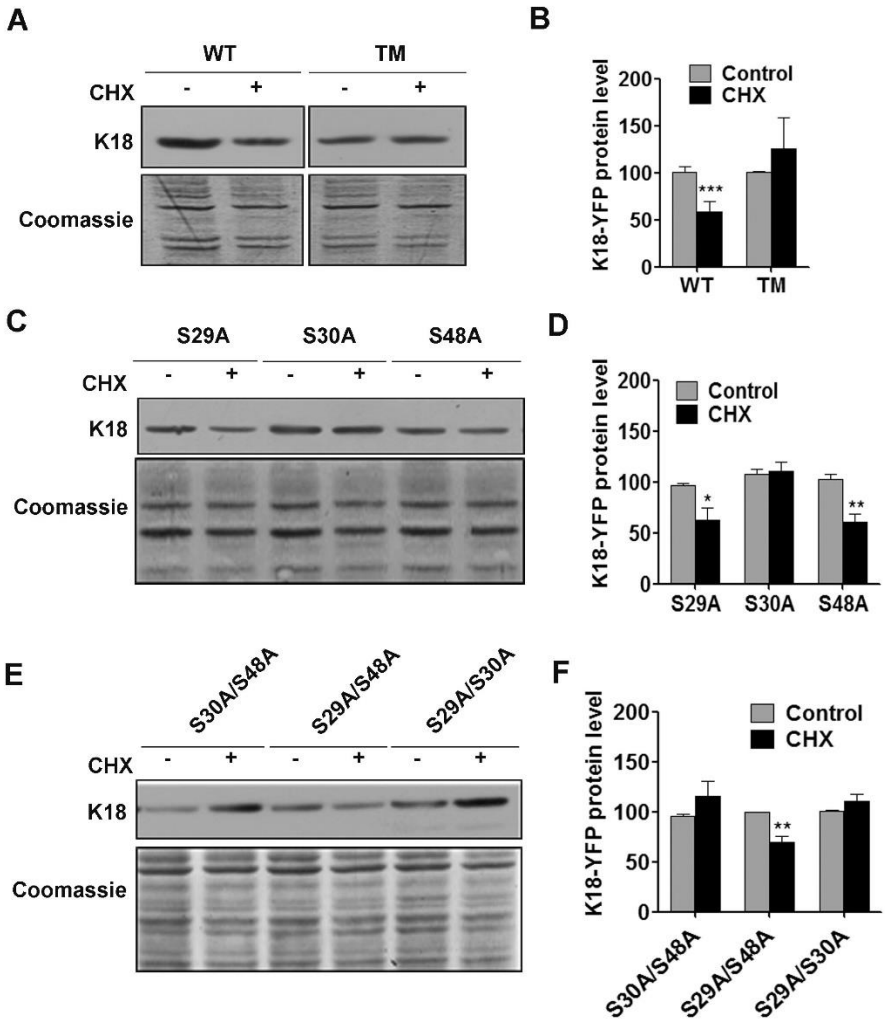


Figure 4.3. *O*-GlcNAcylation at Ser30 regulates the stability of K18. A, C, and E, K18 immunoblots of equally loaded total proteins from CHX-treated (200 µM for 24 h) or control HHL17 cells stably expressing K18-WT or TM (A) or single O-GlcNAc mutants (C) of K18 and double O-GlcNAc mutants of K18 (E). Coomassie staining of blots serves as a loading control. B, D, and F, Densitometric quantification (average of three independent experiments, including A, C,

and E) indicating changes in total keratin 18 levels in control and CHX-treated cells (***, $p < 0.001$ (*B*); *, $p < 0.05$ (*D*); **, $p < 0.01$ (*F*); unpaired Student's *t* test).

4.2.3 O-GlcNAcylation at S30 regulates glycosylation mediated turnover of K18:

Increase in O-GlcNAcylation of K18 resulted in its decreased stability by targeting it for ubiquitin proteasome mediated degradation. However, K18-TM, which lacks all sites of glycosylation, gets stabilised to PUGNAc-mediated degradation. This indicates that O-GlcNAcylation of K18 is prerequisite for its degradation (17). We treated K18 WT and TM with PUGNAc, a potent O-GlcNAcase inhibitor. PUGNAc treatment is known to cause increased O-GlcNAcylation and subsequent degradation of K8/18 (17). PUGNAc treatment at different time points (0, 24 and 48 hrs.) to K18-WT showed K18 degradation at 48 hrs. However, K18-TM exhibited resistance for degradation (Fig. 4.4, A and B). To test whether increasing O-GlcNAcylation at Ser30 could reduce the stability of K18, K18-WT and various O-GlcNAc mutant-expressing cells were treated with PUGNAc. Both K18 mutants with the S30A mutation (K18-TM and K18-S30A) exhibited no reduction upon PUGNAc treatment, whereas K18 with Ser30 (K18-WT, K18-S29A, and K18-S48A) showed a marked reduction in protein levels (Fig. 4.4, C–F). These results conclusively indicate that O-GlcNAcylation specifically at Ser30 determines the stability of K18.

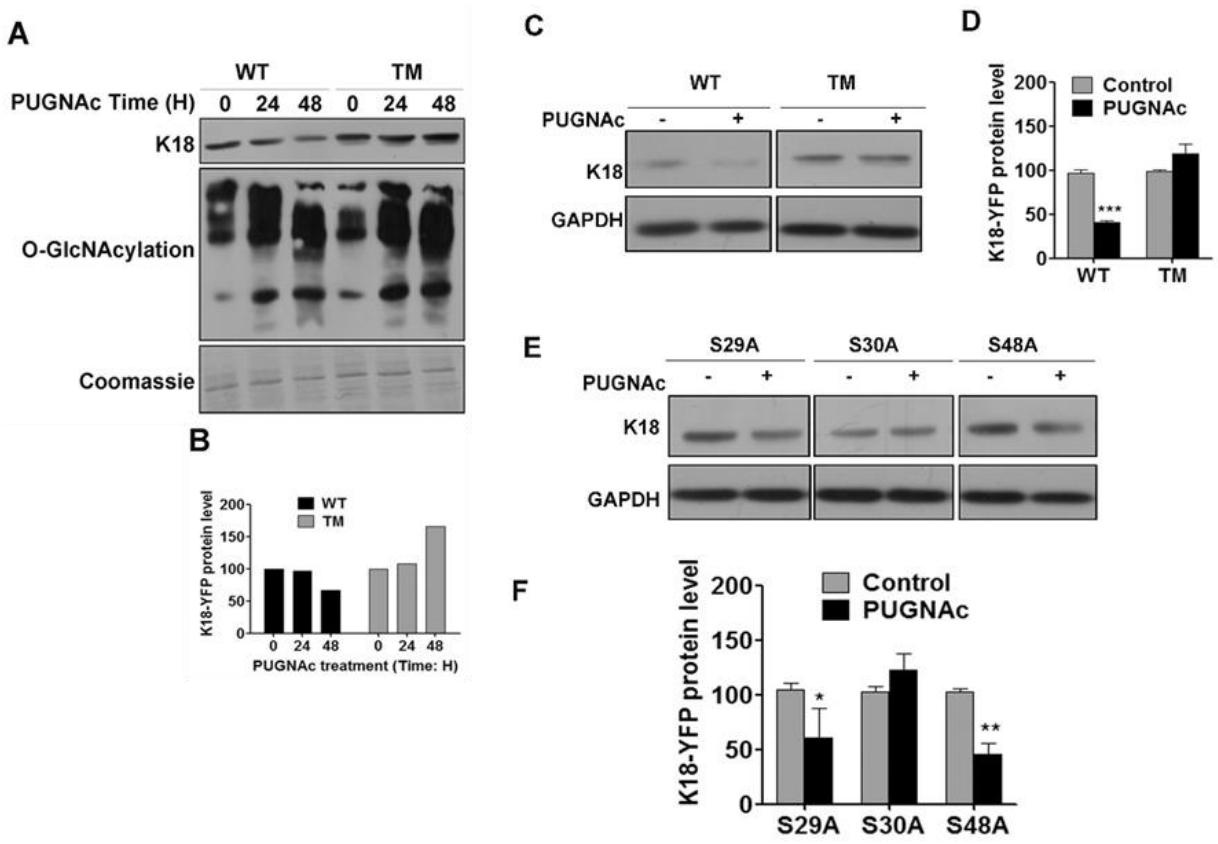


Figure 4.4 *O*-GlcNAcylation of K18 at Ser30 is required for its degradation.

A, Equal total proteins from untreated and PUGNAc treated HHL17 cells stably expressing K18-WT and K18-TM immunoblotted with K18 and O-GlcNAc. Coomassie stained blot served as loading control. B, Densitometric analysis of K18 levels in A. C and E. K18 immunoblot of equal total proteins from PUGNAc-treated (100 µM for 48 h) or control HHL17 cells expressing K18-WT or TM (C) and single *O*-GlcNAc mutants (E) of K18. The GAPDH immunoblot served as a loading control. D and F, densitometric quantification of C and E, indicating changes in total keratin 18 levels in control and PUGNAc-treated cells (average of three independent experiments, including C and E) (***, $p < 0.001$ (*H*); *, $p < 0.05$; **, $p < 0.01$ (*J*); unpaired Student's *t* test). Error bars represent mean ±S.E.

4.2.4 O-GlcNAcylation at serine 30 is essential for maintaining normal keratin 18 filament organization:

O-GlcNAcylation similar to phosphorylation, is major determinant of keratin filament organization. O-GlcNAcylation of K18 is required for normal filament organization (17). Therefore, we investigated whether, similar to phosphorylation, can O-GlcNAcylation also regulate keratin filament organization in site specific manner. Intensity quantification of K18-WT filaments across the cell reveal that filament density is highest around the nucleus and gradually decreases toward the cell periphery (Fig. 4.5, A and B) (19). This organization was severely affected in K18-TM, which displayed increased filament accumulation around the nucleus with collapse of peripheral filaments (Fig. 4.5 C and D). In order to investigate O-GlcNAcylation site which regulates K18 filament organization, we investigated K18-YFP filament intensity in O-GlcNAcylation site specific single and double mutants. Interestingly, similar to K18-TM, K18-S30A exhibited severely affected K18 filament organization, whereas the filament organization of the K18-S29A and K18-S48A mutants was similar to K18-WT (Fig.4. 6, A and B). Moreover, rescue of O-GlcNAcylation at Ser30 in K18-S29A/ S48A could restore the abnormal filament organization of K18-TM (Fig. 4.6, C and D), implying a key role for gSer30 in supporting the filament organization of K18.

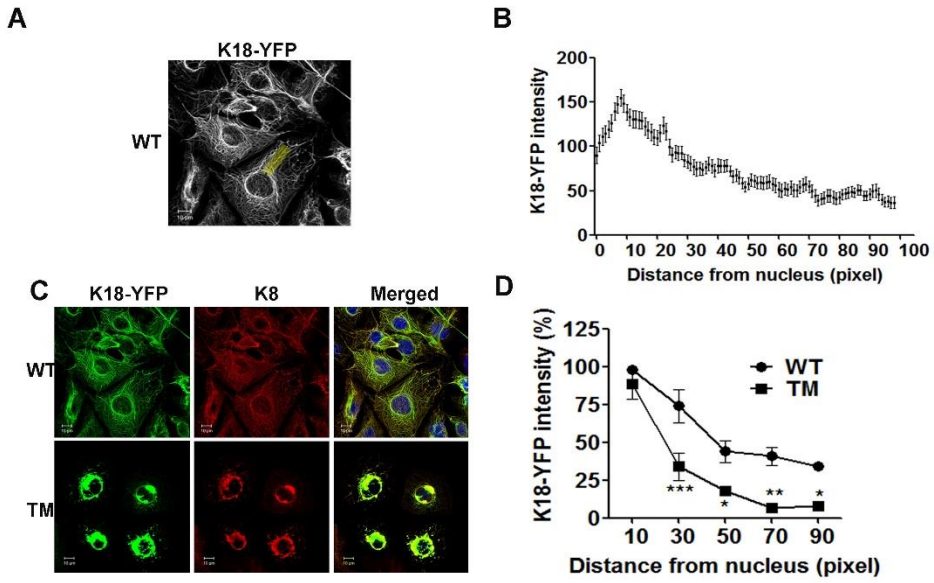


Figure 4.5. O-GlcNAcylation of K18 is essential for maintaining normal filament organization. *A*, immunofluorescence image of cells expressing WT-K18, demonstrating the method adopted to quantitate K18-YFP intensity from the perinuclear region to the cell periphery. Fluorescence intensity across a straight line of uniform length and width (100 and 30 pixels, respectively) was measured at three different regions per cell. *B*, fluorescence intensity of the keratin network across a cell (perinuclear region to cell periphery). Each point represents mean ±S.E. of three independent experiments with 20 cells/ experiment. *C*, immunofluorescence images of cells expressing K18-WT and K18-TM. Images show K18-YFP (green), K8 (red), and merged images with DAPI (blue). Scale bars=10 µm. D, quantification of fluorescence intensity of the K18-YFP filament network of cells in *C* (average of three independent experiments; 20 cells/experiment; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way analysis of variance followed by Bonferroni post-tests). Error bars represent mean ±S.E.

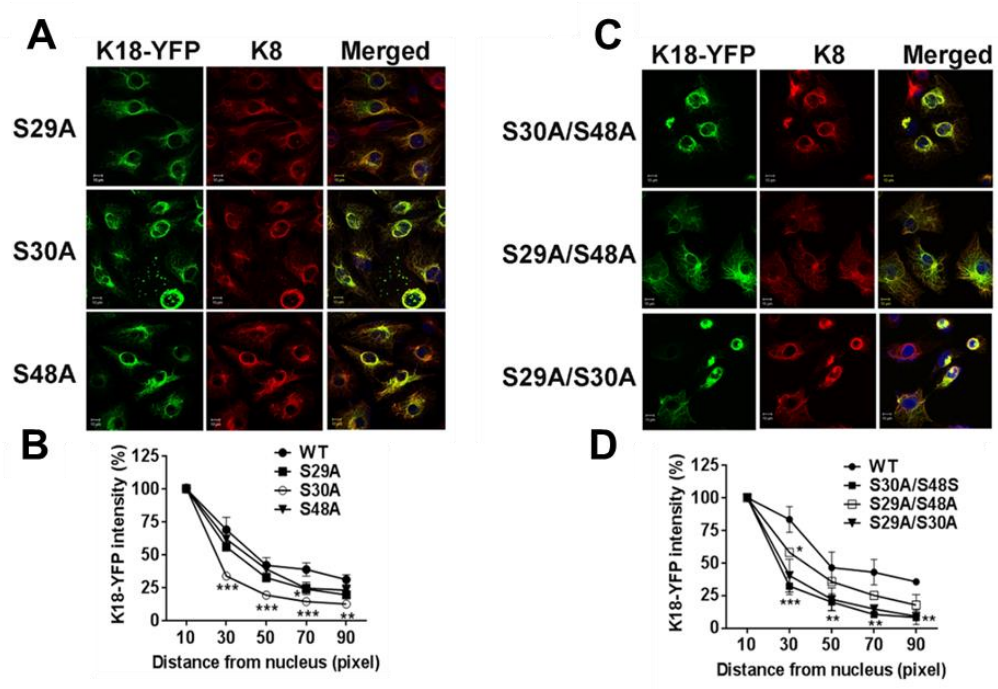


Figure 4.6 *O*-GlcNAcylation of K18 at Ser30 is essential for maintaining normal filament organization. A and C, immunofluorescence images of cells expressing O-GlcNAc site specific (A), single mutants (*C*), double mutants of K18. Images show K18-YFP (*green*), K8 (*red*), and merged images with DAPI (*blue*). *Scale bars*=10 µm. *B* and D, quantification of fluorescence intensity of the K18-YFP filament network of cells in A and C, (average of three independent experiments; 20 cells/experiment; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way analysis of variance followed by Bonferroni post-tests). *Error bars* represent mean ±S.E.

4.3 Objective :2

To investigate whether O-GlcNAcylation regulates phosphorylation on K18, and if yes then which site/(s) of glycosylation regulate phosphorylation.

4.3.1 O-GlcNAcylation and phosphorylation exhibit both a cooperative and antagonistic relationship on K8 and K18:

O-GlcNAcylation and phosphorylation are dynamic modifications and often regulate each other in a reciprocal manner. In case of keratin 18, both the modifications correlate positively with its solubility (17). However, there is no such evidence that these two modifications of K18 regulate each other. To address whether these two modifications on keratin 18 exhibit cross-talk or act independently to regulate K18 properties, we first investigated whether these two modifications coexist on K18. To achieve this, keratin 8 and keratin 18 were separated, purified, and renatured from preparative SDS-PAGE gels (as described under "methods"). The purified K8 and K18 fractions were clean, with no detectable cross-contamination of keratin 8 and 18 isoforms (Fig. 7A). O-GlcNAcylated proteins from these fractions were purified on WGA beads, followed by immunoprecipitation to enrich O-GlcNAcylated K8 and K18. Purified O-GlcNAcylated K8 and K18 showed notable levels of phosphorylation (Fig. 4.7B), suggesting co-existence of these two modifications on K8 and K18. We further aimed to investigate the relationship/cross-talk between these two modifications on K18. There are two phosphorylation sites (S33 and S52) adjacent to three O-GlcNAcylation sites (S29, S30 and S48) on K18. Levels of site-specific phosphorylation (Ser(P)33 and Ser(P)52) on various O-GlcNAc mutants of K18 were assessed. K18-TM showed a significant reduction in basal levels of Ser(P)33, whereas the levels of Ser(P)52 were notably higher compared with K18-WT (Fig. 4.7, C–E), suggesting that O-GlcNAcylation on K18 exhibits a cooperative relationship with Ser(P)33 and a reciprocal relationship with Ser(P)52. (The site-

specific K18-Ser (P)33 and K18-Ser(P)52 phospho-antibodies have been reported previously (5, 40). The specificity of K18-Ser33 phosphoantibody was confirmed using cells expressing phosphorylation mutants of K18 (K18-S33A and S52A) (Fig. 4.7, F).

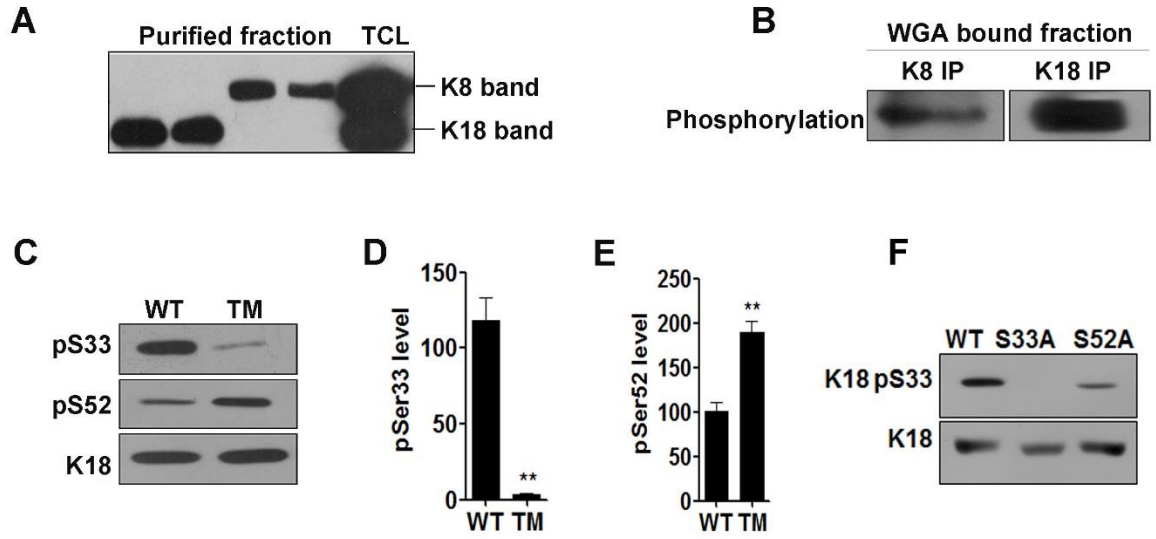


Figure 4.7. *O*-GlcNAcylation of K18 regulates phosphorylation both positive (pS33) and reciprocal (pS52) manner. Immunoblots of K8, K18, *O*-phosphorylation, Ser(P)33 (IB4), and Ser(P)52 (3055) are shown as indicated. *A*, K8 and K18 immunoblot of purified keratin 8 and 18 fractions (as described under "methods") to ensure the purity of the preparation. Total cell lysate (*TCL*) served as a marker to indicate the molecular weight of K8 and K18. *B*, K8 and K18 were immunoprecipitated from WGA-bound K8 or K18 fractions and immunoblotted with phospho-Ser/Thr. *C*, total cell lysates of HHL17 cells stably expressing K18-WT and TM were immunoblotted with K18Ser(P)33 (IB4) or K18Ser(P)52 (3055) antibodies respectively. *D*, densitometric quantification (average of three independent experiments, including *C*, showing levels of K18 Ser(P)33 and K18 Ser(P)52 (**, $p < 0.01$, unpaired Student's *t* test (D). *Error bars* represent mean ±S.E.

4.3.2 O-GlcNAcylation at S48 regulates phosphorylation at S52 in reciprocal manner while O-GlcNAcylation at S30 regulates phosphorylation at S33 in positive manner:

O-GlcNAcylation on K18 exhibits a cooperative relationship with Ser(P)33 and a reciprocal relationship with Ser(P)52. Hence, we investigated which site of O-GlcNAcylation is responsible

for regulation of each of the phosphorylation site of K18. The existence of an inverse relationship between gSer48 and Ser(P)52 on K18 has been shown earlier in in vitro studies (44). Investigation of Ser(p)52 in O-GlcNAcylation single, double and triple mutants of K18 suggested that, O-GlcNAcylation at S48 indeed regulates phosphorylation at S52 of K18 (Fig. 4.8 A-D). To investigate the O-GlcNAc site/s on K18 that aid in phosphorylation of Ser33, we assessed the basal levels of Ser(P)33 in various single and double O-GlcNAc mutants of K18. Interestingly, all O-GlcNAc mutants of K18 harbouring S30A exhibited significantly lower levels of Ser(P)33 (Fig. 4.8, E–H), suggesting a dependence on O-GlcNAcylation of K18 at Ser30 for the occurrence of phosphorylation at Ser33.

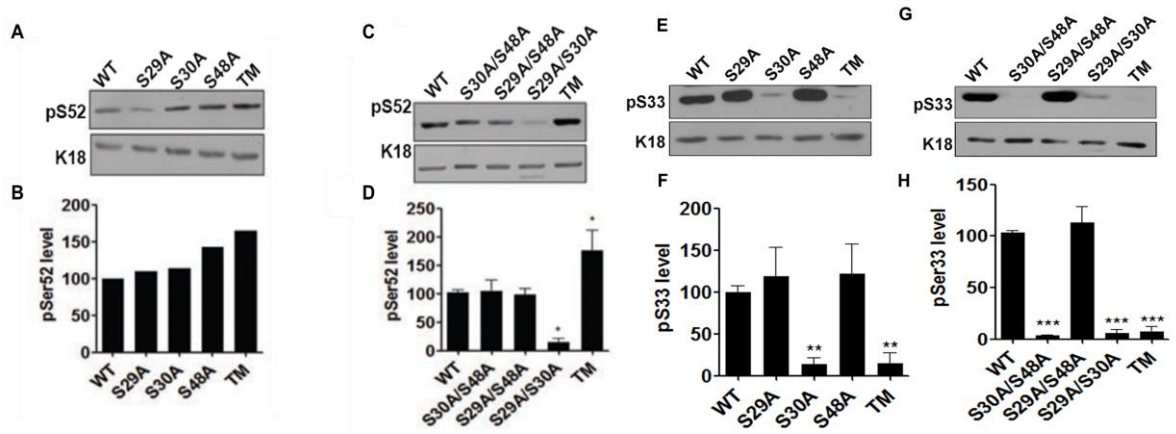


Figure 4.8. K18 *O*-GlcNAcylation at serine 30 positively regulates phosphorylation at serine 33 while O-GlcNAcylation at serine 48 reciprocally regulates phosphorylation at serine 52. Immunoblots of K18, Ser(P)52 (3055) and Ser(P)33 (IB4), are shown as indicated. *A*, *C*, E, and G, total cell lysates of HHL17 cells stably expressing K18- single *O*-GlcNAc mutants of K18 (*A*), and double *O*-GlcNAc mutants (*C*), of K18 were immunoblotted with K18Ser(P)52 (3055) and K18-sigle O-GlcNAc mutants of K18 (E), double O-GlcNAc mutants (G) of K18 were immunostained with K18Ser(P)33 (antibody clone IB4) antibodies respectively. B, D, F, and H, Densitometric quantification (average of three independent experiments, including A, C, E and G)

showing levels of K18 Ser(P)52 and K18 Ser(P)33; *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$; one-way analysis of variance. *Error bars* represent mean ±S.E.

4.3.3 O-GlcNAcylation at Ser30 Determines the Occurrence of Phosphorylation at Ser33 on K18:

Since abrogation of O-GlcNAcylation of K18 at Ser30 prevents its phosphorylation at Ser33, we set to investigate whether this cooperative relationship can be observed when O-GlcNAc levels on K18 are altered dynamically. PUGNAc treatment of K18-WT-expressing cells led to a time-dependent increase in total O-GlcNAcylation with a concomitant increase in phosphorylation of K18 at Ser33 (Fig. 4.9, A and B). This PUGNAc-mediated increase in Ser(P)33 levels was seen only in K18 mutants where Ser30 O-GlcNAcylation site was available, viz. K18-S29A and K18-S48A (Fig. 4.9, C and D). These results confirm that O-GlcNAcylation of K18 at Ser30 can positively influence phosphorylation at Ser33.

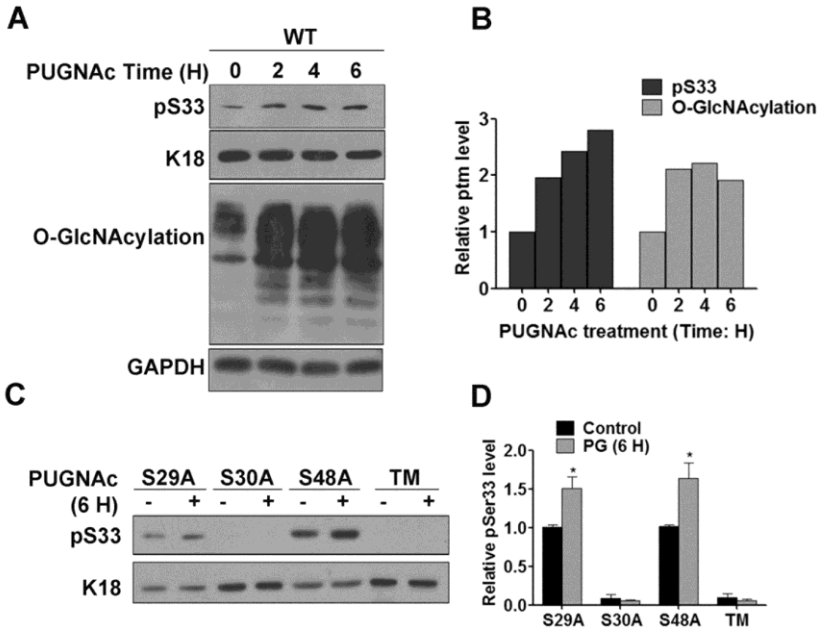


Figure 4.9. ***O*-GlcNAcylation of keratin 18 at serine 30 supports dynamic phosphorylation at serine 33.** *A* and *C*, immunoblot of equally loaded total proteins from HHL-17 cells expressing WT or *O*-GlcNAc mutants. *A*, cells expressing K18-WT were treated with PUGNAc for different

times (0, 2, 4 and 6 h). Shown are cells expressing single and triple *O*-GlcNAc mutants treated with PUGNAc for 6 h (*C*). *B*, Densitometric analysis of total *O*-GlcNAcylation and K18 phosphorylation at Ser33 in *A*. *D*, Densitometric analysis of Ser(P)33 levels on K18 (average of three independent experiments, including *C*; *, $p < 0.05$; Student's *t* test). *Error bars* represent mean ± S.E.

4.3.4 O-GlcNAcylation at Ser30 Determines the Occurrence of Phosphorylation at Ser33 on K18 and is unidirectional:

Because the dynamics of phosphorylation is regulated by the activities of specific kinases and phosphatases acting on that site, we predicted that inhibition of phosphatases by OA could restore Ser(P)33 levels in K18-S30A mutants. OA treatment led to a notable increase in Ser(P)33 levels on K18-WT and K18 O-GlcNAc mutants where Ser30 O-GlcNAcylation can occur, viz. K18-S29A and K18-S48A (Fig. 4.10, A and B). Whereas, K18 S30A and TM did not exhibit pSer33 even after OA treatment. This suggests that O-GlcNAcylation of K18 at Ser30 is essential for the occurrence of phosphorylation at serine 33. It may be possible that O-GlcNAcylation at S30 may regulate interaction of specific kinases to K18 at S33. We aimed to further investigate whether this cooperativity is mutual, with Ser(P)33 regulating O-GlcNAcylation at Ser30. To address this, we used stable cells where the K18 mutant can be O-GlcNAcylated only at serine 30 (K18-S29A/S48A) because antibodies recognizing site-specific O-GlcNAcylation on keratin 18 are not available. It was interesting to note that treating these cells with OA led to an increase in Ser(P)33 with no notable change in O-GlcNAcylation at serine 30 (Fig. 4.10, C and D). This suggests that the cooperative interplay between modifications at these two sites is one-sided, with gSer30 acting as an upstream switch for phosphorylation at Ser33.

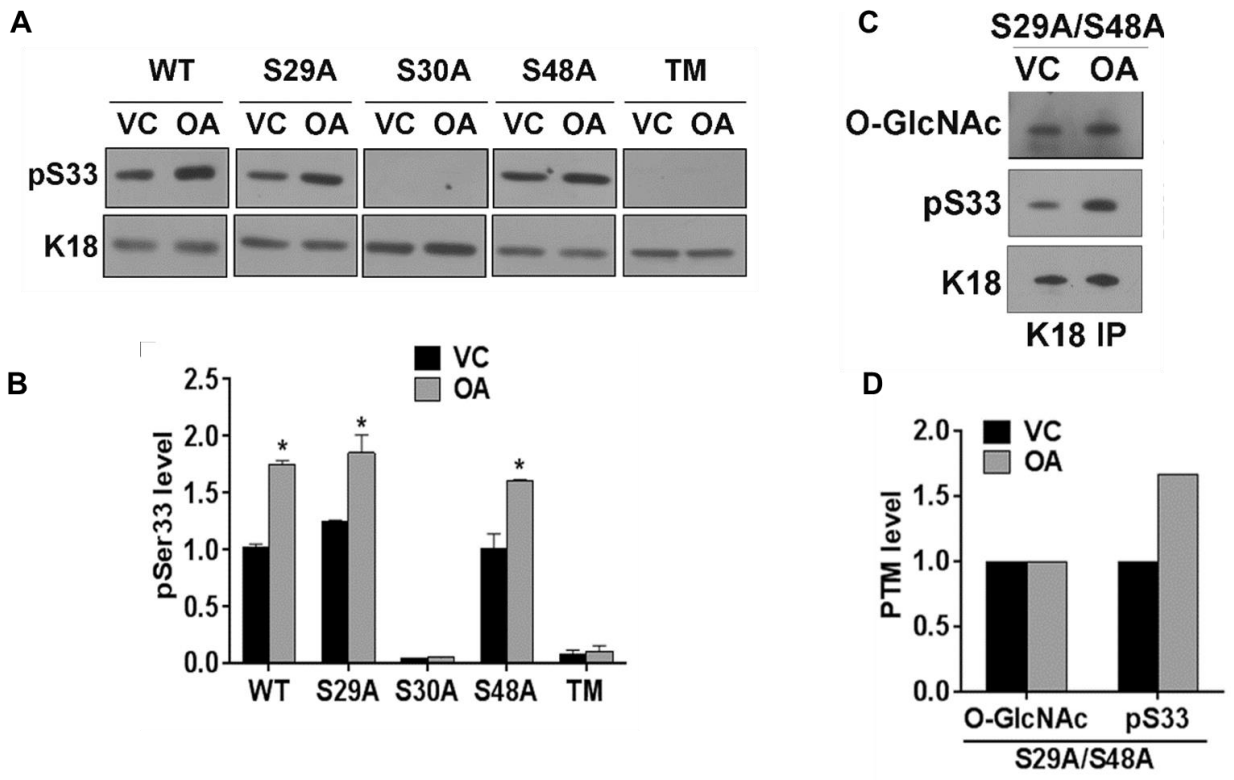


Figure 4.10. *O*-GlcNAcylation of keratin 18 at serine 30 regulates phosphorylation at serine 33, may be by regulating its kinases in a unidirectional manner. *A*, immunoblot of equal total proteins from HHL-17 cells expressing WT or *O*-GlcNAc mutants. *VC*, vehicle control. Shown are cells expressing WT, single and triple *O*-GlcNAc mutants treated with OA for 2 h. *B*, Densitometric analysis of Ser(P)33 levels on K18 (average of three independent experiments, including A; *, $p < 0.05$; Student's *t* test). *Error bars* represent mean ± S.E. C, immunoprecipitate (*IP*) of the double *O*-GlcNAc mutant K18-S29A/S48A immunoblotted with *O*-GlcNAc, Ser(P)33, and K18. D, densitometric analysis of *O*-GlcNAcylation and Ser(P)33 on K18. The K18 immunoblot served as a loading control.

4.3.5 O-GlcNAcylation-mediated Phosphorylation of Keratin 18 at Serine 33 Regulates Its Stability:

O-GlcNAcylation at Ser30 induces solubility and subsequent degradation of K18 along with increased phosphorylation at Ser33 (Figs. 4.2– 4.10). Hence, it is possible that Ser(P)33 mediates the effect of gSer30 on the solubility and stability of K18. Although, phosphorylation at Ser33 has

been shown previously to regulate solubility (5, 45) and shear stress-induced reorganization of keratin 8/18 filaments (14), its role in regulating the stability of keratin 18 is still unclear (46). To investigate the role of site-specific phosphorylation on K18 in regulating its stability, cells expressing K18-WT and phosphorylation mutants (K18-S33A and K18- S52A) were treated with cycloheximide. Although K18-WT and K18-S52A exhibited reduced K18 levels upon cycloheximide treatment, the protein levels of K18-S33A remained unchanged, suggesting a role for Ser (P)33 in regulating the stability of K18 (Fig. 4.11, A and B). It is possible that O-GlcNAcylation at serine 30 can influence the stability of K18 either directly by aiding ubiquitination or indirectly by stabilizing Ser (P) 33, which would lead to increased solubility and subsequent degradation. To understand this, stable cells expressing K18 phosphorylation mutants (K18-S33A and K18-S52A) were treated with PUGNAc to increase O-GlcNAcylation on K18. PUGNAc treatment led to reduced levels of K18-S52A but not K18-S33A (Fig. 4.11, C and D). These results indicate that O-GlcNAcylation at Ser30 regulates the solubility and stability of keratin 18 mainly by stabilizing Ser(P)33.

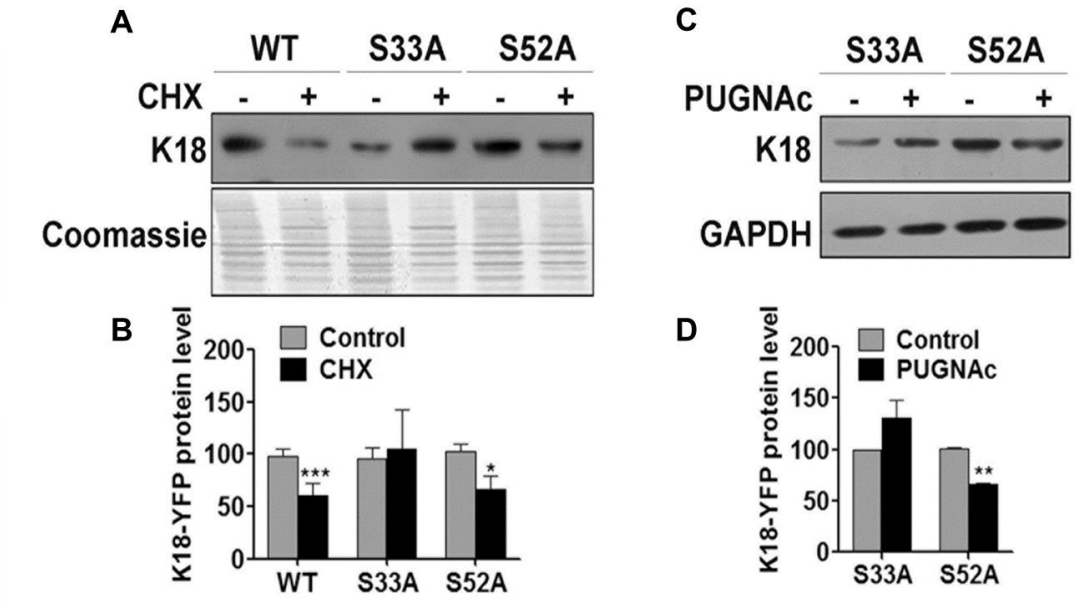


Figure 4.11. Phosphorylation at Ser33 regulates the stability of keratin 18. A, equal total proteins from untreated or CHX-treated (200 µM for 24 h) cells expressing WT, S33A, and S52A mutants of K18 immunoblotted with K18. C, untreated or PUGNAc-treated (100 µM for 48 h) cells expressing phosphomutants (S33A and S52A) of K18 were immunoblotted with K18 and GAPDH as indicated. The Coomassie-stained blot in A and GAPDH in C served as a loading control. B and D, densitometric analysis indicating relative levels of K18 (average of three independent experiments, including *A* and C; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Student's *t* test). *Error bar* represents mean ±S.E.

4.3.6 Phosphorylation at S33 regulates filament organization of K18:

As mutation of O-GlcNAcylation at S30 (S30A, S29A/S30A, S30A/S48A and TM) led to reduce phosphorylation of S33, we predicted that loss of Ser(P)33 could phenocopy the filament organization of K18- S30A and K18-TM. As expected, the K18-S33A mutant exhibited perinuclear aggregation and loss of peripheral filaments, whereas the filament organization of the K18-S52A mutant was similar to K18-WT (Fig. 4.12, A-B). This observation is also supported by previous reports that show that K18-Ser(P)33 plays a key role in regulating filament organization

and that loss of phosphorylation at this site (K18-S33A) causes the collapse of filaments around the nucleus (5).

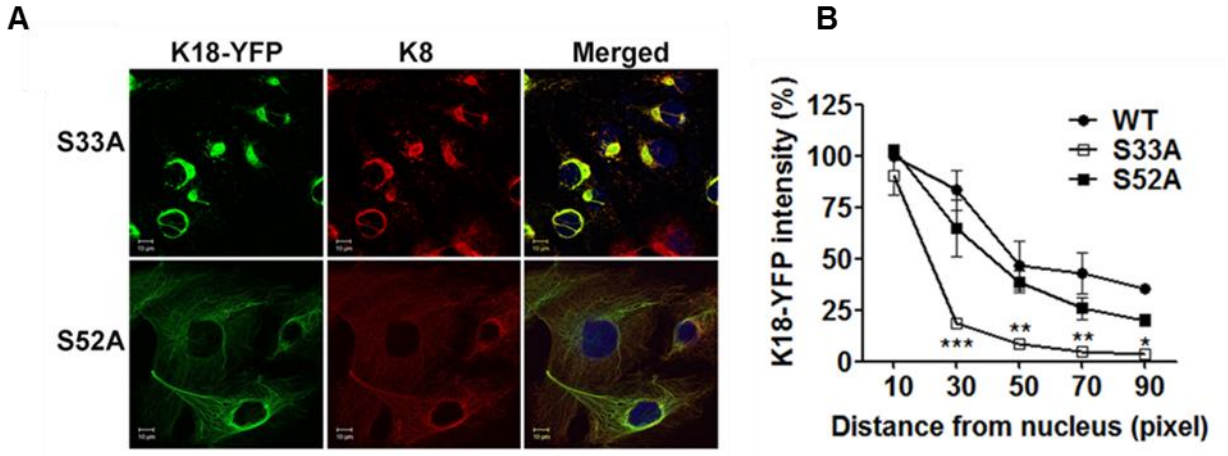


Figure 4.12. Phosphorylation of K18 at Ser33 is essential for maintaining normal filament organization. A, immunofluorescence image of cells expressing K18 S33A and S52A phosphomutants *B,* fluorescence intensity of the keratin network across a cell (perinuclear region to cell periphery). Each point represents mean ±S.E. of three independent experiments with 20 cells/ experiment. Images show K18-YFP (green), K8 (red), and merged images with DAPI (blue). *Scale bars*=10 µm. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way analysis of variance followed by Bonferroni post-tests). *Error bars* represent mean ±S.E.

4.3.7 Collapsed keratin filaments do not alter ability to phosphorylate:

S33 phosphorylation is dependent on S30 glycosylation (Figure 6-9). We inferred that the defective architecture (collapsed filament) in S30A mutants is due to loss of phosphorylation at S33 as loss of phosphorylation at S33 is known to cause collapse of filaments Fig 4.12 and (1). Further the ability of collapsed filaments to get modified was investigated. Therefore, to address ability of defective keratin filaments to get modified, we immuno-stained the filaments with site-specific phosphorylation antibody (K18-pS52) to check if collapsed filaments could be phosphorylated. We found that collapsed filaments in K18-TM (triple glycosylation mutant), K18-

S30A and K18-S33A exhibited pS52 levels similar to K18-WT (Fig. 4.13). This observation along with the evidence that glycosylation at S30 is a prerequisite for S33 phosphorylation, compelled us to infer that loss of phosphorylation at S33 is the major reason for defective filament architecture in S30A mutants.

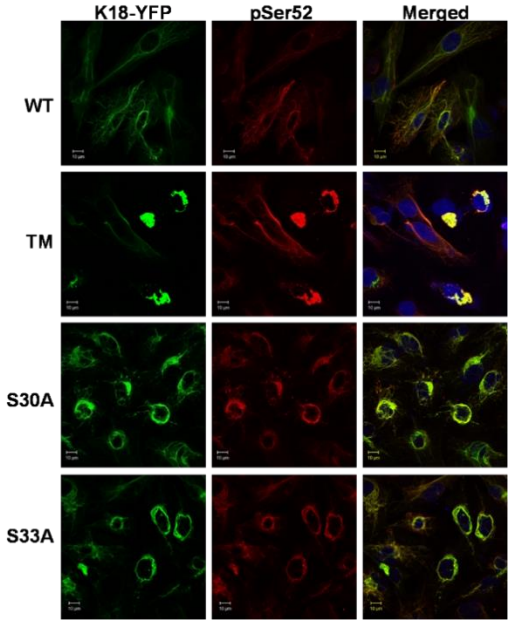


Figure 4.13. Defective keratin filaments do not alter ability to phosphorylate: Immunofluorescence images of cells expressing K18 WT, TM, S30A and S33A. Images show K18-YFP (green), K18-pSer52 (red), and merged images with DAPI (blue). Scale bars=10 µm.

4.3.8 Phosphomimetic mutation of S33 does not restore mutational defects of S30A:

As the biochemical evidence (Figure 4.6-4.12) indicates that S33 phosphorylation is dependent on S30 glycosylation, we inferred that the defective architecture (collapsed filaments) in S30A mutants is due to loss of phosphorylation at S33 as loss of phosphorylation at S33 is known to cause collapse of filaments Fig 4.12 and (1). Similarly K18 stability is determined by S33 phosphorylation dependent O-GlcNAcylation at S30. To confirm this, we assessed the stability of K18 after rescuing the phosphorylation at Ser33 by substituting with a phosphomimetic mutation

(S33D). However, both K18-S33D and K18-S30A/ S33D showed higher stability, as assessed by cycloheximide treatment, suggesting that S33D does not substitute for phosphorylation at Ser33 and therefore could not rescue the enhanced stability of K18 upon loss of phosphorylation at Ser33 (Fig. 4.14, A and B). Similarly, we also assessed the filament organization in the K18-S33D and K18-S30A/ S33D phosphomimetic mutants. The collapsed filament organization was not rescued in either of these stable lines, suggesting that S33D is unable to compensate for phosphorylation at Ser33 (Fig. 4.14, C and D). These results imply that gSer30-dependent phosphorylation of Ser33 is essential for maintaining the normal filament organization of keratin 18.

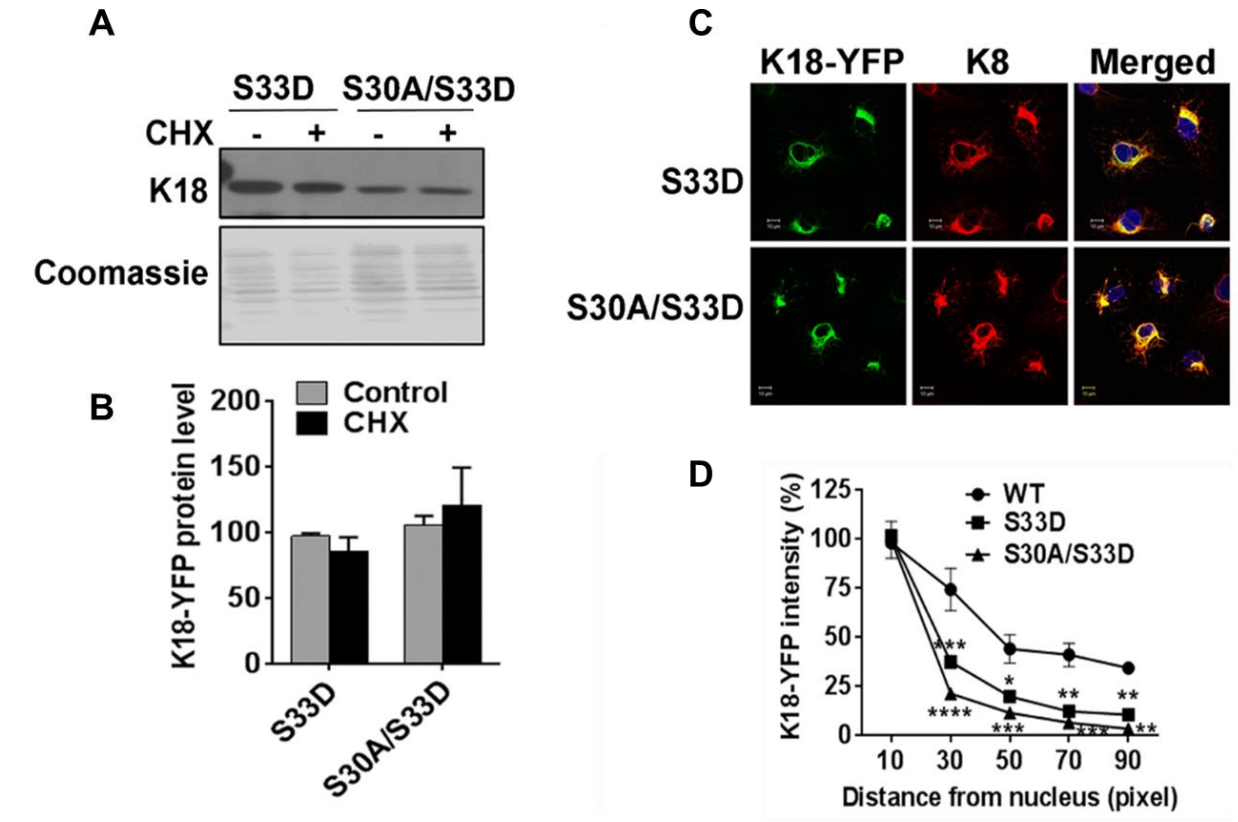


Figure 4.14. Phosphomimetic mutation is unable to restore S30A mutational defects. *A*, equally loaded total proteins from untreated or CHX-treated (200 μM for 24 h) cells expressing S33D and S30A/S33D mutants of K18 immunoblotted with K18 antibody. The Coomassie-stained blot in *A* served as a loading control. *B*, densitometric analysis indicating relative levels of K18

(average of three independent experiments, including A. Error bars represent mean ±S.E. C, immunofluorescence images of cells expressing phosphomimetic mutant of K18. Images show K18-YFP (green), K8 (red), and merged images with DAPI (blue). Scale bars=10 μm. D, quantification of fluorescence intensity of the K18-YFP filament network of cells in *C*, (average of three independent experiments; 20 cells/experiment; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way analysis of variance followed by Bonferroni post-tests). *Error bars* represent mean ±S.E.

4.4 Objective: 3

To investigate the role of O-GlcNAcylation on K18 in the cellular processes like cell adhesion, spreading and motility.

4.4.1 O-GlcNAcylation and phosphorylation of K18 regulate cellular spreading on fibronectin without affecting its ability of adhesion:

We further aimed to investigate the role of O-GlcNAcylation and phosphorylation in regulation of cellular processes mediated by K18. As described in material and methods, HHL17 cells expressing site specific O-GlcNAc single, triple and single phosphorylation mutants were cultured on fibronectin coated plate to study cellular adhesion. O-GlcNAcylation and phosphorylation mutations of K18 did not alter extent of cellular adhesion on fibronectin (Fig. 4.15, A-B). Further, cellular spreading was investigated in these mutant cells. Interestingly, significant reduction in cellular spreading was found in HHL17 cells expressing O-GlcNAc mutants S30A, triple mutant and phosphorylation mutant S33A when compared to the WT, as seen by laser confocal microscopy (Fig. 4.15, C-F). The cytoplasmic to nuclear area was used to quantitate the relative spreading. These results together suggest that O-GlcNAcylation at S30 and phosphorylation at S33 regulates cellular spreading mediated by K18.

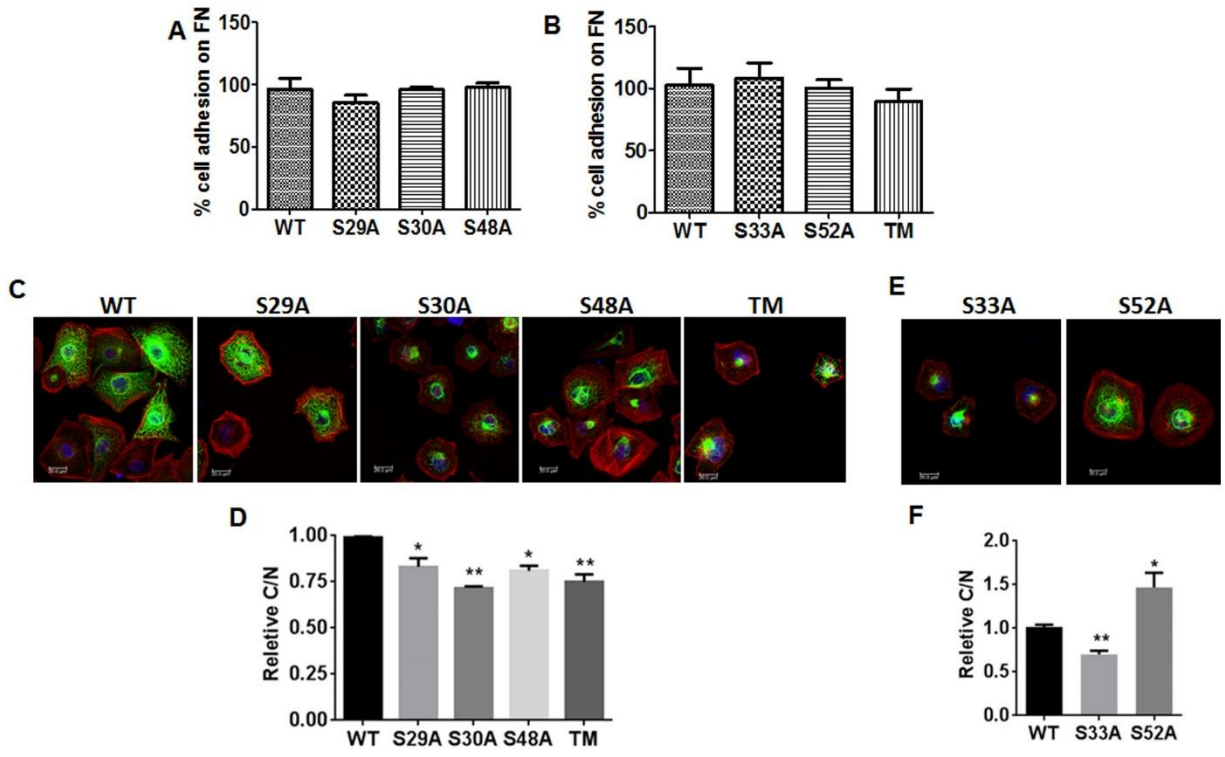
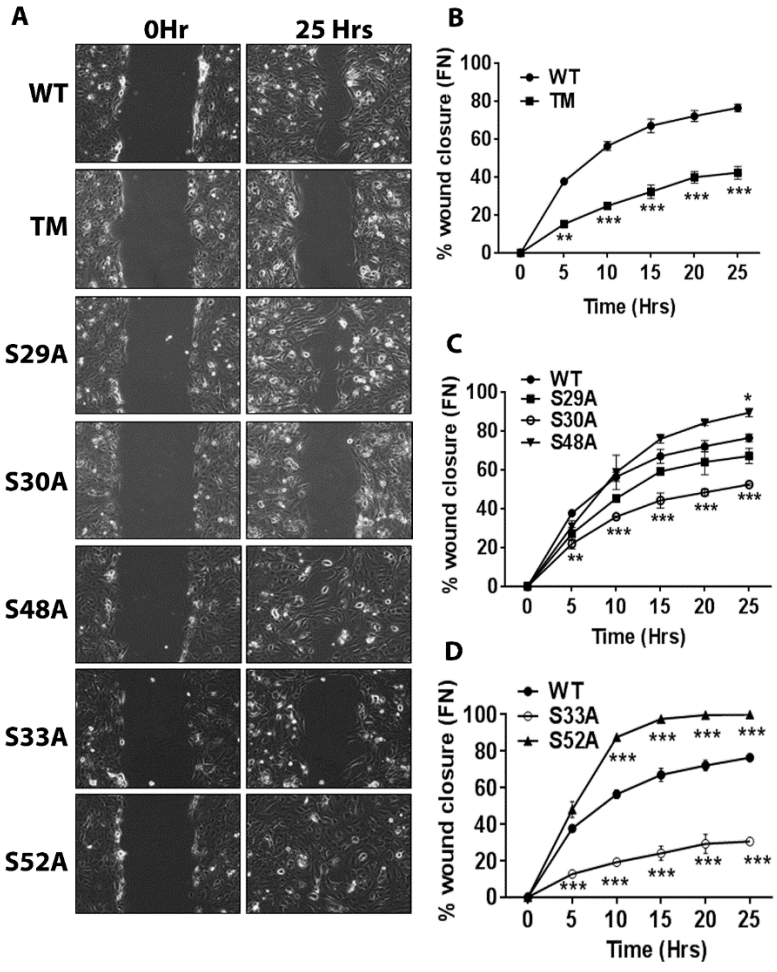


Figure 4.15. O-GlcNAcylation and phosphorylation of K18 do not affect ability to adhere on fibronectin whereas, regulate cellular spreading on fibronectin A and B, Adhesion of HHl17 cells stably expressing WT, O-GlcNAcylation and phosphorylation site specific mutants of K18 on fibronectin. Values are mean ± SE of three independent experiments performed in triplicates. C, Confocal imaging of cell spreading by Phalloidin-TRITC stained HHL17 cells stably expressing K18 WT, O-GlcNAcylation and phosphorylation mutants. Images show YFP (*green*), Phalloidin-TRITC (*red*), and merged images with DAPI (*blue*). Scale bars=20 µm. D and E, quantification of cellular spreading on fibronectin (FN) by ratio of cytoplasmic to nuclear area of three independent experiments, 20 cells/experiment. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way analysis of variance. *Error bars* represent mean ±S.E.

4.4.2 O-GlcNAcylation and phosphorylation regulate K18 mediated cellular migration on fibronectin:

O-GlcNAcylation at S30 cooperates with phosphorylation at S33 to regulate K18 filament organization. Keratin filament organization provides dynamic flexibility needed during cellular migration. Because, K8/18 deficient cells migrate slow on substratum such as fibronectin, we

investigated the cellular impact of the cooperativity between gSer30 and Ser(P)33 on migration on fibronectin. We assessed the migration of site-specific O-GlcNAcylation and phosphorylation mutants on fibronectin substrate in a scratch wound assay. It is interesting to note that only S30A and S33A O-GlcNAc and phosphomutants of K18, respectively, exhibited reduced migration and wound closure compared to the WT (Fig. 4.16, A–D). The other O-GlcNAcylation and phosphorylation mutants of K18 (S29A, S48A, and S52A) showed migration similar to K18-WT (Fig. 4.16, A–D). Similarly, relative rate of wound healing was also regulated by O-GlcNAcylation at S30 and phosphorylation at S52 of K18 (Fig. 4.16, E-G). These results further highlight the importance of Ser30 O-GlcNAcylation-mediated Ser33 phosphorylation on keratin 18 in modulating cellular processes like migration.



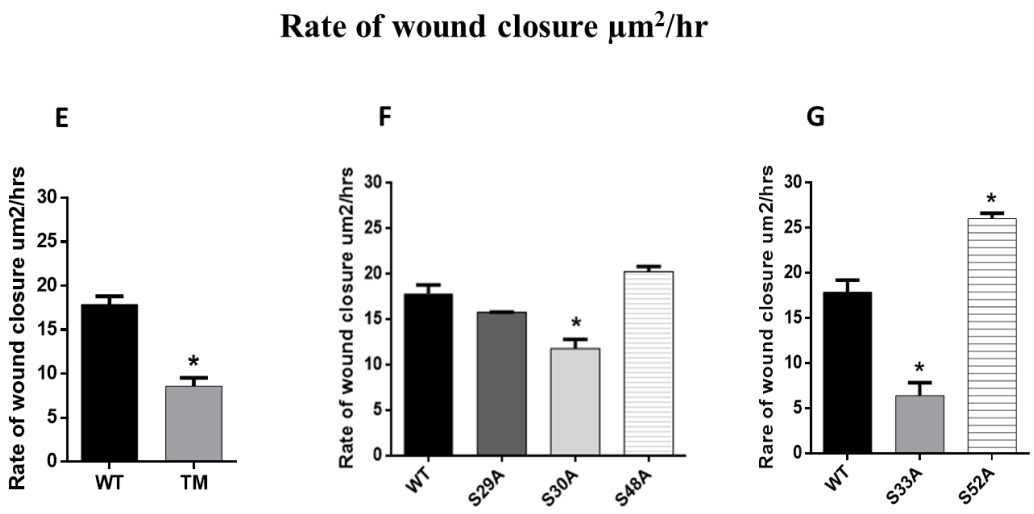


Figure 4.16. *O*-GlcNAcylation and phosphorylation of K18 regulate the cellular migration on fibronectin. *A*, comparison of migration of HHL17 cells stably expressing K18-WT and the indicated *O*-GlcNAcylation and phosphorylation mutants of K18 by scratch wound assay on fibronectin (FN). *B*, *C*, and *D*, quantitation of percent wound closure of two independent experiments, each experiment at three different points. E, F and G, rate of wound closure µm^2/hr on fibronectin. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; two-way analysis of variance followed by Bonferroni post-tests). *Error bars* represent mean ± S.E.

Chapter 5: Discussion

O-GlcNAcylation regulates number of cellular functions by modulating protein properties, among them, regulation of protein phosphorylation is major mechanism. Both O-GlcNAcylation and phosphorylation are abundant (10–20% of all proteins are O-GlcNAcylated and 40–60% are phosphorylated) and dynamically modify Ser/Thr residues of cellular proteins. On most phospho-glyco proteins discovered so far, O-GlcNAcylation and phosphorylation antagonize each other in site occupancy (191). Because O-GlcNAc is a bulky residue, (the Stokes radius of O-GlcNAc is five times larger than phosphorylation), negative charge of phosphate group and conformational changes in modifying protein, these two PTMs reciprocally regulate each other at adjacent sites. However, this seems unclear for K18 as both these modifications exhibit similar changes under certain conditions such as mitotic arrest (277) and heat stress (278), while during induced hepatotoxicity they exhibit inverse relationship (phosphorylation gets increased and O-GlcNAcylation gets reduced) (279). Of the two phosphorylation sites mapped on keratin 18, pS33 causes increased solubility during mitosis and filament reorganization in response to shear stress (5,14). In basal condition, phosphorylation at S33 is maintained at low level whereas, during mitosis and stress when K8/18 filaments are solubilised at higher rate, level of pS33 is significantly increased (5). In addition, S33 phosphorylation also regulates binding of 14-3-3 family proteins during mitosis, which is important for G2/M progression (50). On the other hand, pS52 is a physiological phosphorylation site, which is known to regulate keratin filament reorganization under stress conditions (115). It is also involved in maintenance of basolateral polarity of keratin filaments (Omary 1997). Previously our lab has shown that, mutating the three known O-GlcNAcylation sites of K18 (S29, 30, 48 to A) led to altered filament organization and decrease in both solubility and degradation of keratin 18 (17). However, it was still unclear whether this regulatory role of O-GlcNAcylation is site specific and if it is dependent on phosphorylation of

K18. Here we have demonstrated hitherto unreported, positive crosstalk between S30 O-GlcNAcylation and S33 phosphorylation on K18. This cross talk is responsible for regulating K18 solubility, filament organization and stability (Fig. 6.1).

Disassembly of K8/18 filament network in to soluble form is prerequisite for its ubiquitin proteasome mediated degradation. Filamentous or aggregated K8/18, although ubiquitinated, cannot be degraded by proteasomes unless they are disassembled in to soluble form (280). In case of Mallory-Denk bodies (MDB), a hepatotoxin induced K8>K18 ratio, and keratin crosslinking by transglutaminase-2 led to formation of cytoplasmic aggregates. Hyper phosphorylated and ubiquitinated MDB cannot be efficiently cleared/degraded by proteasome, because of their insoluble nature (92). Phosphorylation at serine 33 plays an important role in solubility and reorganization of keratin 8/18 filament network in conditions such as, mitosis and shear stress, where keratin filaments get entirely disassembled (5,14). Moreover, it is also a binding site for 14-3-3 family proteins which sequester keratins in to soluble form (50). In addition to this, Okadaic Acid (OA) mediated inhibition of phosphatases led to an increase in pS33 levels along with enhanced binding to 14-3-3 in HT-29 epithelial cells (5,50), whereas abrogation of phosphorylation at S33 had no effect on OA induced solubility of K18-S33A mutant when expressed in NIH3T3 fibroblasts (5). Although, these reports strongly indicate a role for pS33 in mediating K18 solubility, they also point to varied impact of modifications on the solubility of keratin 18 in different cell types (5,50). Of the three sites of O-GlcNAcylation, it was interesting to observe that loss of gS30 was enough to stall the phosphorylation induced solubility during OA treatment (Fig. 4.2). Further to this, the loss of gS30 also led to an increased stability of K18 (Fig. 4.3 and Fig. 4.4). Investigation of K18 relative half-life by Cycloheximide (by inhibiting new protein synthesis) (Fig. 4.3) and by PUGNAc treatment (enrichment of O-GlcNAcylation and

subsequent degradation of K18) (Fig. 4.4) indicate that O-GlcNAcylation is enough to target K18 for degradation. Taken together, these observations indicate that, O-GlcNAcylation at S30 could be central to not only phosphorylation dependent solubility but also subsequent degradation. On most proteins, O-GlcNAcylation protects against proteasomal degradation either directly or by antagonizing phosphorylation. Some of the examples of such proteins are, p53, Δ-Lactoferrin, Snail1 and ERβ (193). O-GlcNAcylation could also target the protein for degradation as in the case of the kinase CK2, where O-GlcNAcylation at Ser347 leads to degradation by antagonizing phosphorylation at Thr344 (194). In either of the cases O-GlcNAcylation regulates protein stability by antagonizing phosphorylation. Interestingly, this seemed contrary on K18, as phosphorylation induced solubility and degradation were totally dependent on O-GlcNAcylation at serine 30 (Fig. 4.2, 4.3 and 4.4).

During, solubility or filament reorganization of K8/18, O-GlcNAcylation and phosphorylation are concomitantly increased on K18. Hence, co-existence of both these modifications on K18 seems plausible. However, possibilities such as detection of simultaneous existence of O-GlcNAcylation and phosphorylation on two different molecules of K18 or simultaneous existence of O-GlcNAcylation and phosphorylation separately on K8 and K18 of a tetramer cannot be ruled out. Therefore, we investigated O-GlcNAcylation and phosphorylation on a separately purified K8 and K18 molecules (Fig. 4.7A). Indeed, presence of notable phosphorylation on O-GlcNAcylated K8 and K18 species confirms the co-occurrence of these modifications (Fig. 4.7B). However, due to the presence of multiple sites of both these modifications, they could exhibit mutually exclusive relationship at proximal sites but can still co-exist at distal sites. Further analysis using site specific K18 O-GlcNAc mutants along with site specific K18 phosphorylation antibodies indicated both cooperative and antagonistic interplay between these modifications at proximal sites (Fig. 4.7 C-

E). As there are 2 major phosphorylation sites on K18, S33 and S52, which are adjacent to O-GlcNAcylation sites, S29, S30 and S48, we investigated levels of these two phosphorylation in O-GlcNAcylation mutants of K18. Surprisingly, our results showed that, O-GlcNAcylation at S30 promotes its adjacent phosphorylation at S33 (Fig. 4.8E-H) while, O-GlcNAcylation at S48 antagonises phosphorylation at S52 (Fig. 4.8A-D). A previous study also supports the presence of reciprocal relationship between gS48 and pS52 (281). The existence of promotive/cooperative relationship between gS30 and pS33 is both interesting and novel. Further, global high-throughput phospho-proteomics upon elevated O-GlcNAc levels supports the prevalence of such non-reciprocal, co-operative interplay on many proteins (36). Such type of synergistic relationship between O-GlcNAcylation and phosphorylation exists in the catalytic cleft of the CAMKIV kinase, albeit at distal sites wherein mutation of the glycosylation sites T57/S58 to Alanine on CAMKIV causes a drastic reduction in basal activating phosphorylation at T200 (35).

Crosstalk between O-GlcNAcylation at S30 and phosphorylation at S33 is cooperative and dynamic in nature as pS33 levels alter in response to acute pharmacological reagents (by enrichment of cellular O-GlcNAcylation :treatment of PUGNAc (Fig. 4.9 A-D) and by enrichment of cellular phosphorylation :treatment of OA) (Fig. 4.10 A-B). Additionally, O-GlcNAcylation at S30 acts as a switch for phosphorylation at S33 (Fig. 10 C-D) indicating, this interplay is unidirectional. Thus, O-GlcNAcylation at S30 can facilitate phosphorylation at S33 either by aiding or hindering interactions with kinases and phosphatases respectively. The possibility of gS30 facilitating interaction with kinase(s) seems more probable, as the loss of pS33 in K18-TM and S30A mutants couldn't be restored upon inhibiting phosphatases (Fig. 4.10A-B). It will be interesting to validate effect of gS30 on affinity of pS33 specific kinases. But, multiple kinases phosphorylate S33 (i.e. Raf1, CDC2 and PKCζ) and kinase such as, Raf1which phosphorylates

both S33 and Ser52 of K18 makes the study complex. However, investigation of extent of S33 phosphorylation in S30A/S52A phospho-glyco double mutant may validate this observation. It is also important to note that OA induced enhanced association of 14-3-3 with K18 is marginally reduced but not completely diminished in triple glycosylation mutant when expressed in BHK fibroblasts (5). Although this suggests that S33 could still get basally phosphorylated in the absence of glycosylation at S30, it also hints at the possibility of different kinases involved in phosphorylating S33 in different cell systems (5,14,282). Overall, these results suggest that gS30 controls solubility and degradation of K18 by stabilizing phosphorylation at S33. This seems plausible as phosphorylation on many proteins acts as a signal for ubiquitination mediated proteasomal degradation (192). Protein phosphorylation can regulate ubiquitination by, regulating activity of E3 ligase. Phosphorylation increases affinity for E3 ligase towards target protein by creating a phosphodegron (a short motif for phosphorylation mediated recognition of proteins), and lastly, by regulating substrate/ligase interaction at the level of subcellular compartmentalization. Even on K8, shear stress induced phosphorylation at S73 enables E2 ligases to mediate proteasomal degradation (102). Indeed, loss of phosphorylation at S33 increased the stability of K18 which could not be rescued by increasing O-GlcNAcylation on K18 (Fig. 4.11 A-D) suggesting that O-GlcNAcylation at S30 regulates K18 degradation mainly through S33 phosphorylation. Although, substitution with negatively charged residues like aspartate (D) rescues loss of ser/thr phosphorylation on many proteins, such as keratin 18, S33D does not seem to substitute for phosphorylation (Fig. 4.14 A-B) (5). These findings imply that O-GlcNAcylation at S30 predominantly aids in the phosphorylation at S33 which in turn causes increased solubility and subsequent degradation of K18.

Both, cultured cells and in vivo studies on hepatocytes have demonstrated that, phosphorylation at S33 is also a key regulator of K18 solubility and filament organization (5,50,116). In general, keratins 8/18 exhibit a gradient of filament network, with highly bundled filament near the nucleus and fine, thin filaments at cell periphery (Fig. 4.5 A-D) (110). As reported previously, expression of K18-S33A mutant led to disruption of filament organization, visualised as highly concentrated perinuclear filaments and collapsed peripheral filament network, whereas the architecture of K8/18 is unaltered in K18-S52A mutants or K18-WT expressing cells (Fig. 4.12 A-B) (5). Among the O-GlcNAcylation mutants, only K18-S30A mimicked the perinuclear collapsed network of K18 as seen in K18-S33A phosphomutant (Fig. 4.6 A-D). The defects in filament organization in K18-S33A and K18-S30A could not be rescued by S33D mutations (Fig. 14 C-D), suggesting that similar to K18 stability, S33D cannot substitute for pS33 to rescue filament organization. These observations further strengthen the regulatory role of gS30 in promoting pS33 which in turn dictates the solubility, filament organization and turnover of keratin 18.

A tight regulation of epithelial cell adhesion and migration is crucial for physiological and pathological processes, such as, wound healing and tumor metastasis (283). Interaction of integrin with ECM elicits signalling cascade which transduces intracellular signalling events, leading to cellular migration. Integrin engagement with ECM is modulated by intracellular molecules which modulate attachment and spreading efficiency of cells (283,284). K8/18 filaments are also important determinant in regulation of such events in simple epithelial cells. Because, hepatocytes express solely the K8/K18 pair as cytoplasmic IFs, they serve as a model of choice to address the role of K8/K18 in cellular behaviour, including cellular adhesion, spreading and migration (43,285). Disturbed filament organization due to O-GlcNAcylation and phosphorylation mutation at S30 and S33 of K18 respectively showed reduced cellular spreading on fibronectin without

affecting its ability to create initial attachments on fibronectin (Fig. 4.15). Previously, Marceau et.al. have demonstrated in K8-knockout hepatocytes and K8-knockdown rat hepatoma cells that, K8/18 filament deficient hepatocytes exhibited reduced capacity to spread on fibronectin (43). However, localization of integrin on cell surface and interaction with ECM remained unaffected. Lack of K8/18 filament showed altered recruitment of focal adhesion (FAs) complex proteins and activity of FAs signalling molecules such as, PKC and FAK (44). The dynamic reorganization of keratin 8/18 filaments is essential for the plasticity of keratin filament network during many cellular processes including cell migration (286). Rapid changes in keratin network dynamics are initiated especially in the lamellipodia of migrating cells (110). It is also important to note that phosphorylation of keratin 8 alone can play a critical role in mediating the migration of epithelial tumor cells and hepatocytes (9,44). Interestingly, loss of O-GlcNAcylation at S30 or phosphorylation at S33 on K18 was sufficient to reduce the migrating potential of hepatocytes indicating that both gS30 and pS33 act cooperatively to impact filament dynamics during cell migration (Fig. 4.16). Interestingly, mutation of S52 phosphorylation (S52A) leads to increased cellular spreading and migration potential on fibronectin. Although, S52 phosphorylation of K18 is involved in regulation of keratin filament organisation, its mutation in to S52A does not lead to defects in K8/18 filaments. This suggests that, phosphorylation of S52 does not exclusively required for filament organization. Hence, the underlined mechanism in gain of function mutation of S52A is need to be study.

Taken together these results demonstrate a synergism between S30 O-GlcNAcylation and S33 phosphorylation on keratin 18 in regulating its functional properties like solubility, filament organization and stability (Fig. 6.1). The cycling of O-GlcNAc on cellular proteins is tightly linked to glucose metabolism (287,288) and is often deregulated in multiple pathological conditions like

diabetes and cancer (32). These findings raise the possibility of factors like glucose metabolism to regulate keratin 8/18, mainly in metabolically active cells like hepatocytes. The positive regulation of phosphorylation by proximal O-GlcNAcylation opens up new avenue to understand functional regulation of phosphoglyco proteins. Understanding how O-GlcNAcylation can facilitate phosphorylation on K18 will be important, as this type of interplay might be as abundant as reciprocal crosstalk.

As demonstrated here, O-GlcNAcylation of K18 at Ser30 determines phosphorylation at Ser33. This unidirectional interplay regulates functional properties of K18. In this regard, there is need to develop strategies which can target both O-GlcNAcylation and phosphorylation in a site specific manner to deal with pathological conditions associated with altered K8/18 functions.

Further, O-GlcNAcylation is known to regulate number of signalling pathways which may be deregulated in various pathological conditions related to immune system, diabetes mellitus, cancer, cardiovascular diseases, neurodegenerative diseases etc. In these diseased conditions, global as well as site specific alterations of O-GlcNAcylation are reported in cellular proteins. To cure such disease conditions, targeting cellular O-GlcNAcylation by modulating activity of their cyclic enzymes can lead to global changes in cellular O-GlcNAcylation. Hence, investigating and targeting specific O-GlcNAcylation sites of cellular proteins may lead to development of targeted therapies for these diseases.

Chapter 6: Summary and Conclusion

6.1 Summary:

- O-GlcNAcylation of K18 at Ser30 is critical for regulation of its solubility and subsequent degradation.
- O-GlcNAcylation at Ser30 is critical for regulation of normal keratin filament organization.
- O-GlcNAcylation and phosphorylation coexist on K8 and K18 molecules.
- O-GlcNAcylation of K18 regulates its phosphorylation in site dependent manner.
    - Phosphorylation at Ser52 is regulated by O-GlcNAcylation at Ser48 in reciprocal manner.
    - Phosphorylation at Ser33 is regulated by O-GlcNAcylation at Ser30 in positive manner.
- O-GlcNAcylation at S30 acts as upstream switch for phosphorylation at S33 (unidirectional regulation).
- Similar to O-GlcNAcylation at S30, phosphorylation at Ser33 regulates K18 solubility and filament organization.
- O-GlcNAcylation of K18 at Ser30 regulates its stability and filament organization by regulating phosphorylation at Ser33.
- Phosphomimetic mutation of K18 S33D does not substitute phosphorylation at S33.
- Positive correlation at S30 O-GlcNAcylation and phosphorylation at S33 is important in regulation of cellular processes such as cellular spreading and migration on Fibronectin.

6.2 Conclusion:

Dynamic O-GlcNAcylation regulates protein properties by diverse mechanisms, among them, antagonistic regulation of protein phosphorylation is major mechanism. These PTMs driven simple regulatory mechanism is known to govern vital cellular processes. Interestingly, the present study has shown that, O-GlcNAcylation can even support proteins phosphorylation on K18, which could prove as a novel regulatory mechanism for vital cellular processes.

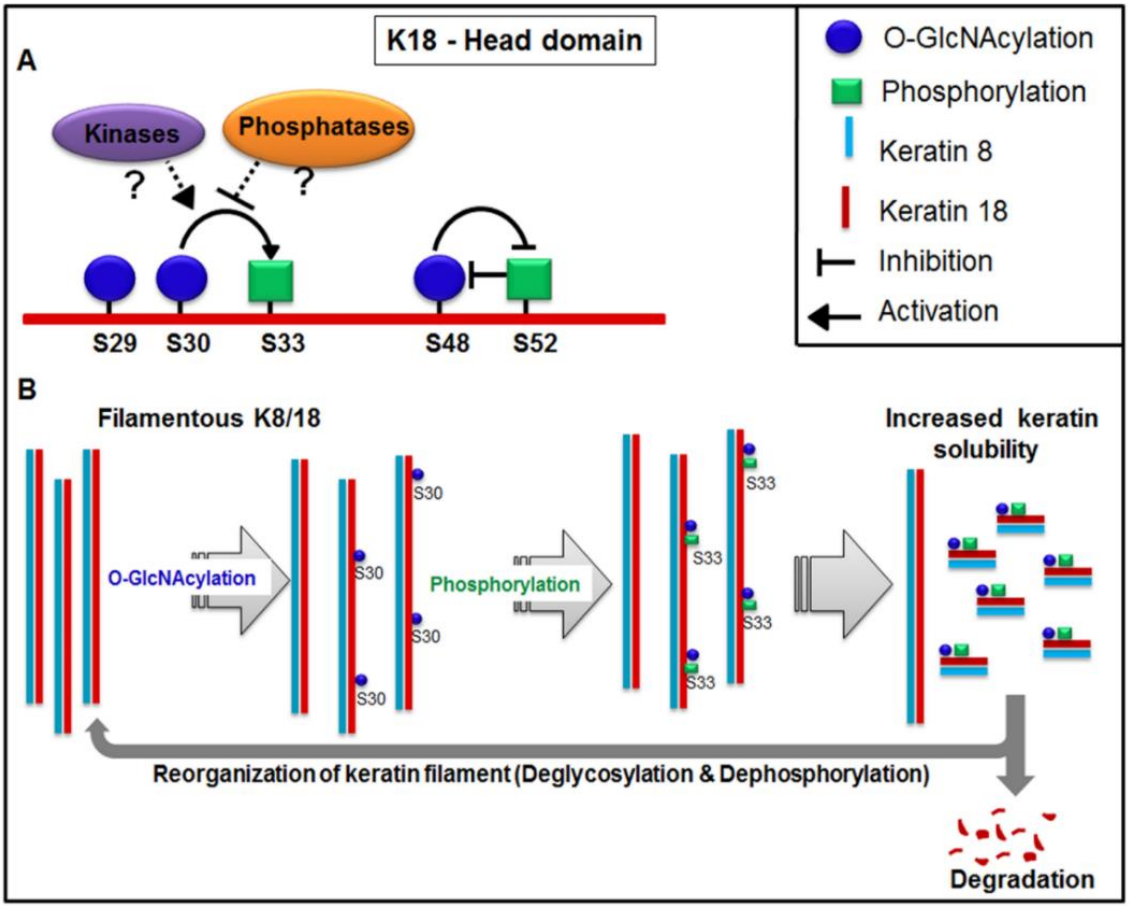


Figure 6.1. Model depicting cross-talk between *O*-GlcNAcylation and phosphorylation at proximal sites on keratin 18 to regulate the solubility, degradation, and reorganization of the keratin filament network. A, *O*-GlcNAcylation at serine 30 facilitates proximal phosphorylation at serine 33 either by aiding the interaction with specific kinases or hindering the interaction with phosphatases. *O*-GlcNAcylation at serine 48 exhibits a mutually exclusive relationship with

phosphorylation at serine 52. B, serine 30 *O*-GlcNAcylation of keratin 18 filaments promotes stabilization of serine 33 phosphorylation, which leads to filament disassembly and a concomitant increase in soluble keratin 18. The soluble keratin 18 subunits are either targeted for proteasomal degradation or incorporated into the filament network upon loss of *O*-GlcNAcylation and phosphorylation at serine 30 and 33, respectively.

SUMARY AND CONCLUSION

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Functional Implications of *O*-GlcNAcylation-dependent Phosphorylation at a Proximal Site on Keratin 18*[S]

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Keratins 8/18 (K8/18) are phosphoglycoproteins and form the major intermediate filament network of simple epithelia. The three *O*-GlcNAcylation (Ser^{29}, Ser^{30}, and Ser^{48}) and two phosphorylation (Ser^{33} and Ser^{52}) serine sites on K18 are well characterized. Both of these modifications have been reported to increase K18 solubility and regulate its filament organization. In this report, we investigated the site-specific interplay between these two modifications in regulating the functional properties of K18, like solubility, stability, and filament organization. An immortalized hepatocyte cell line (HHL-17) stably expressing site-specific single, double, and triple *O*-GlcNAc and phosphomutants of K18 were used to identify the site(s) critical for regulating these functions. Keratin 18 mutants where *O*-GlcNAcylation at Ser^{30} was abolished (K18-S30A) exhibited reduced phosphorylation induced solubility, increased stability, defective filament architecture, and slower migration. Interestingly, K18-S30A mutants also showed loss of phosphorylation at Ser^{33}, a modification known to regulate the solubility of K18. Further to this, the K18 phosphomutant (K18-S33A) mimicked K18-S30A in its stability, filament organization, and cell migration. These results indicate that *O*-GlcNAcylation at Ser^{30} promotes phosphorylation at Ser^{33} to regulate the functional properties of K18 and also impact cellular processes like migration. *O*-GlcNAcylation and phosphorylation on the same or adjacent sites on most proteins antagonize each other in regulating protein functions. Here we report a novel, positive interplay between *O*-GlcNAcylation and phosphorylation at adjacent sites on K18 to regulate its fundamental properties.

Keratins are the major intermediate filament proteins of epithelia. They form a 10-nm filamentous cytoskeleton made up of a non-covalent obligate heteropolymer of type I and type II keratins and are expressed in a tissue-specific manner (1, 2). Keratin pair 8/18 forms the intermediate filament scaffold predominantly in simple epithelia such as the lining of the alimentary canal, liver, and pancreas (2, 3). The expression pattern of keratins is highly regulated among various epithelia, suggesting a cell type-specific role for various keratins (3). Ectopic expression of K8/18 in other epithelia is highly associated with a malignant phenotype (4). Keratins 8/18 provide mechanical support for cellular integrity and are central to various non-mechanical functions like protein biosynthesis, protection from apoptosis, regulation of cell cycle progression, motility, and organelle transport (5–10). Their biological roles are majorly dependent on their functional properties like solubility, filament organization, and dynamics (11, 12). Keratins 8/18 undergo several posttranslational modifications like phosphorylation, *O*-GlcNAcylation, acetylation, sumoylation, and transamidation (13). Of these, site-specific phosphorylation is very well characterized for its role in regulating the functional properties, like solubility and filament organization, of both keratin 8 and 18 (5, 9, 14–16). Recent evidence shows that these functional properties are also regulated by other modifications, like *O*-GlcNAcylation, sumoylation of keratins 8/18, and acetylation of keratin 8 (17–19). However, the site-specific roles of *O*-GlcNAcylation and its cross-talk with phosphorylation are yet to be uncovered.

The addition of a single GlcNAc to serine and threonine residues to nuclear and cytoplasmic proteins, termed *O*-GlcNAcylation, was first identified in 1984 by Torres and Hart (20). Two enzymes, *O*-GlcNAc transferase and *O*-GlcNAcase, regulate the cycling of *O*-GlcNAc on proteins (21, 22). Unlike complex classical glycosylation, which is static in nature, *O*-GlcNAcylation is a dynamic modification similar to phosphorylation (23). A wide range of nuclear and cytoplasmic proteins get abundantly modified with this single sugar modification, *viz.* transcription factors, nuclear pore proteins, enzymes, and cytoskeletal proteins (21). On most proteins, *O*-GlcNAcylation exhibits cross-talk with phosphorylation to regulate essential properties of proteins like protein-protein/DNA-protein interactions (24), subcellular localization (25), and proteasome-mediated protein degradation (26, 27), thereby controlling cellular processes like transcription, signal transduction, stress response, and cell cycle progression (28, 29). Aberrant *O*-GlcNAcylation is associated with several pathological conditions like cancer, diabetes, and neurodegenerative diseases (30–33).

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There is no consensus sequence for *O*-GlcNAc modification. However, it is often observed on the same or proximal Ser/Thr residues that are used for phosphorylation, thereby negatively regulating phosphorylation (23). These two modifications can regulate each other by competitively blocking the site (34), sterically hindering the addition at an adjacent or proximal site (26), or by influencing their respective enzymes (35, 36). This type of reciprocal interplay between *O*-GlcNAcylation and phosphorylation to regulate protein functions is observed on several proteins, including intermediate filaments like neurofilaments (neurofilament–M) and cytoskeleton-associated proteins like Tau (37, 38). With recent advances in mass spectrometry, more complex and extensive cross-talk between *O*-GlcNAcylation and phosphorylation on many cellular proteins has been discovered (36).

Although *O*-GlcNAcylation (gSer^{29},[5] gSer^{30}, and gSer^{48}) (39) and phosphorylation (Ser(P)^{33} and Ser(P)^{52}) (5, 40) occur at proximal sites on keratin 18, their mutual interplay in regulating its functional properties is largely unexplored. In our previous report, we showed that total *O*-GlcNAcylation on keratins 8/18 can regulate solubility, filament organization, and stability (17). In this study, we aim to understand the site-specific role of K18 *O*-GlcNAcylation in regulating proximal phosphorylation and functional properties. Using a panel of site-specific *O*-GlcNAcylation and phosphorylation mutants of K18 along with antibodies that can detect site-specific phosphorylation, we demonstrate that these two modifications exhibit substantial cross-talk at proximal sites on K18 to regulate solubility, stability, and filament organization and also cellular processes such as migration. We also uncover a novel role for *O*-GlcNAcylation to promote phosphorylation at proximal sites. These findings represent the first detailed characterization of site-specific interplay between *O*-GlcNAcylation and phosphorylation of intermediate filament protein along with identification of a unique, phosphorylation-promoting role for *O*-GlcNAcylation.

Experimental Procedures

Reagents—HHL17 (human hepatocyte line 17) cells were a gift from Dr. A. H. Patel (Institute of Virology, Glasgow, UK) (41). The K18 phosphorylation-specific antibodies K18-Ser(P)^{33} (clone IB4) and anti K18-Ser(P)^{52} (clone 3055) were a gift from Prof. Bishr Omary (Michigan Medical School). Cell culture reagents were obtained from Invitrogen. The antibodies used in this study were mouse keratin 18 clone CY-90 (C8541), mouse keratin 8 clone M20 (C5301), and mouse HRP (A4416) (Sigma); mouse anti-*O*-GlcNAc clone RL-2 (MA1-072, Affinity Bioreagents); mouse Ser/Thr phosphorylation clone 22a (612549, BD Biosciences, Clontech); anti-GAPDH clone ABM22C5 (10-10011, Abgenex); rabbit HRP (sc-2004, Santa Cruz Biotechnology); and mouse Alexa Fluor 568 (A11004, Molecular Probes, Invitrogen). Cycloheximide, okadaic acid, PUGNAc, and fibronectin were purchased from Sigma-Aldrich (St. Louis, MO). WGA-Sepharose beads, the PVDF membrane, and the ECL kit were purchased from Amersham Biosciences. Cultureware was obtained from Nunclon. Phusion polymerase and restriction enzymes were from New England Biolabs. All other chemicals were purchased locally and were of analytical grade.

Cell Culture—The HHL17 cell line was routinely cultured and maintained in DMEM containing 0.03% glutamine, 10 units/ml penicillin G-sodium, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B, and 10% FBS at 37 °C and 5% CO_2. For different drug/inhibitor treatments, cycloheximide, PUGNAc, and okadaic acid were used at concentrations of 200 μM/ml, 100 μM/ml, and 400 nM/ml, respectively, for various times.

Plasmids, Site-directed Mutagenesis, Cloning, and Stable Expression—Human K18 WT-YFP cDNA (a gift from Dr. Rudolf Leube and Dr. Reinhard Windoffer, Johannes Gutenberg University) and K18 *O*-GlcNAcylation triple mutant (TM) (S29A/S30A/S48A) cDNA (a gift from Prof. Bishr Omary, Michigan Medical School) were used to generate site-specific (Ser-to-Ala) single and double *O*-GlcNAc mutants of K18 using the QuikChange II site-directed mutagenesis kit according to the protocol of the manufacturer. K18-WT and all mutants were further cloned into EYFP-lentiviral vector (42). Using the QuikChange II site-directed mutagenesis kit, K18-S33A, K18-S52A, K18-S33D, and K18-S30A/S33D were generated from EYFP-K18-WT cloned in the lentiviral vector. The primers used for site-directed mutagenesis are listed in supplemental Table 1.

Infectious lentivirus to express K18-WT and various mutants was produced as described previously (42). To generate K18-WT and various K18 mutants expressing stable cell lines, HHL-17 cells were infected with infectious lentivirus in the presence of Polybrene (8 μg/ml) at 80–90% confluence. After infection (24 h), stable cells expressing the transgene were selected and maintained in complete medium containing puromycin (0.8 μg/ml).

Protein Isolation and Immunoblotting—Total cell lysates were prepared by solubilizing HHL17 cells in 2% SDS cell lysis buffer (62.5 mM Tris and 2% SDS (pH 6.8)). Differential extraction of soluble and filamentous keratin was performed as described in Ref. 17. In brief, soluble keratins were extracted by resuspending cells in phosphate-buffered saline containing 1% Nonidet P40, 5 mM EDTA, protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 25 μg/ml aprotinin), and phosphatase inhibitor mixture for 20 min at 4 °C, followed by centrifugation (16,000 × *g*, 1 h.). The supernatant was collected as the soluble fraction, and the resulting insoluble pellet was solubilized in 2% SDS cell lysis buffer (pellet). Proteins from total, soluble, and insoluble lysates in 1× Laemmli sample buffer were resolved on 12% SDS-PAGE, transferred to PVDF membranes, and probed with antibodies as indicated. Membranes were washed three times with Tris-buffered saline (20 mM Tris and 500 mM NaCl (pH 7.5)) containing 0.1% Tween 20 for 30 min, incubated with anti-mouse or anti-rabbit secondary antibodies coupled to horseradish peroxidase, and visualized using enhanced chemiluminescence. After Western blotting, the membrane was stained with Coomassie Brilliant Blue R-250, which served as a control

[5] The abbreviations used are: gSer^{29}, *O*-GlcNAcylated Ser^{29}; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate; WGA, wheat germ agglutinin; TM, triple mutant; EYFP, enhanced YFP; OA, okadaic acid; CHX, cycloheximide.

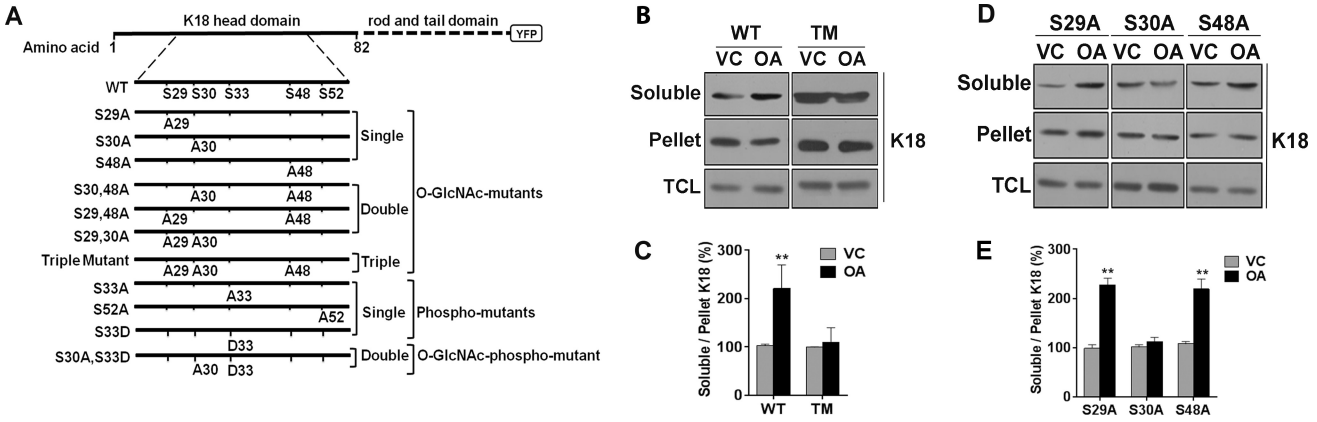

FIGURE 1. **Phosphorylation-dependent solubility is impaired in Ser^{30} *O*-GlcNAc mutants of keratin 18.** *A*, schematic outlining YFP-tagged keratin 18, the WT, and various mutants of *O*-GlcNAcylation sites (gSer^{29}, gSer^{30}, and gSer^{48}) and phosphorylation sites (Ser(P)^{33} and Ser(P)^{52}) used to generate stable HHL17 lines. *B* and *D*, similar numbers of cultured cells were treated for 2 h either with DMSO vehicle control (*VC*) or OA to extract the soluble and pellet fraction or total cell lysate (*TCL*) in identical volumes. Three parts of the soluble fraction (45 μl) and one part each of pellet and total cell lysate (15 μl) were immunoblotted for K18. B, Fractions of HHL17 cells expressing K18-WT and K18-TM and D, Fractions of HHL17 cells expressing single *O*-GlcNAc mutants of K18 (S29A, S30A, and S48A). *C* and *E*, densitometric quantification (average of three independent experiments, including *B* and *D*) indicating the percent change in keratin 18 solubility, which is calculated as the ratio of K18 in soluble and pellet fractions. The ratios in *VC* were taken as 100%. **, $p < 0.01$; unpaired Student's *t* test. *Error bars* represent mean ± S.E.

for equal loading. For immunoprecipitation, cells were lysed in phosphate buffer (PBS (150 mM NaCl and 10 mM phosphate (pH 7.4)) containing 2% Empigen, phosphatase, and protease inhibitor mixture, followed by immunoprecipitation as described in Ref. 17.

Purification of the Glycosylated Form of K8 and 18—To differentially extract cellular K8 and K18 fractions, total cell lysates were resolved on 10% PAGE along with a prestained protein ladder. After electrophoresis, the gel was incubated in 4 M sodium acetate solution for 2 h at −20 °C. Proteins in the gel can be visualized as clear transparent bands against the white opaque gel because of precipitation of free SDS in the gel. The protein bands in the region of K8 (52 kDa) and keratin 18 (48 kDa) were separately collected in microcentrifuge tubes by excising the gel with reference to standard molecular weight markers. The gel pieces were crushed in 500 μl of extraction buffer (0.5% SDS, 150 mM NaCl, and 62.5 mM Tris (pH 6.8)), and the tubes were vortexed vigorously for 45 min at room temperature. Following this, the samples were centrifuged for 5 min at 10,000 × *g*, and the supernatant was collected into a fresh tube. SDS from the extracted samples was removed by adding 150 mg of activated SM-2 Biobeads, followed by incubation at room temperature for 2 h with gentle rocking. The sample was centrifuged at 2000 × *g* for 5 min at room temperature to pellet the SM-2 Biobeads, and the supernatant containing cellular fractions of either keratin 8 or 18 was collected and stored at −20 °C. *O*-GlcNAcylated proteins from these fractions were extracted using WGA-Sepharose beads as described previously (17). Glycosylated K8 and 18 from these samples were immunoprecipitated using K8- and K18-specific monoclonal antibodies as described previously (17).

Immunostaining and Fluorescence Imaging of Cells Expressing EYFP-K18—HHL17 cells stably expressing EYFP-K18-WT; single, double, or triple *O*-GlcNAcylation mutants; and phosphomutants were immunostained for K8 as described previously (17). Images were acquired on a Zeiss LSM510 META confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) using an oil immersion ×63 Plan Apochromat phase-contrast objective (numerical aperture, 1.4) and processed using LSM510 imaging software, version 4.2.

Scratch Healing Assay—For wound healing assays, 6-well culture dishes were coated overnight with 10 μg/ml fibronectin in serum-free DMEM at 4 °C. HHL17 cells stably expressing *O*-GlcNAcylation and phosphorylation site-specific mutants were seeded at a density of 1 million cells/ml of complete medium and incubated at 37 °C for 24 h in a CO_2 incubator. The cells were serum-starved for 24 h for inhibiting cell proliferation. A straight, uniform wound (~400 μm in width) was made using a micropipette tip on the monolayer, and the cells were maintained in serum-free DMEM. The wound closure of cells in response to the immobilized fibronectin was measured for 25 h by time-lapse imaging of at least three different positions across the length of the wound using a Carl Zeiss inverted microscope at ×10 magnification.

Densitometry Quantitation and Statistical Analysis—Densitometric quantitation of scanned images was performed by ImageJ 1.43 software (National Institutes of Health). Band intensities were normalized to respective loading controls. Statistical analysis was performed using GraphPad Prism 5. Significance was analyzed by unpaired Student's *t* test for two samples and two-way analysis of variance for grouped data. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ were considered significant. Photoshop (CS2, Adobe) was used for preparing the figures.

Results

Phosphorylation-induced Solubility of Keratin 18 Is Dependent on Its O-GlcNAcylation at Serine 30—Because both *O*-GlcNAcylation and phosphorylation on keratin 18 are known to increase its solubility (17), we investigated whether perturbing keratin 18 *O*-GlcNAcylation alters its phosphorylation-dependent solubility. For this purpose, we generated stable lines of HHL17 cells expressing various *O*-GlcNAcylation and phosphorylation mutants of K18, as depicted in Fig. 1*A*. The expression of the YFP-tagged K18 transgene in these stable

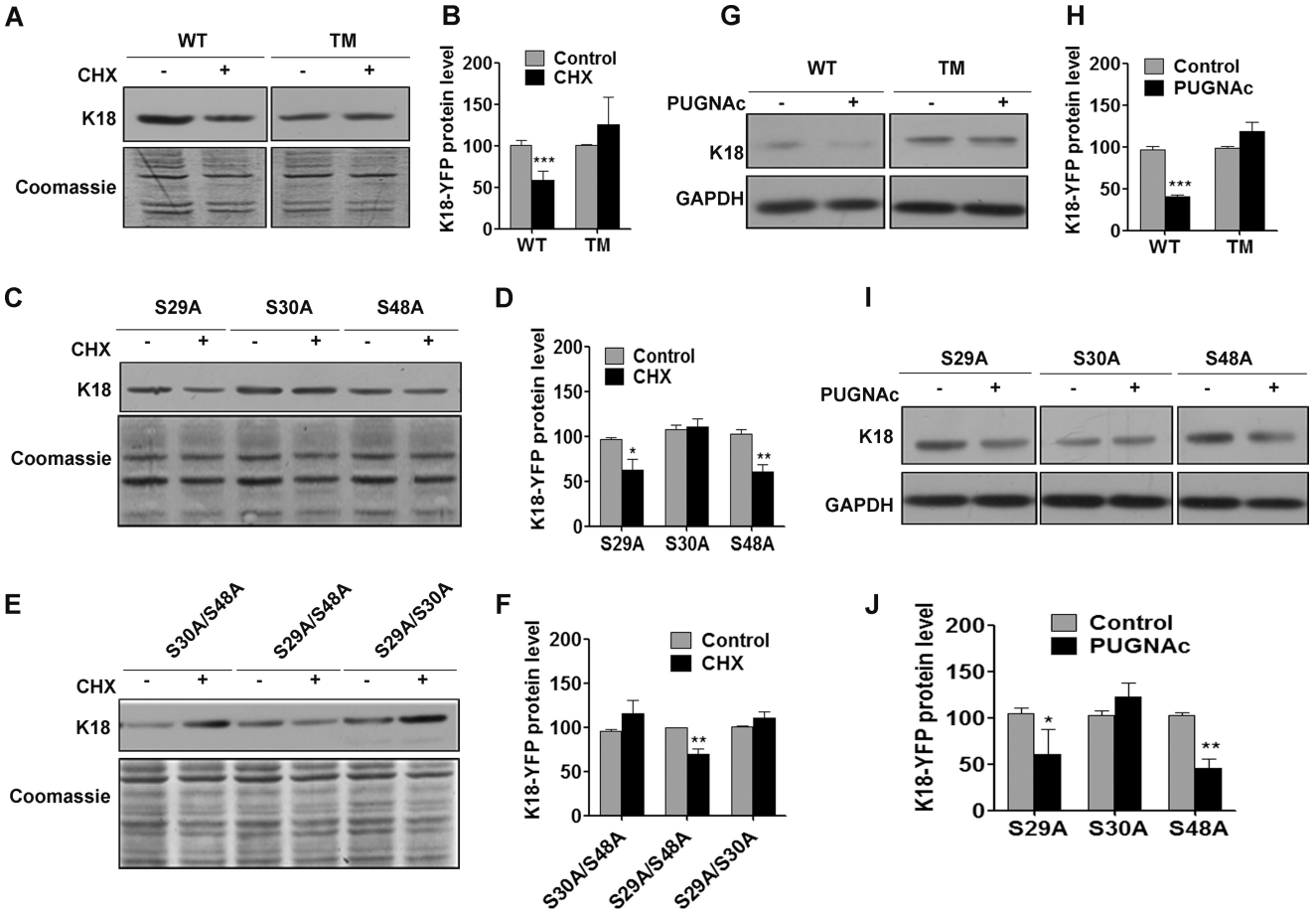

FIGURE 2. ***O*-GlcNAcylation at Ser[30] regulates the stability of K18.** *A*, *C*, and *E*, K18 immunoblots of equal total proteins from CHX-treated (200 μM for 24 h) or control HHL17 cells stably expressing K18-WT or TM (*A*) or single *O*-GlcNAc mutants (*C*) of K18 and double *O*-GlcNAc mutants of K18 (*E*). Coomassie staining of blots serves as a loading control. *B*, *D*, and *F*, densitometric quantification (average of three independent experiments, including *A*, *C*, and *E*) indicating changes in total keratin 18 levels in control and CHX-treated cells (***, $p < 0.001$ (*B*); *, $p < 0.05$ (*D*); **, $p < 0.01$ (*F*); unpaired Student's *t* test). *G* and *I*, K18 immunoblot of equal total proteins from PUGNAc-treated (100 μM for 48 h) or control HHL17 cells expressing K18- WT or TM (*G*) and single *O*-GlcNAc mutants (*I*) of K18. The GAPDH immunoblot served as a loading control. *H* and *J*, densitometric quantification of *G* and *I*, indicating changes in total keratin 18 levels in control and PUGNAc-treated cells (average of three independent experiments, including *G* and *I*) (***, $p < 0.001$ (*H*); *, $p < 0.05$; **, $p < 0.01$ (*J*); unpaired Student's *t* test). *Error bars* represent mean ± S.E.

lines was confirmed by immunofluorescence and immunoblotting (data not shown). To assess phosphorylation-induced solubility, we treated stable cell lines with OA, a broad-spectrum phosphatase inhibitor that increases phosphorylation and thereby the solubility of keratin 8/18 filaments. OA treatment led to a notable increase in solubility of K18-WT, whereas the solubility of K18-TM (S29A/S30A/S48A) was unaltered (Fig. 1, *B* and *C*). Similarly, stable HHL17 cells expressing K18-S29A and K18-S48A mutants exhibited an increase in solubility, whereas mutation of Ser[30] prevented OA-induced solubility of K18 (K18-S30A) (Fig. 1, *D* and *E*), similar to the triple *O*-GlcNAc mutant of K18 (Fig. 1, *B* and *C*). This suggests that phosphorylation-induced solubility of K18 is dependent on its *O*-GlcNAcylation at Ser[30].

O-GlcNAcylation at Serine 30 Regulates the Stability of K18—O-GlcNAcylation of keratin 18 reduces its stability by mediating ubiquitination-dependent proteasomal degradation (17). Because disassembly and sequestration of keratin 18 subunits into a soluble pool could be a prerequisite for its degradation (43), we predicted that K18 *O*-GlcNAcylation at serine 30 could target it for degradation and reduce its stability. The stability of the WT and various K18 *O*-GlcNAcylation mutants was assessed after inhibiting protein synthesis with cycloheximide. Compared with K18-WT, which shows a significant reduction upon CHX treatment, the level of K18-TM remained unchanged (Fig. 2, *A* and *B*). It was interesting to note that, among all K18 *O*-GlcNAc mutants, those that harbored S30A were highly stable with an unaltered protein level upon cycloheximide treatment (Fig. 2, *C–F*). To test whether increasing *O*-GlcNAcylation at Ser[30] could reduce the stability of K18, K18-WT and various *O*-GlcNAc mutant-expressing cells were treated with PUGNAc, a potent *O*-GlcNAcase inhibitor. PUGNAc treatment is known to cause increased *O*-GlcNAcylation and subsequent degradation of K8/18 (17). However, K18-TM, which lacks all sites of glycosylation, exhibited resistance to PUGNAc-mediated degradation (data not shown) (17). Both K18 mutants with the S30A mutation (K18-TM and K18-S30A) exhibited no reduction upon PUGNAc treatment, whereas K18 with Ser[30] (K18-WT, K18-S29A, and K18-S48A) showed a marked reduction in protein levels (Fig. 2, *G–J*). These results conclusively indicate that *O*-GlcNAcylation specifically at Ser[30] determines the stability of K18.

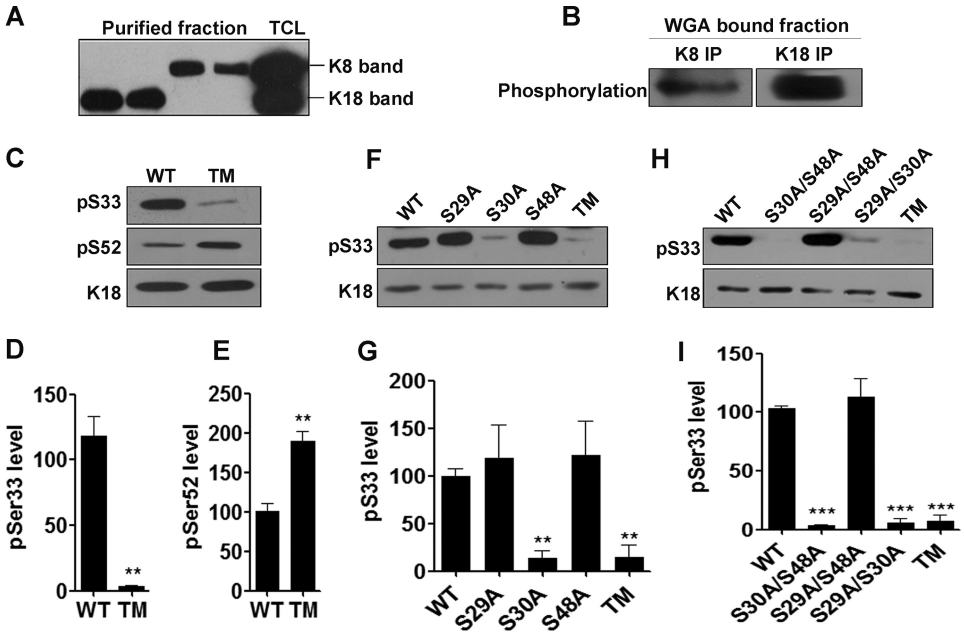

FIGURE 3. ***O*-GlcNAcylation at serine 30 positively regulates phosphorylation of K18 at serine 33.** Immunoblots of K8, K18, *O*-phosphorylation, Ser(P)^{33} (IB4), and Ser(P)^{52} (3055) as indicated. *A*, K8 and K18 immunoblot of purified keratin 8 and 18 fractions (as described under "Experimental Procedures") to ensure the purity of the preparation. Total cell lysate (*TCL*) served as a marker to indicate the molecular weight of K8 and K18. *B*, K8 and K18 were immunoprecipitated from WGA-bound K8 or K18 fractions and immunoblotted with phospho-Ser/Thr. *C*, *F*, and *H*, total cell lysates of HHL17 cells stably expressing K18-WT and TM (*C*), single *O*-GlcNAc mutants of K18 (*F*), and double *O*-GlcNAc mutants (*H*) of K18 were immunoblotted with K18Ser(P)33 (IB4) or K18Ser(P)52 (3055) antibodies. *D*, *E*, *G*, and *I*, densitometric quantification (average of three independent experiments, including *C*, *F*, and *H*) showing levels of K18 Ser(P)^{33} and K18 Ser(P)^{52} (**, $p < 0.01$, unpaired Student's *t* test (D and E); **, $p < 0.01$; *** $p < 0.001$; one-way analysis of variance (*G* and *I*). *Error bars* represent mean ± S.E.

O-GlcNAcylation and Phosphorylation Exhibit Both a Cooperative and Antagonistic Relationship at Proximal Sites on K18—Both *O*-GlcNAcylation and phosphorylation on keratin 18 correlate positively with solubility (17). To address whether these two modifications on keratin 18 exhibit cross-talk or act independently to regulate K18 properties, we first investigated whether these two modifications coexist on K18. To achieve this, keratin 8 and keratin 18 were separated, purified, and renatured from preparative SDS-PAGE gels (as described under "Experimental Procedures"). The purified K8 and K18 fractions were clean, with no detectable cross-contamination of keratin 8 and 18 isoforms (Fig. 3*A*). *O*-GlcNAcylated proteins from these fractions were purified on WGA beads, followed by immunoprecipitation to enrich *O*-GlcNAcylated K8 and K18. Purified *O*-GlcNAcylated K8 and K18 showed notable levels of phosphorylation (Fig. 3*B*), suggesting co-existence of these two modifications on K8 and K18. We further aimed to investigate the relationship/cross-talk between these two modifications on K18 by assessing the levels of site-specific phosphorylation (Ser(P)^{33} and Ser(P)^{52}) on various *O*-GlcNAc mutants of K18. The site-specific K18-Ser(P)^{33} and K18-Ser(P)^{52} phosphoantibodies have been reported previously (5, 40). The specificity of K18-Ser^{33} phosphoantibody was further confirmed using cells expressing phosphorylation mutants of K18 (K18-S33A and S52A) (data not shown). K18-TM showed a significant reduction in basal levels of Ser(P)^{33}, whereas the levels of Ser(P)^{52} were notably higher compared with K18-WT (Fig. 3, *C–E*), suggesting that *O*-GlcNAcylation on K18 exhibits a cooperative relationship with Ser(P)^{33} and a reciprocal relationship with Ser(P)^{52}. The existence of an inverse relationship between gSer^{48} and Ser(P)^{52} on K18 has been indicated earlier in *in vitro* studies (44). To investigate the *O*-GlcNAc site/s on K18 that aid in phosphorylation of Ser^{33}, we assessed the basal levels of Ser(P)^{33} in various single and double *O*-GlcNAc mutants of K18. Interestingly, all *O*-GlcNAc mutants of K18 harboring S30A exhibited significantly lower levels of Ser(P)^{33} (Fig. 3, *F–I*), suggesting a dependence on *O*-GlcNAcylation of K18 at Ser^{30} for the occurrence of phosphorylation at Ser^{33}.

O-GlcNAcylation at Ser^{30} Determines the Occurrence of Phosphorylation at Ser^{33} on K18—Because abrogation of *O*-GlcNAcylation of K18 at Ser^{30} prevents its phosphorylation at Ser^{33}, we set to investigate whether this cooperative relationship can be observed when *O*-GlcNAc levels on K18 are altered dynamically. PUGNAc treatment of K18-WT-expressing cells led to a time-dependent increase in total *O*-GlcNAcylation with a concomitant increase in phosphorylation of K18 at Ser^{33} (Fig. 4, *A* and *B*). This PUGNAc-mediated increase in Ser(P)^{33} levels was seen only in K18 mutants where Ser^{30} *O*-GlcNAcylation site was available, *viz.* K18-S29A and K18-S48A (Fig. 4, *C* and *D*). These results confirm that *O*-GlcNAcylation of K18 at Ser^{30} can positively influence phosphorylation at Ser^{33}. Because the dynamics of phosphorylation are regulated by the activities of specific kinases and phosphatases acting on that site, we predicted that inhibition of phosphatases by OA could restore Ser(P)^{33} levels in K18-S30A mutants. OA treatment led to a notable increase in Ser(P)^{33} levels on K18-WT and K18 *O*-GlcNAc mutants where Ser^{30} *O*-GlcNAcylation can occur, *viz.* K18-S29A and K18-S48A (Fig. 4, *E* and *F*). This suggests that *O*-GlcNAcylation of K18 at Ser^{30} is essential for the occurrence of phosphorylation at serine 33. We aimed to further investigate whether this cooperativity is mutual, with Ser(P)^{33} regulating *O*-GlcNAcylation at Ser^{30}. To address

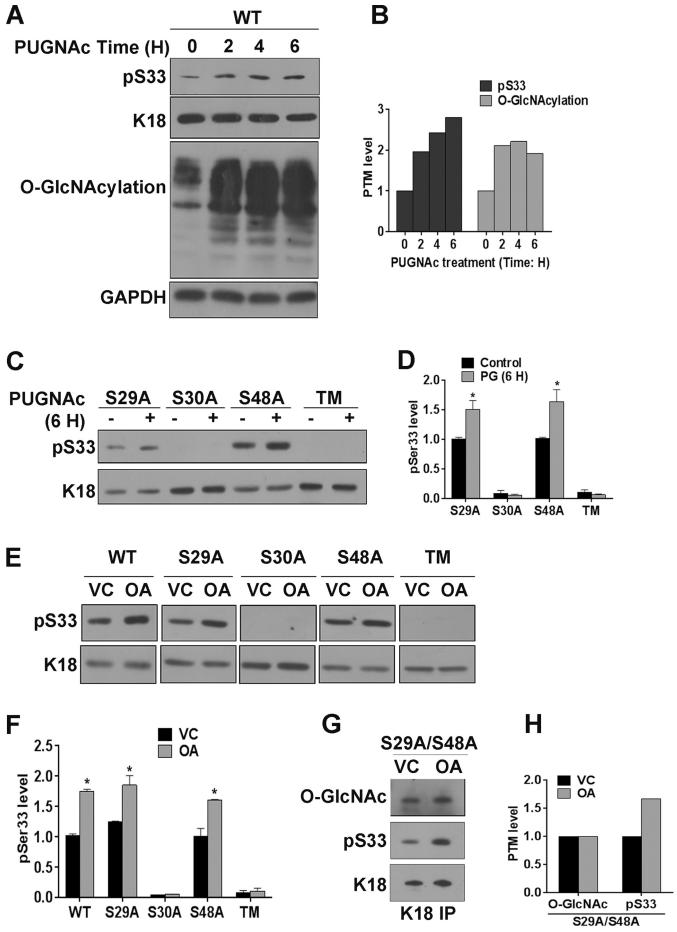

FIGURE 4. ***O*-GlcNAcylation of keratin 18 at serine 30 supports dynamic phosphorylation at serine 33.** *A*, *C*, and *E*, immunoblot of equal total proteins from HHL-17 cells expressing WT or *O*-GlcNAc mutants. *A*, cells expressing K18-WT were treated with PUGNAc for different times (0, 2, 4 and 6 h). *VC*, vehicle control. Shown are cells expressing single and triple *O*-GlcNAc mutants treated with PUGNAc for 6 h (*C*) and OA for 2 h (*E*). *B*, densitometric analysis of total *O*-GlcNAcylation and K18 phosphorylation at Ser^{33} in *A*. *D* and *F*, densitometric analysis of $Ser(P)^{33}$ levels on K18 (average of three independent experiments, including *C* and *E*; *, $p < 0.05$; Student's *t* test). *Error bars* represent mean ± S.E. *G*, immunoprecipitate (*IP*) of the double *O*-GlcNAc mutant K18-S29A/S48A immunoblotted with *O*-GlcNAc, $Ser(P)^{33}$, and K18. *H*, densitometric analysis of *O*-GlcNAcylation and $Ser(P)^{33}$ on K18. The K18 immunoblot served as a control for loading.

this, we used stable cells where the K18 mutant can be *O*-GlcNAcylated only at serine 30 (K18-S29A/S48A) because antibodies recognizing site-specific *O*-GlcNAcylation on keratin 18 are not available. It was interesting to note that treating these cells with OA led to an increase in $Ser(P)^{33}$ with no notable change in *O*-GlcNAcylation at serine 30 (Fig. 4, *G* and *H*). This suggests that the cooperative interplay between modifications at these two sites is one-sided, with $gSer^{30}$ acting as an upstream switch for phosphorylation at Ser^{33}.

O-GlcNAcylation-mediated Phosphorylation of Keratin 18 at Serine 33 Regulates Its Stability—Because *O*-GlcNAcylation at Ser^{30} induces solubility and subsequent degradation of K18 along with increased phosphorylation at Ser^{33} (Figs. 1–4), it is possible that $Ser(P)^{33}$ mediates the effect of $gSer^{30}$ on the solubility and stability of K18. Although phosphorylation at Ser^{33} has been shown previously to regulate solubility (5, 45) and shear stress-induced reorganization of keratin 8/18 filaments (14), its role in regulating the stability of keratin 18 is still unclear (46). To investigate the role of site-specific phosphorylation on K18 in regulating its stability, cells expressing K18-WT and phosphorylation mutants (K18-S33A and K18-S52A) were treated with cycloheximide. Although K18-WT and K18-S52A exhibited reduced K18 levels upon cycloheximide treatment, the protein levels of K18-S33A remained unchanged, suggesting a role for $Ser(P)^{33}$ in regulating the stability of K18 (Fig. 5, *A* and *B*). It is possible that *O*-GlcNAcylation at serine 30 can influence the stability of K18 either directly by aiding ubiquitination or indirectly by stabilizing $Ser(P)^{33}$, which would lead to increased solubility and subsequent degradation. To understand this, stable cells expressing K18 phosphorylation mutants (K18-S33A and K18-S52A) were treated with PUGNAc to increase *O*-GlcNAcylation on K18. PUGNAc treatment led to reduced levels of K18-S52A but not K18-S33A (Fig. 5, *C* and *D*). These results indicate that *O*-GlcNAcylation at Ser^{30} regulates the solubility and stability of keratin 18 mainly by stabilizing $Ser(P)^{33}$. To confirm this, we assessed the stability of K18 after rescuing the phosphorylation at Ser^{33} by substituting with a phosphomimetic mutation (S33D). However, both K18-S33D and K18-S30A/S33D showed higher stability, as assessed by cycloheximide treatment, suggesting that S33D does not substitute for phosphorylation at Ser^{33} and therefore could not rescue the enhanced stability of K18 upon loss of phosphorylation at Ser^{33} (Fig. 5, *E* and *F*).

O-GlcNAcylation at Serine 30 Is Essential for Maintaining Normal Keratin 18 Filament Organization and Cell Migration—Because $gSer^{30}$ and $Ser(P)^{33}$ cooperate to regulate the solubility and stability of K18, we hypothesized that modifications at these two sites could also regulate filament organization of K18. Intensity quantification of K18-WT filaments across the cell reveal that filament density is highest around the nucleus and gradually decreases toward the cell periphery (Fig. 6, *A* and *B*) (19). This organization was severely affected in both K18-TM and K18-S30A, which displayed increased filament accumulation around the nucleus with collapse of peripheral filaments, whereas the filament organization of the K18-S29A and K18-S48A mutants was similar to K18-WT (Fig. 6, *C–F*). Moreover, rescue of *O*-GlcNAcylation at Ser^{30} in K18-S29A/S48A could restore the abnormal filament organization of K18-TM (Fig. 6, *G* and *H*), implying a key role for $gSer^{30}$ in supporting the filament organization of K18. Because $gSer^{30}$ on K18 leads to phosphorylation at Ser^{33}, we predicted that loss of $Ser(P)^{33}$ could phenocopy the filament organization of K18-S30A and K18-TM. As expected, the K18-S33A mutant exhibited perinuclear aggregation and loss of peripheral filaments, whereas the filament organization of the K18-S52A mutant was similar to K18-WT (Fig. 6, *I* and *J*). This is also supported by previous reports that show that K18-$Ser(P)^{33}$ plays a key role in regulating filament organization and that loss of phosphorylation at this site (K18-S33A) causes the collapse of filaments around the nucleus (5). To confirm these results, we assessed the filament organization in the K18-S33D and K18-S30A/S33D phosphomimetic mutants. The collapsed filament organization was not rescued in either of these stable lines, suggesting that S33D is unable to compensate for phosphorylation at

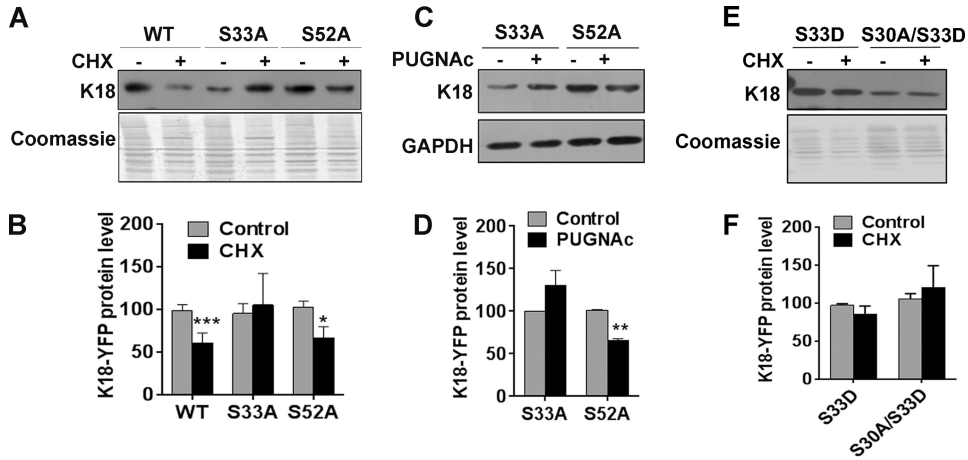

FIGURE 5. **Phosphorylation at Ser^{33} regulates the stability of keratin 18.** *A* and *E*, equal total proteins from untreated or CHX-treated (200 μM for 24 h) cells expressing WT, S33A, and S52A (*A*) and S33D and S30A/S33D (*E*) mutants of K18 immunoblotted with K18. *C*, untreated or PUGNAc-treated (100 μM for 48 h) cells expressing phosphomutants (S33A and S52A) of K18 were immunoblotted with K18 and GAPDH as indicated. The Coomassie-stained blot in *A* and *E* and GAPDH in *C* served as a loading control. *B*, *D*, and *F*, densitometric analysis indicating relative levels of K18 (average of three independent experiments, including *A*, C, and *E*; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Student's *t* test). *Error bars* represents mean ± S.E.

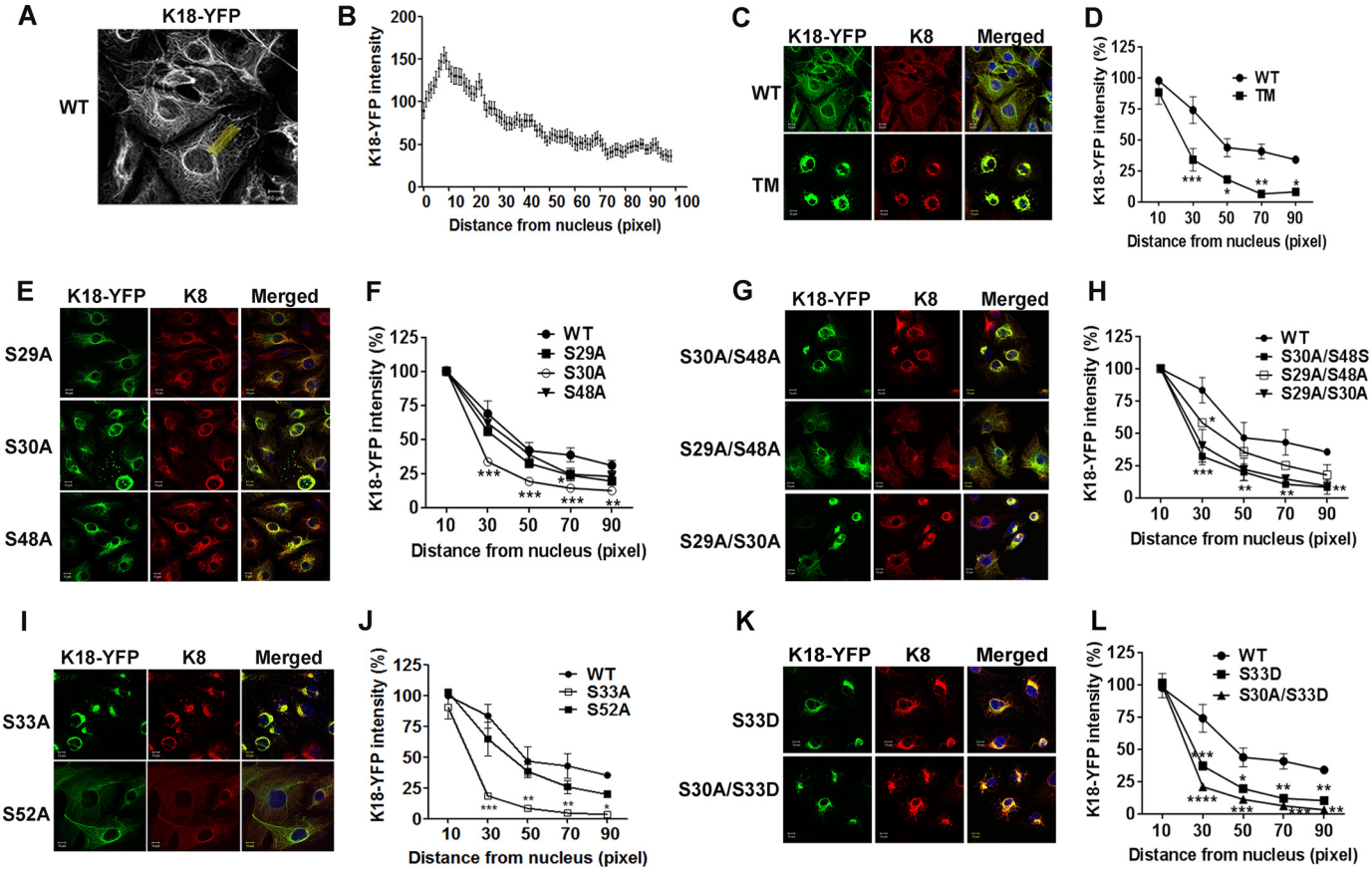

FIGURE 6. ***O*-GlcNAcylation of K18 at Ser^{30} is essential for maintaining normal filament organization.** *A*, immunofluorescence image of cells expressing WT-K18, demonstrating the method adopted to quantitate K18-YFP intensity from the perinuclear region to the cell periphery. Fluorescence intensity across a straight line of uniform length and width (100 and 30 pixels, respectively) was measured at three different regions per cell. *B*, fluorescence intensity of the keratin network across a cell (perinuclear region to cell periphery). Each point represents mean ± S.E. of three independent experiments with 20 cells/experiment. *C*, *E*, *G*, *I*, and *K*, immunofluorescence images of cells expressing K18-WT and K18-TM (*C*), single *O*-GlcNAc mutants (*E*), double mutants (*G*), phosphomutants (*I*), and phosphomimetic mutant (*K*) of K18. Images show K18-YFP (*green*), K8 (*red*), and merged images with DAPI (*blue*). *Scale bars* = 10 μm. *D*, *F*, *H*, *J*, and *L*, quantification of fluorescence intensity of the K18-YFP filament network of cells in *C*, *E*, *G*, *I*, and *K* (average of three independent experiments; 20 cells/experiment; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; two-way analysis of variance followed by Bonferroni post-tests). *Error bars* represent mean ± S.E.

Ser^{33} (Fig. 6, *K* and *L*). These results imply that gSer^{30}-dependent phosphorylation of Ser^{33} is essential for maintaining the normal filament organization of keratin 18. To further investigate the cellular impact of the cooperativity between gSer^{30} and Ser(P)^{33}, we assessed the migration of site-specific *O*-GlcNAcylation and phosphorylation mutants on fibronectin substrate in a scratch wound assay. It is interesting to note that only S30A and S33A *O*-GlcNAc and phosphomu-

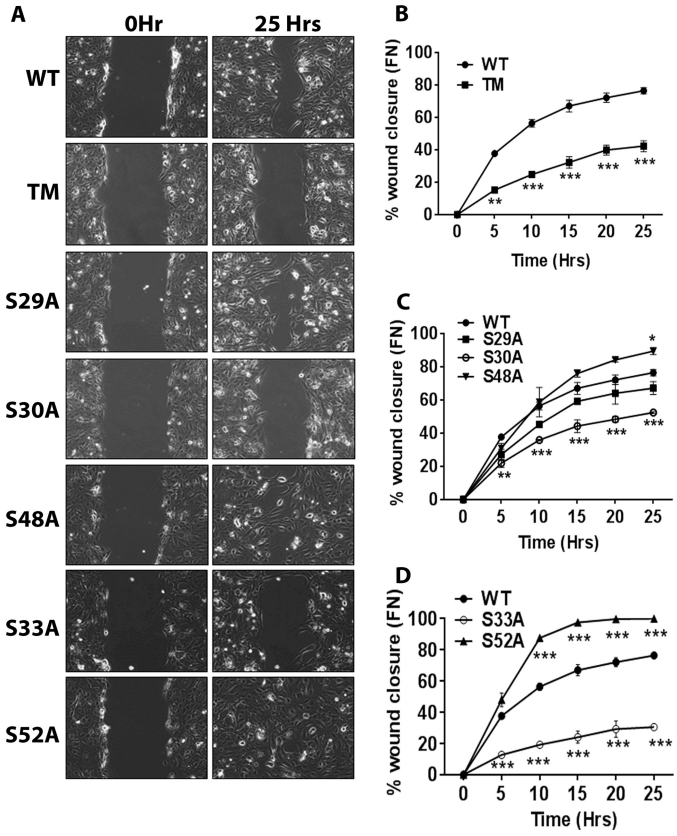

FIGURE 7. ***O*-GlcNAcylation and phosphorylation of K18 regulate the cellular migration on fibronectin.** *A*, comparison of migration of HHL17 cells stably expressing K18-WT and the indicated *O*-GlcNAcylation and phosphorylation mutants of K18 by scratch wound assay on fibronectin (*FN*). *B*, *C*, and *D*, quantitation of percent wound closure of two independent experiments, each experiment at three different points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; two-way analysis of variance followed by Bonferroni post-tests). *Error bars* represent mean ± S.E.

tants of K18, respectively, exhibited reduced migration and wound closure compared with the WT (Fig. 7, *A–D*). The other *O*-GlcNAcylation and phosphorylation mutants of K18 (S29A, S48A, and S52A) showed migration similar to K18-WT (Fig. 7, *A–D*). These results further highlight the importance of Ser^{30} *O*-GlcNAcylation-mediated Ser^{33} phosphorylation on keratin 18 in modulating cellular processes like migration.

Discussion

On most phosphoglycoproteins discovered so far, *O*-GlcNAcylation and phosphorylation antagonize each other in site occupancy and regulation of protein functions (47). However, this seems unclear for K18 because both of these modifications exhibit similar changes under certain conditions, like mitotic arrest (48) and heat stress (49), whereas, during induced hepatotoxicity, they exhibit an inverse relationship (50). Of the two phosphorylation sites mapped on keratin 18, $Ser(P)^{33}$ causes increased solubility during mitosis and filament reorganization in response to shear stress (5, 14), whereas $Ser(P)^{52}$ is known to regulate filament reorganization under stress conditions (51). Mutating the three known *O*-GlcNAcylation sites of K18 (Ser^{29}, Ser^{30}, and Ser^{48} to Ala) led to altered filament organization and decreased both solubility and degradation of keratin 18 (17). However, it is still unclear whether this regulatory role of *O*-GlcNAcylation is site-specific and whether it is dependent on its cross-talk with phosphorylation. Here we demonstrate a hitherto unreported, positive cross-talk between Ser^{30} *O*-GlcNAcylation and Ser^{33} phosphorylation on K18, and this cross-talk is responsible for regulating K18 solubility, filament organization, and stability (Fig. 8).

Solubility of the K8/18 network is known to play an essential role in degradation because filament or aggregated K8/18, although ubiquitinated, cannot be degraded by proteasomes unless they are disassembled into soluble form (43). Phosphorylation at serine 33 plays an important role in the solubility and reorganization of keratin 8/18 filament network mainly during mitosis and shear stress (5, 14). Moreover, it is also a binding site for the 14-3-3 class of proteins, which further aids in the solubility of K8/18 filaments (45). In addition to this, OA-mediated inhibition of phosphatases led to an increase in $Ser(P)^{33}$ levels along with enhanced binding to 14-3-3 in HT-29 epithelial cells (5, 45), whereas abrogation of phosphorylation at Ser^{33} had no effect on OA-induced solubility of the K18-S33A mutant when expressed in NIH3T3 fibroblasts (5). Although these reports strongly indicate a role for $Ser(P)^{33}$ in mediating K18 solubility, they also point to varied effects of modifications on the solubility of keratin 18 in different cell types (5, 45). Of the three sites of *O*-GlcNAcylation, it was interesting to observe that loss of $gSer^{30}$ was enough to stall the phosphorylation-induced solubility during OA treatment (Fig. 1). Further to this, the loss of $gSer^{30}$ also led to an increased stability of K18 (Fig. 2), suggesting that *O*-GlcNAcylation at Ser^{30} could be central to not only phosphorylation-dependent solubility but also subsequent degradation. On most proteins, *O*-GlcNAcylation protects against proteasomal degradation either directly or by antagonizing phosphorylation (52). *O*-GlcNAcylation could also target the protein for degradation, as in the case of the kinase CK2, where *O*-GlcNAcylation at Ser^{347} leads to degradation by antagonizing phosphorylation at Thr^{344} (53). In either of the cases, *O*-GlcNAcylation regulates protein stability by antagonizing phosphorylation. Interestingly, this seemed contrary on K18 because phosphorylation-induced solubility and degradation were totally dependent on *O*-GlcNAcylation at serine 30 (Figs. 1 and 2).

The co-existence of both these modifications on K18 seems plausible because they exhibit similar changes during conditions associated with increased solubility and filament reorganization. Indeed, the presence of notable phosphorylation on *O*-GlcNAcylated K8 and K18 species confirms the co-occurrence of these modifications (Fig. 3*B*). However, because of the presence of multiple sites of both these modifications, they could exhibit a mutually exclusive relationship at proximal sites but can still co-exist at distal sites. Further analysis using site-specific K18 *O*-GlcNAc mutants along with site-specific K18 phosphorylation antibodies indicate both a cooperative and antagonistic interplay between these modifications at proximal sites (Fig. 3, *C–E*). Although *O*-GlcNAcylation at Ser^{30} promotes phosphorylation at Ser^{33} (Fig. 3, *F–I*), *O*-GlcNAcylation at Ser^{48} antagonizes phosphorylation at Ser^{52} (data not shown). Although a previous study supports the presence of a reciprocal relationship between $gSer^{48}$ and $Ser(P)^{52}$ (44), the existence of a promotive/cooperative relationship

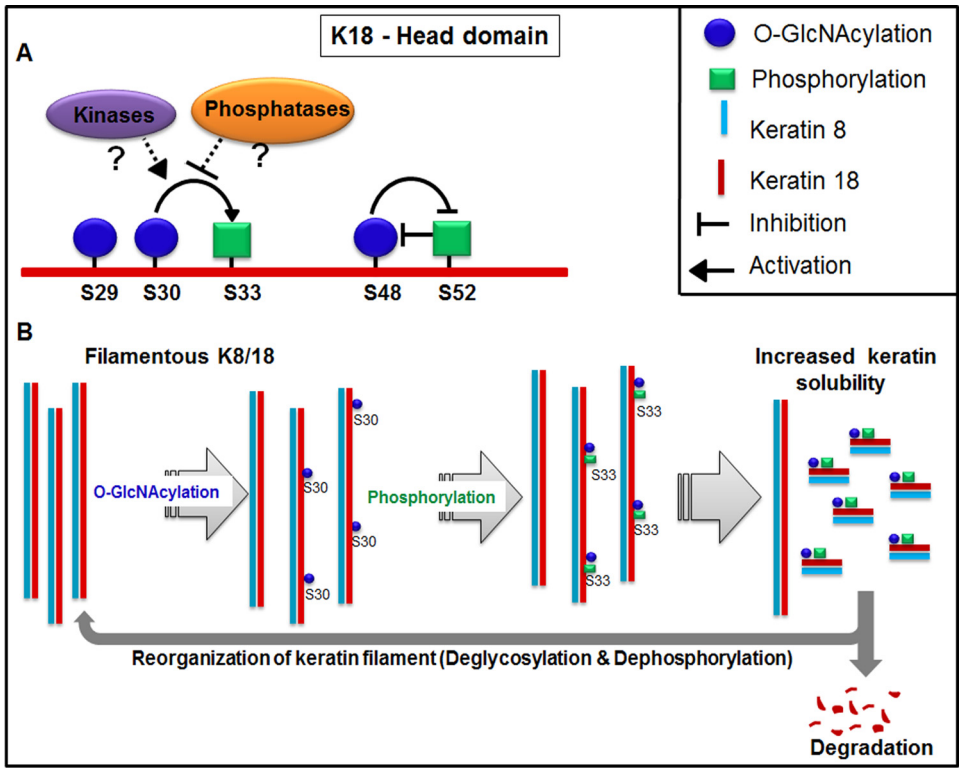

FIGURE 8. **Model depicting cross-talk between *O*-GlcNAcylation and phosphorylation at proximal sites on keratin 18 to regulate the solubility, degradation, and reorganization of the keratin filament network.** *A*, *O*-GlcNAcylation at serine 30 facilitates proximal phosphorylation at serine 33 either by aiding the interaction with specific kinases or hindering the interaction with phosphatases. *O*-GlcNAcylation at serine 48 exhibits a mutually exclusive relationship with phosphorylation at serine 52. *B*, serine 30 *O*-GlcNAcylation of keratin 18 filaments promotes stabilization of serine 33 phosphorylation, which leads to filament disassembly and a concomitant increase in soluble keratin 18. The soluble keratin 18 subunits are either targeted for proteasomal degradation or incorporated into the filament network upon loss of *O*-GlcNAcylation and phosphorylation at serines 30 and 33, respectively.

between gSer^{30} and Ser(P)^{33} is both interesting and novel. Furthermore, global high-throughput phosphoproteomics upon elevated *O*-GlcNAc levels does support the prevalence of such a non-reciprocal, co-operative interplay on many proteins (36). Such a type of synergistic relationship between *O*-GlcNAcylation and phosphorylation exists in the catalytic cleft of the calcium/calmodulin-dependent kinase IV (CAMKIV) kinase, albeit at distal sites, where mutation of the glycosylation sites Thr^{57}/Ser^{58} to alanine on calcium/calmodulin-dependent kinase IV causes a drastic reduction in basal activating phosphorylation at Thr^{200} (35).

This cooperative interplay between gSer^{30} and Ser(P)^{33} not only responds to an acute pharmacological increase in *O*-GlcNAcylation but also seems to be unidirectional, with gSer^{30} acting as a switch for phosphorylation at Ser^{33} (Fig. 4). Thus, *O*-GlcNAcylation at Ser^{30} can facilitate phosphorylation at Ser^{33} either by aiding or hindering interactions with kinases and phosphatases, respectively. The possibility of gSer^{30} facilitating interaction with kinase(s) seems more probable because the loss of Ser(P)^{33} in K18-TM and S30A mutants could not be restored upon inhibiting phosphatases (Fig. 4, *E* and *F*). It is also important to note that OA-induced enhanced association of 14-3-3 with K18 is marginally reduced but not completely diminished in the triple glycosylation mutant when expressed in baby hamster kidney fibroblasts (5). Although this suggests that Ser^{33} could still be basally phosphorylated in the absence of glycosylation at Ser^{30}, it also hints at the possibility of different kinases involved in phosphorylating Ser^{33} in different cell systems (5, 14, 54). Overall, these results suggest that gSer^{30} controls the solubility and degradation of K18 by stabilizing phosphorylation at Ser^{33}. This seems plausible because phosphorylation on many proteins acts as a signal for ubiquitination-mediated proteasomal degradation (55). Even on K8, shear stress-induced phosphorylation at Ser^{73} enables E2 ligases to mediate proteasomal degradation (56). Indeed, loss of phosphorylation at Ser^{33} increased the stability of K18, which could not be rescued by increasing *O*-GlcNAcylation on K18 (Fig. 5, *A–D*) or by phosphomimetic substitution at Ser^{33} (Fig. 5, *E* and *F*). Although substitution with negatively charged residues like aspartate rescues loss of Ser/Thr phosphorylation on many proteins, on keratin 18, S33D does not seem to substitute for phosphorylation (Fig. 5, *E* and *F*) (5). These findings imply that *O*-GlcNAcylation at Ser^{30} predominantly aids in the phosphorylation at Ser^{33}, which in turn causes increased solubility and subsequent degradation of K18.

Phosphorylation at Ser^{33} is also a key regulator of K18 solubility and filament organization both in cultured cells and hepatocyte *in vivo* (5, 45, 57). In general, keratins 8/18 exhibit a gradient of filament network with highly bundled filament near the nucleus and fine, thin filaments at the cell periphery (58). As reported previously, expression of K18-S33A mutant led to disruption of filament organization, visualized as highly concentrated perinuclear filaments and a collapsed peripheral filament network, whereas the architecture of K8/18 is unaltered in K18-S52A mutants or K18-WT-expressing cells (Fig. 6, *C*, *D*, *I*, and *J*) (5). Among the *O*-GlcNAcylation mutants, only K18-S30A

mimicked the perinuclear collapsed network of K18, as seen in K18-S33A phosphomutant (Fig. 6, *C–H*). The defects in filament organization in K18-S33A and K18-S30A could not be rescued by S33D mutations, suggesting that, similar to K18 stability, S33D cannot substitute for Ser(P)^{33} to rescue filament organization. These observations further strengthen the regulatory role of gSer^{30} in promoting Ser(P)^{33}, which in turn dictates the solubility, filament organization, and turnover of keratin 18.

The dynamic reorganization of keratin 8/18 filaments is essential for the plasticity of the keratin filament network during many cellular processes, including cell migration. Rapid changes in keratin network dynamics are initiated especially in the lamellipodia of migrating cells (58). It is also important to note that phosphorylation of keratin 8 alone can play a critical role in mediating the migration of epithelial tumor cells and hepatocytes (9, 59). Interestingly, loss of *O*-GlcNAcylation at Ser^{30} or phosphorylation at Ser^{33} on K18 was sufficient to reduce the migrating potential of hepatocytes, indicating that both gSer^{30} and gSer^{33} act cooperatively to impact filament dynamics during cell migration (Fig. 7).

Taken together these results demonstrate a synergism between S30 *O*-GlcNAcylation and Ser^{33} phosphorylation on keratin 18 in regulating its functional properties like solubility, filament organization, and stability (Fig. 7). The cycling of *O*-GlcNAc on cellular proteins is tightly linked to glucose metabolism (60, 61) and is often deregulated in multiple pathological conditions like diabetes and cancer (32). These findings raise the possibility of factors like glucose metabolism to regulate keratin 8/18, mainly in metabolically active cells like hepatocytes. The positive regulation of phosphorylation by proximal *O*-GlcNAcylation opens up new avenue to understand the functional regulation of phosphoglycoproteins. Understanding how *O*-GlcNAcylation can facilitate phosphorylation on K18 will be important because this type of interplay might be as abundant as reciprocal cross-talk.

Author Contributions—P. S. K. performed the experiments, generated mutants, designed the experiments, analyzed the data, interpreted the results, and wrote the manuscript. S. B. conceived and supervised the work, generated the mutants, performed the purification and immunoprecipitation of WGA-bound (glycosylated) keratins, designed the experiments, supervised data analysis, interpreted the results, and wrote the manuscript. R. D. K. conceived and supervised the work, designed the experiments, supervised data analysis, and wrote the manuscript. M. M. V. supervised the work and wrote the manuscript.

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Supplementary table 1. Primer sequences used for site directed mutagenesis: Site directed mutagenesis primers 5'-3' forward and reverse primer sequences, template used for PCR reaction and mutant product after reaction. Nucleotide sequences in bold red letters indicate point mutations. WT= Wild Type, TM= Triple mutant, S= Serine, A= Alanine, D= Aspartate.

Supplementary Table 1

Sr. No.	Mutation	Template	Primer	Sequence (5'-3')	PCR Product
1	S29A	K18 WT	Forward	GGCCGGTC**GC**CAGCGCGGCCAGCG	S29A
			Reverse	CGCTGGCCGCGCTG**GC**GACCGGCC	
2	S30A	K18 WT	Forward	CCGGCCGGTCAGC**GC**CGCGGCC	S30A
			Reverse	GGCCGCG**GC**GCTGACCGGCCGG	
3	S48A	K18 WT	Forward	CGGATCTCCGTG**G**CCCGCTCCACCA	S48A
			Reverse	TGGTGGAGCGGG**C**CACGGAGATCCG	
4	A29S	K18 TM	Forward	CCGGCCGGTC**AG**CGCCGCGGCC	S30A/S48A
			Reverse	GGCCGCG**GC**GCTGACCGGCCGG	
5	A30S	K18 TM	Forward	GGCCGGTCGCC**AG**CGCGGCCAGCG	S29A/S48A
			Reverse	CGCTGGCCGCGCTG**GC**GACCGGCC	
6	A48S	K18 TM	Forward	CGGATCTCCGTG**T**CCCGCTCCACCA	S29A/S30A
			Reverse	TGGTGGAGCGGG**A**CACGGAGATCCG	
7	S33A	K18 WT	Forward	CAGCAGCGCGGCC**GC**CGTCTATGCAGGC	S33A
			Reverse	GCCTGCATAGACG**GC**GGCCGCGCTGCTG	
8	S52A	K18 WT	Forward	GTCCCGCTCCACC**GC**CTTCAGGGGCGGC	S52A
			Reverse	GCCGCCCCTGAAG**GC**GGTGGAGCGGGAC	
9	S33D	K18 WT	Forward	CAGCAGCGCGGCC**GA**CGTCTATGCAGGC	S33D
			Reverse	GCCTGCATAGACG**GA**GGCCGCGCTGCTG	
10	S33D	K18 S30A	Forward	CAGCAGCGCGGCC**GA**CGTCTATGCAGGC	S30A/S33D
			Reverse	GCCTGCATAGACG**GA**GGCCGCGCTGCTG	

Functional Implications of *O*-GlcNAcylation-dependent Phosphorylation at a Proximal Site on Keratin 18

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