UNIVERSITY OF CALIFORNIA SAN DIEGO

Spatial Organization of Glycolytic Enzymes on Mitochondria via O-GlcNAcylation

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science
in
Biology

by

Haoming Wang

Committee in Charge:

Professor Gulcin Pekkurnaz, Chair
Professor Yimin Zou, Co-Chair
Professor Enfu Hui

2018

The thesis of Haoming Wang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

DEDICATION

I dedicate my Thesis to my parents, Zhitai Wang and Jinping Gao, for bringing me to this world and giving me love and support.

EPIGRAPH

“You cannot connect the dots looking forward; you can only connect them looking backward. So, you have to trust that the dots will somehow connect in your future."

Steve Jobs

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
DMSO	Dimethyl sulfoxide
HEK239T	Human embryonic kidney
hMilton1 (TRAK1)	Human trafficking kinesin-binding protein 1
HK	Hexokinase
HK1	Hexokinase-1
HK2	Hexokinase-2
HK3	Hexokinase-3
IP	Immunoprecipitation
GCK	Glucokinase
G6P	Glucose-6-Phosphate
GFP	Green Fluorescent Protein
Km	Michaelis constant
Ki	Inhibitor constant
OGT	O-GlcNAc Transferase
OGA	O-GlcNAcase
O-GlcNAc	O-linked β-N-acetyl glucosamine
Pi	Inorganic phosphorus
TMG	Thiamet-G
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine

VDAC1	Voltage-dependent anion-selective channel protein 1

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to acknowledge Professor Gulcin Pekkurnaz for her support as the chair of my committee. Through multiple drafts and many long nights, her guidance has proved to be valuable.

I would also like to acknowledge Seungyoon Blenda Yu, Nathalie Djaja, Chloe Nguyen, Teva Bracha, Nirosh Mataraarachchi, Norah Al-Azzam, Corbin Frohreich from Pekkurnaz Lab, without them my research would have no doubt taken ten times as long. It is their support that helped me in an immeasurable way.

I would also like to acknowledge for Dr. Majid Ghassemian and Molecular Mass Spectrometry Facility (MMSF) of UCSD for helping with mass spectrometry

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

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In the brain, glucose is the major energy source and its metabolism starts with the activity of the first rate-limiting enzyme Hexokinase (HK). HK1 is the dominant isoform in the brain, and is mostly associated with the mitochondrial outer membrane. The positioning of HK1 on mitochondria is critical, because it couples glucose metabolism to the energy production of mitochondria. Here, we report a new molecular mechanism that regulates the mitochondrial positioning and activity of HK-1 via the metabolic sensor enzyme O-GlcNAc transferase (OGT). OGT catalyzes a reversible posttranslational modification of proteins by adding a GlcNAc sugar moiety to serine and threonine residues. The catalytic activity of OGT is regulated by intracellular UDP-GlcNAc concentrations, which fluctuate proportionally in response to nutrient flux through the hexosamine pathway. In this study, we show that HK1 is dynamically modified with O-GlcNAc at its regulatory domain. O-GlcNAcylation of HK1 is elevated when cells are transfected with OGT or treated with Thiamet-G (TMG), an inhibitor of de-GlcNAcyating enzyme. We further characterized that O-GlcNAc modification of HK1 recruits it on mitochondria and increases its enzymatic activity. Our findings may reveal key molecular pathways that couple neuronal metabolism to mitochondrial function via OGT, and how their dysregulation leads to neurological disorders.

INTRODUCTION

Cellular metabolism is comprised of complex reactions, whose fine-tuned control is critical to cellular health and function. It is necessary to study participating enzymes in order to understand the fine-tuning of the metabolic reactions. A number of quantitative studies have focused on metabolic control through modulating enzyme concentrations and their turnover rates (Amar et al., 2008; Grima and Schnell, 2006). However, these studies lack details on the spatial organization of enzymes within the cell. In fact, cells can modulate their metabolism by organizing metabolic enzymes in response to spatially and temporally varying nutrient availability and energy demands. In mammalian neurons, which can have axons up to a meter long, nutrient availability and metabolic demand are spatially and temporally heterogeneous. For example: Glucose availability can vary in different regions of axons (Ferreira et al., 2011; Hall et al., 2012; Weisova et al., 2009); glucose transporters were proposed to localize near narrow regions around the nodes of Ranvier (Harris and Attwell, 2012; Magnani et al., 1996; Rosenbluth, 2009). Metabolically demanding regions of neurons such as presynaptic boutons have rapid ATP turnover rates (Rangaraju et al., 2014). Synaptic activity and neuronal firing require large amounts of ATP (Ferreira et al., 2011; Shulman et al., 2004; Weisova et al., 2009).

In the brain, glucose is the main energy source and its metabolism heavily affects neuronal functions (Peppiatt and Attwell, 2004). The first step of glucose metabolism catalyzed by the enzyme Hexokinase (HK), also the first rate-limiting step. HK catalyze Glucose to Glucose-6-Phosphate (G6P) by transferring a

phosphoryl group from ATP. HK consists of a family of proteins with four isoforms: hexokinase-1 (HK1), hexokinase-2 (HK2), hexokinase-3 (HK3) and glucokinase (GCK). These isoforms differ in their enzymatic activity and subcellular localization. HK1, 2 and 3 can be inhibited by their product G6P, but only the inhibition of HK1 can be antagonized by P_i. As for HK2 and 3, P_i causes additional inhibition. The changing of G6P and P_i levels are tightly related by nutrient availability and energy demand. It has been proposed that the response of HK1 to G6P and P_i levels are an adaptation of catabolic functions to meet energy demands. HK2 and 3 are responsible for primarily anabolic functions by providing G6P for glycogen synthesis and other metabolic uses. HK1, 2 and GCK have been most widely studied compared to HK3. This may be due to the fact that HK3 expression is low in most tissues and difficult to isolate for functional assays.

HK1 is the dominant isoform in the brain and other insulin sensitive tissues (Aubert-Foucher et al., 1984; Walters and McLean, 1968). 70% of HK1 was demonstated to colocalize with mitochondria in brain tissue (Grossbard and Schimke, 1966; Katzen et al., 1970). While the exact mechanism regulating the mitochondrial positioning of HK1, it has been proposed that HK1 binds mitochondria through Voltage-dependent anion-selective channel protein 1 (VDAC1), the protein that forms channels through the mitochondrial outer membrane (Thomas et al., 1993). When HK1 binds to mitochondria, it gains the privilege to have direct access of ATP generated by mitochondrial oxidative phosphorylation (Arora and Pedersen, 1988). The ADP produced by mitochondrially bound HK1 can also be recruited again for re-phosphorylation

(Bessman and Geiger, 1980; Polakis and Wilson, 1985; Viitanen et al., 1984), which then leads to increase in ATP binding affinity, decrease in the sensitivity of G6P and release of HK1 from mitochondria (Wilson, 1980). Based on this knowledge, it might be advantageous for HK1 to localize on mitochondria to upregulate its enzymatic activity. Moreover, binding of HK1 to mitochondria may provide additional advantage for enzymatic activity regulation in response to spatially varying glucose availability and energy demands.

Post-translational modification O-GlcNAcylation has emerged as a major molecular and cellular metabolic-sensing signaling mechanism in the recent years. O-linked β-N-acetyl glucosamine (O-GlcNAc) is an uncharged sugar group, which can be added or removed from target proteins by OGT and O-GlcNAcase (OGA) respectively. O-GlcNAcylation is tightly regulated by intracellular UDP-GlcNAc concentrations (Bond and Hanover, 2015), which fluctuate proportionally in response to nutrient flux through the hexosamine biosynthetic pathway. There are more than 4,000 proteins that have been identified and regulated by O-GlcNAcylation. O-GlcNAcylation plays an important role in mitochondria motility regulation (Pekkurnaz et al., 2014), cell stress response (Guinez et al., 2007; Guinez et al., 2006), protein turn over (Guinez et al., 2008) and insulin resistance (Ma and Hart, 2013).

In this study, we studied the molecular mechanisms regulating mitochondrial positioning of HK1 on mitochondria and how it couples glucose metabolism to the energy production pathways. Here, we report a new molecular

mechanism that regulates the mitochondrial positioning and activity of HK-1 via the metabolic sensor enzyme O-GlcNAc transferase (OGT). We showed that binding of HK1 to mitochondria is regulated by O-GlcNAcylation, and its enzymatic activity is increased when O-GlcNAcylation is increased. The present study may reveal key molecular pathways that couple neuronal metabolism to mitochondrial function via OGT and may be vital for optimizing novel molecules for treating neurodegenerative diseases and other neurological disorders.

RESULTS

O-GlcNAcylation Increases Mitochondrial Hexokinase-1 in Cortical Neurons

To examine the role of O-GlcNAc cycling on cortical neuron mitochondria, DIV 15 cortical neurons were either treated with 1μM TMG, an inhibitor of the enzyme OGA (Yuzwa et al., 2008) or DMSO, 18 hr prior to cell lysis. Transient inhibition of OGA enhances O-GlcNAcylated proteins in cells and leads to mitochondrial motility arrest. We used gradient centrifugation to pellet mitochondria from cortical neurons and immunoprecipitated O-GlcNAcylated proteins by using anti-O-GlcNAc antibody (RL2) (Holt et al., 1987; Snow et al., 1987). Previous studies showed that inhibiting OGA activity increases O-GlcNAcylated hMilton-1 (Pekkurnaz et al., 2014). Therefore, hMilton-1 was used as a positive control. As expected, O-GlcNAcylated hMilton-1 significantly increased when OGA function was inhibited (Figure 1A and 1C). We started analyzing glycolytic enzyme positioning on mitochondria and specifically focused on HK1. OGA inhibition increased the level of HK1 in O-GlcNAc immunoprecipitation from mitochondrial fraction (Figure 1A and 1B).

To further elucidate the changes in total mitochondrial HK1, equal amounts of mitochondrial input, which is saved before performing immunoprecipitation, and cytosolic input, were loaded. Antibodies were used to probe HK1, ATP5B and GAPDH; the latter two were used as loading controls. Upon inhibition of OGA activity, mitochondrial HK1 is increased (Figure 1D and E) and cytosolic HK1 is decreased (Figure 1D and F). HK1 was identified to bind with mitochondria by interacting with VDAC1 (Felgner et al., 1979; Lindén et al., 1982; Fiek et al., 1982).

VDAC1 was also probed, but its expression level was not affected by OGA inhibition (Figure 1D and G). Thus, inhibiting OGA activity will increase mitochondrial HK1 and decrease cytosolic HK1, but will not change VDAC1's expression level.

Increasing O-GlcNAcylation Recruits Hexokinase-1 on Mitochondria

We hypothesized that increasing OGT activity and inhibition OGA activity is a fundamental mechanism regulating HK1 recruitment on mitochondria. We therefore examined the effect of OGT and TMG treatment in HEK293T cells, where HK1 is mostly localized in cytosol. We transfected HEK239T cells with full-length nucleocytoplasmic isoform of OGT together with TMG treatment. Indeed, OGT overexpression and OGA inhibition recruited more endogenous HK1 on mitochondria (Figure 2A-C). Inhibiting OGA activity by treating cells with TMG also recruited more endogenous HK1 on mitochondria (Figure 2B and 2C). There was no significant difference between TMG treatment alone and overexpression OGT with TMG treatment.

We also ectopically expressed full-length HK1 tagged with GFP and OGT plus TMG treatment in HEK293T cells. Consistent with previous results, compared to control conditions, OGT overexpression and OGA inhibition recruited more HK1-GFP on mitochondria, (Figure 4A-C) and inhibition of OGA activity alone also recruited HK1-GFP on mitochondria (Figure 4B and 4C). Thus, localization of HK1 with mitochondria is regulated by O-GlcNAcylation.

OGT Directly Modify Hexokinase-1 O-GlcNAcylation

To test whether the HK1 is a direct substrate of OGT, we immunoprecipitated endogenous HK1 with or without TMG treatment and probed with anti-O-GlcNAc antibody. The O-GlcNAcylation level of endogenous HK1 was altered by OGA inhibition (Figure 5A and 5B), but the expression of endogenous HK1 was not altered (Figure 5A)

We also examined O-GlcNAcylation of HK1-GFP upon OGT overexpression or OGA inhibition. Anti-O-GlcNAc antibodies detected a band that co-migrates with HK1 in anti-GFP immunoprecipates from HEK293T cells expressing GFP-tagged full-length HK1 (Figure 5C). Intensity of this band was increased with overexpression of OGT and inhibition of OGA by TMG (Figure 5D), but the intensity of expression of HK1-GFP was not changed. Thus, O-GlcNAcylation sites on HK 1 are not saturated in control conditions, and the level of their modification is likely to be determined by the fine balance and regulation of OGT and OGA activity.

Hexokianse-2 is not a Direct Substrate of OGT

HK2 can also binds with mitochondria (Azoulay-Zohar et al., 2004; Fiek et al., 1982; Lindén et al., 1982), therefore, we also examined O-GlcNAcylation of HK2-GFP to determine whether HK2 can be O-GlcNAcylated. Anti-O-GlcNAc antibodies detected a band that co-migrates with HK2 in anti-GFP immunoprecipates from HEK293T cells expressing GFP-tagged full-length HK2 (Figure 6A). However, the intensity of this band was not altered by inhibition of

OGA (Figure 6B). Thus, GlcNAcylation sites on HK2 are saturated in control conditions, and the level of their modification is not determined by OGT and OGA activity.

O-GlcNAcylation is Necessary for Hexokinase-1 recruitment on Mitochondria

To identify O-GlcNAcylation sites in HK1, HK1 was immunoprecipitated from HEK293T cells and resolved on an SDS-PAGE gel. Peptides isolated from a tryptic digestion were analyzed by using electron transfer dissociation (ETD) MS/MS, which preserves labile glycosidic linkages. We identified one peptide on HK1 (Figure 7C) that was modified with O-GlcNAc, threonine 259. This amino acid is modified by O-GlcNAcylation and is not conserved in human HK2 or human HK3 (Figure 7A), but is conserved in human glucokinase, rat HK1, and mouse HK1 (Figure 7B). To determine the potential role of this site in mitochondrial recruitment of HK1, we expressed HK1 constructs in HEK293T cells wherein the putative O-GlcNAcylation site was mutated to alanine. We measured its O-GlcNAcylation levels relative to control. Mutation of that site abolished the amount of GlcNAc immunoreactivity recycling on HK1 (Figure 7D and 7E). We compared the results of its expression to that of full-HK1-GFP when OGT was co-expressed and TMG treated in HEK293T cells. There was no significant difference between HEK293T cells containing the T259A mutated HK1 construct upon treatment with or without TMG, and endogenous OGT or overexpression (Figure 8A-C). Understanding the

mechanism of how this site regulates HK1 localization on mitochondria requires further research.

O-GlcNAcylation Increases Hexokinase-1 Activity

Identification of the required O-GlcNAcylation sites on HK1 allowed us to determine the role of this modification on HK1's enzymatic activity. We took advantage of the fact that HK1 localization on mitochondria gives it the privilege to access ATP, (Arora and Pedersen, 1988) allowing G6P levels to increase. We examined G6P level to determine whether O-GlcNAcylation of HK1 affects its enzymatic activity. Cells were transfected with OGT were treated with or without TMG had increased G6P level (Figure 9A). Similarly, ectopic expression of HK1-GFP and OGT with TMG treatment also had increased G6P level (Figure 9B). There was no significant difference of G6P levels in cells transfected with T259A mutated HK1-GFP, in response to OGT and TMG treatment (Figure 9C). To validate that G6P level increases were not caused by expression differences, cell lysates were also probed with anti-HK1 or anti-GFP (Figure 9D-F), and intensity of HK1 or GFP was normalized to tubulin (Figure 9G-I). There was no significant difference of endogenous HK1, HK1-GFP and T259A-HK1-GFP expression level.

DISCUSSION

Cellular metabolism contains a complex system of reactions; its control is vital for cellular function and health. Cells can organize metabolic components in order to meet spatial and temporal varying nutrient availability and energy demands. The localization of metabolic enzymes can therefore influence energy, signaling and metabolism (Saxton and Hollenbeck, 2012). We have now established that (1) Increasing O-GlcNAcylation increases mitochondrial HK1 in cortical neurons; (2) Increasing O-GlcNAcylation recruits more HK1 onto mitochondria; (3) that the enzyme OGT, a putative metabolic sensor, mediates recruitment of HK1 on mitochondria; (4) O-GlcNAcylation of threonine 259 on HK1 is required for recruiting it on mitochondria; (5) this pathway is likely to be relevant for regulating HK1's enzymatic activity.

The O-GlcNAcylation level of HK1 was correlated with its localization on mitochondria and enzymatic activity, whether the level was manipulated by overexpression of OGT, or by pharmacological inhibition of OGA (Figure 9). By mapping and mutating a key site of O-GlcNAc addition in HK1 (T259A-HK1-GFP), we demonstrated that this site is a key and an essential substrate for OGT modulation of HK1 localization and enzymatic activity. Increasing glucose elevates total O-GlcNAcylation, which also position HK1 on mitochondria. HK1 bound to mitochondria have direct access to ATP generated from oxidative phosphorylation within the mitochondria, which might lead to increased enzymatic activity. This may explain how increased O-GlcNAcylation elevates total G6P.

Previous study shows that mitochondria in axon of neuron was arrested at high glucose area (Pekkurnaz et al., 2014). After mitochondria is arrested in high glucose area, HK1 and other glycolytic enzymes may be recruited on mitochondria due to elevated O-GlcNAcylation. This may allow improvement of total metabolic efficiency when energy demand is high along axons.

The detail molecular mechanism of how threonine 259 regulates HK1 localization on mitochondria is not clear yet. Threonine 259 is localized on N-terminal of HK1, which is known as the regulatory domain. We hypothesized that O-GlcNAcylation of threonine 259 may decreases HK1's Km of glucose, which means increasing its binding ability of glucose, or it could increase HK1's Ki of G6P, which can make mitochondria bounded HK1 more stable and not easily released by G6P. Molecular structural studies are needed in future to elucidate this mechanism.

Even though, T259A abolished O-GlcNAcylation recycling of HK1, the intensity of O-GlcNAc of HK1 was not altered when probed with anti-GlcNAc antibody, which may indicate that some O-GlcNAcylation sites turn over very slowly and may primarily serve a structural role, whereas others are actively cycled and likely regulate protein function (Khidekel et al., 2007). TMG inhibition of OGA only caused the level of O-GlcNAcylation to rise when the protein site was constantly recycled (Pekkurnaz et al., 2014; Yang et al., 2017) HK1 O-GlcNAcylation meets this criterion (Figures 7E-G), and OGT overexpression or OGA inhibition recruited HK1 on mitochondria and increased its enzymatic activity (Figure 3, 4 and 9). However, HK2 O-GlcNAcylation does not meet that criterion

(Figure 6) because O-GlcNAcylation is already saturated in control conditions. Therefore, inhibition of OGA does not change its O-GlcNAcylation level. Only the O-GlcNAc site on HK1 is undergoing dynamic regulation and normally exists in a sub-stoichiometric level of modification that allows it to respond to nutrient availability and energy demands.

The O-GlcNAcylation site of threonine 259 on human HK1 is conserved through human, rat, mouse, dog, and cat, (Figure 7B) and many other mammalian animals, but not in *Drosophila* nor *Saccharomyces cerevisiae*. Potentially indicating its important role in regulating HK1 activity and function in mammalian animals. Threonine 259 is not conserved in *Drosophila* nor *Saccharomyces cerevisiae* may suggestion that during evolution mammalian animals may gained more advantages through modulating threonine 259's O-GlcNAcylation to have better control of HK1 enzymatic activity.

Threonine 259 is specific to HK1 and glucokinase, but not other HK isoforms. Glucokinase also can phosphorylate glucose to generate G6P, but it has low-affinity glucose phosphorylation activity and significantly different kinetic properties, functions from other HK isoforms. Glucokinase expression is regulated by O-GlcNAcylation (Baldini et al., 2016), and it was also expressing in brain, specifically in hypothalamic and limbic regions, which also have metabolic-sensing function. This may revolve that O-GlcNAcylation not only can modulate GCK expression, but the conserved threonine 259 may also give its ability to sensing nutrient availability. HK2 does not have threonine 259, which may explain why its

O-GlcNAcylation is saturated in baseline conditions and not constantly recycled, other O-GlcNAcylated sites may serve as primary structure of the protein.

FIGURE LEGENDS

Figure 1. O-GlcNAcylation Increases Mitochondrial Hexokinase-1 in Cortical Neurons.

(A-C) Immunoprecipitation (IP) of O-GlcNAcylated proteins from cortical neuron mitochondria. The blot was probed with HK1 and hMilton-1 to analyze the consequences of TMG treatment. Precipitation with mouse IgG was used as a control. DMSO was used as a treatment control. TMG treatment increased O-GlcNAcylated hMilton1 on mitochondria and was used as a positive control. The result indicates that TMG treatment increased O-GlcNAcylated HK1 on mitochondria. (B)The intensity of HK1 and (C) hMilton1 bands were normalized with mouse IgG. n=3 independent TMG or DMSO treatments per condition.

(D-G) Input lines were loaded with 2.25% of mitochondrial lysates, which were used for the IP, or 2.25% of cytosolic lysates. Mitochondrial lysates were probed with anti-ATP5B antibody as a loading control, cytosolic lysates were probed with anti-GAPDH antibody as a loading control. TMG treatment increased mitochondrial HK1 and decreased cytosolic HK1. VDAC-1's expression was not affected by TMG treatment. (E) The intensity of mitochondrial HK1 bands were normalized with ATP5B. (F) The intensity of cytosolic HK1 bands were normalized with GAPDH. (G) The intensity of VDAC-1 bands were normalized with ATP5B. n=3 independent TMG or DMSO treatments per condition.

All values are shown as mean ± SEM n.s. not significant. **P<0.01, ****P<0.0001; Mann-Whitney U test.

Figure 2. A model of Mitochondrial enrichment of Hexokinase-1

A model showing the relationship between HK1, O-GlcNAcylated HK1 and mitochondria. Increasing OGT activity or Inhibiting OGA activity recruits HK1 and O-GlcNAcylated HK1 on mitochondria.

Figure 3. Enhanced O-GlcNAcylation Recruits Endogenous Hexokinase-1 on Mitochondria.

(A) HEK293T cells were cultured with or without TMG overnight and transfected with mito-DsRed (red) and either with or without OGT. Cells were immunostained with anti-HK1 (green). The presence of OGT and TMG caused the otherwise diffuse HK1 concentrate on mitochondria.

(B-C) Quantitative analysis of colocalization coefficients Manders 1 and 2 (Above auto threshold). Channel 1 represents HK1 and channel 2 represents the mitochondria label. n=8 cells and 3 independent transfections and TMG or DMSO treatments per condition.

All values are shown as mean ± SEM n.s. not significant. **P<0.01; one-way ANOVA.

Figure 4. Enhanced O-GlcNAcylation Recruits HK1-GFP on Mitochondria.

(A) HEK293T cells were cultured with or without TMG overnight and transfected with Full-HK1-GFP, mito-DsRed (red) and either with or without OGT. Cells were immunostained with anti-GFP (green). The presence of OGT and TMG caused the otherwise diffuse Full-HK1-GFP concentrate on mitochondria.

(B-C) Quantitative analysis of colocalization coefficients Manders 1 and 2 (Above auto threshold). Channel 1 represents HK1 and channel 2 represents the mitochondria label. n=4 and 3 independent transfections and TMG or DMSO treatments per condition.

All values are shown as mean ± SEM n.s. not significant. *P<0.05, **P<0.01; one-way ANOVA.

Figure 5. Hexokinase-1 is a Direct Substrate of OGT.

(A-B) Immunoprecipitation (IP) of endogenous HK1 from HEK293T cells to analyze consequences of TMG treatment. Precipitation with rabbit IgG was used as a control. Input lanes were loaded with 3% of the cell lysates used for the IP and also probed with anti-Tubulin antibody as a loading control. TMG treatment increased O-GlcNAcylated HK1 without altering its expression level. (B) O-GlcNAc levels on endogenous HK1 fold changes in response to TMG treatment were calculated, n=1 independent TMG treatment per condition.

(C-D) Immunoprecipitation (IP) of exogenous full-HK1-GFP from HEK293T cells to analyze consequences of OGT overexpression and TMG treatment. Precipitation with rabbit IgG was used as a control. Input lanes were loaded with 3% of the cell lysates used for the IP and also probed with anti-Tubulin antibody as a loading control. OGT overexpression and TMG treatment increased O-GlcNAcylated full-HK1-GFP without altering its expression level. (D) O-GlcNAc levels on full-HK1-GFP fold changes in response to TMG treatment and OGT

overexpression were calculated, n=3 independent transfections and TMG or DMSO treatments per condition.

All values are shown as mean ± SEM n.s. not significant. **P<0.01; Mann-Whitney U test.

Figure 6. Hexokinase-2 O-GlcNAcylation is not Dynamic.

(A-B) Immunoprecipitation (IP) of exogenous full-HK2-GFP from HEK293T cells to analyze consequences of TMG treatment. Precipitation with rabbit IgG was used as a control. Input lanes were loaded with 3% of the cell lysates used for the IP and also probed with anti-Tubulin antibody as a loading control. TMG treatment did not increase O-GlcNAcylated full-HK2-GFP nor did it alter its expression level. (B) O-GlcNAc levels on full-HK2-GFP fold changes in response to TMG treatment were calculated, n=3 independent with or without TMG treatments per condition.

Figure 7. Mapping and Mutating the O-GlcNAcylation Site of Hexokinase-1.

(A) Sequence alignment of O-GlcNAcylation sites (red) in human HK1, HK2, HK3 and GCK. (B) Sequence alignment of O-GlcNAcylation sites (red) in human HK1, Rat HK1, Mouse HK1, Dog HK1 and Cat HK1. (C) Schematic summary of Full-HK1-GFP with O-GlcNAcylation site (red), mitochondria binding domain (M), N-terminal half (N), C-terminal half (C) and GFP. (D-E) Immunoprecipitation (IP) of exogenous full-HK1-GFP and T259A-HK1-GFP from HEK293T cells to analyze consequences of OGT overexpression and TMG treatment. Precipitation with rabbit IgG was used as a control. Input lanes were loaded with 3% of the cell

lysates used for the IP and also probed with anti-Tubulin antibody as a loading control. OGT overexpression and TMG treatment increased O-GlcNAcylated full-HK1-GFP but not T259A-HK1-GFP. (E) O-GlcNAc levels on full-HK1-GFP and O-GlcNAc levels on T259A-HK1-GFP fold changes in response to TMG treatment and OGT overexpression were calculated, n=3 independent transfections and TMG or DMSO treatments per condition.

All values are shown as mean ± SEM n.s. not significant. *P<0.05; Mann-Whitney U test.

Figure 8. Increasing O-GlcNAcylation Cannot Recruit T259A-HK1 on Mitochondria.

(A) HEK293T cells were cultured with or without TMG overnight and transfected with T259A-HK1-GFP, mito-DsRed (red) and either with or without OGT. Cells were immunostained with anti-GFP (green). The presence of OGT and TMG did not recruit T259A-HK1-GFP on mitochondria. (B) Quantitative analysis of colocalization coefficients Manders 1 and 2 (Above auto threshold). Channel 1 represents T259A-HK1-GFP and channel 2 represents the mitochondria label. n=5 cells and 3 independent transfections and TMG or DMSO treatments per condition.

All values are shown as mean ± SEM n.s. not significant. one-way ANOVA.

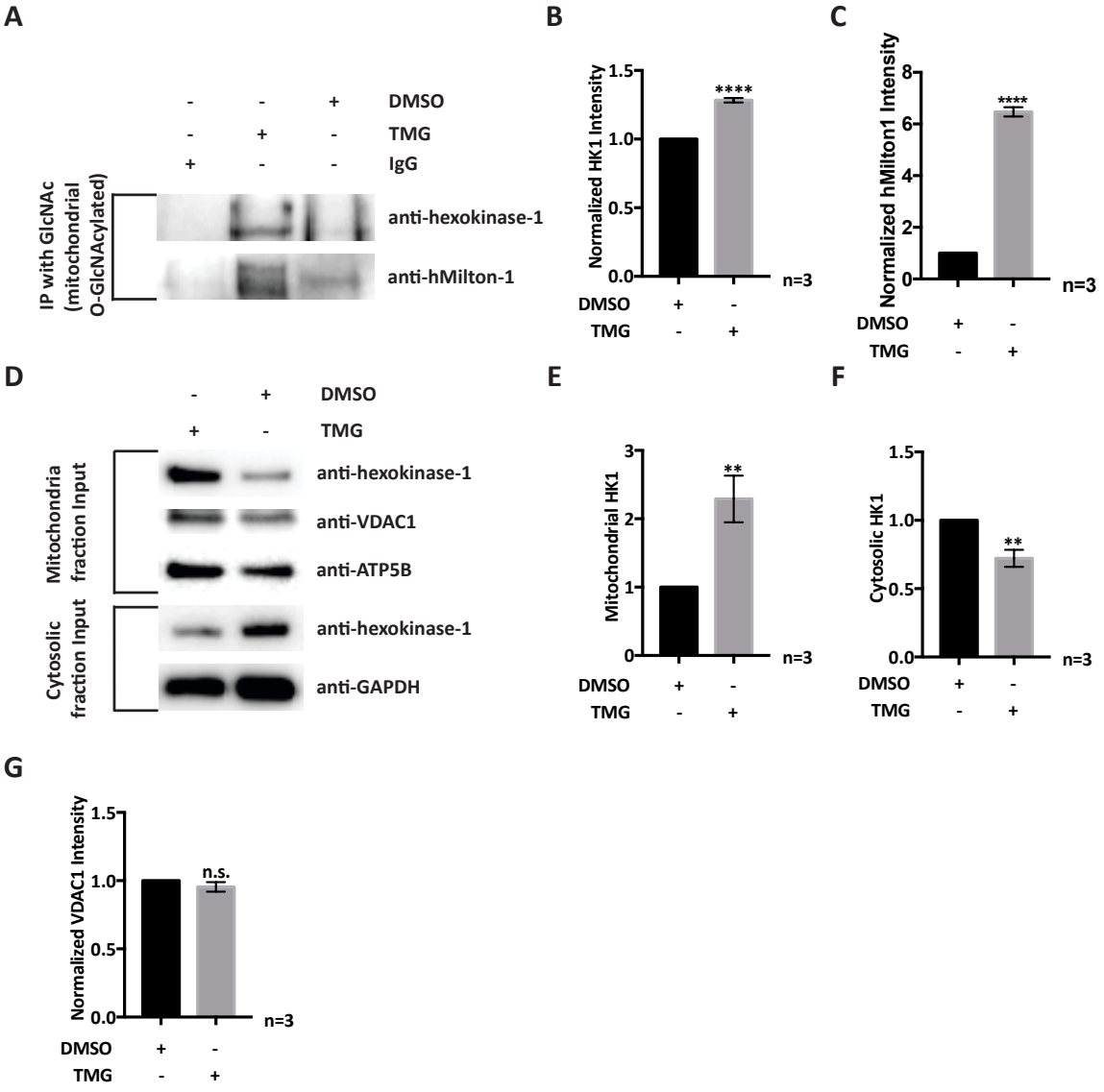
Figure 9. O-GlcNAcylation of Hexokinase-1 Increases its Enzymatic Activity.

(A) G6P levels were measured in HEK293T cells with or without TMG treatment and with or without OGT overexpression. G6P levels were normalized

by total protein. n=6 independent transfections and with or without TMG treatments per condition. (B) G6P levels were measured in HEK293T cells with full-HK1-GFP transfection, with or without OGT transfection and with or without TMG treatment. G6P levels were normalized by total protein. n=8 independent transfections and with or without TMG treatments per condition. (C) G6P levels were measured in HEK239T cells with T259A-HK1-GFP transfection, with or without OGT transfection and with or without TMG treatment. G6P levels were normalized by total protein. n=3 independent transfections and with or without TMG treatments per condition. (D-F) Samples were loaded with 3% of the cell lysates used for G6P measurement and probed with anti-Tubulin antibody as a loading control. OGT overexpression and TMG treatment did not alter (D) endogenous HK1, (E) full-HK1-GFP or (F) T259A-HK1-GFP expression level. (G-I) The intensity of (G) endogenous HK1 bands, (H) exogenous full-HK1-GFP bands and (I) T259A-HK1-GFP bands were normalized to the intensity of Tubulin bands. n=3 or n=6 independent transfections and TMG treatments per condition.

All values are shown as mean ± SEM n.s. not significant. *P<0.05, **P<0.01, ****P<0.0001 one-way ANOVA.

FIGURES



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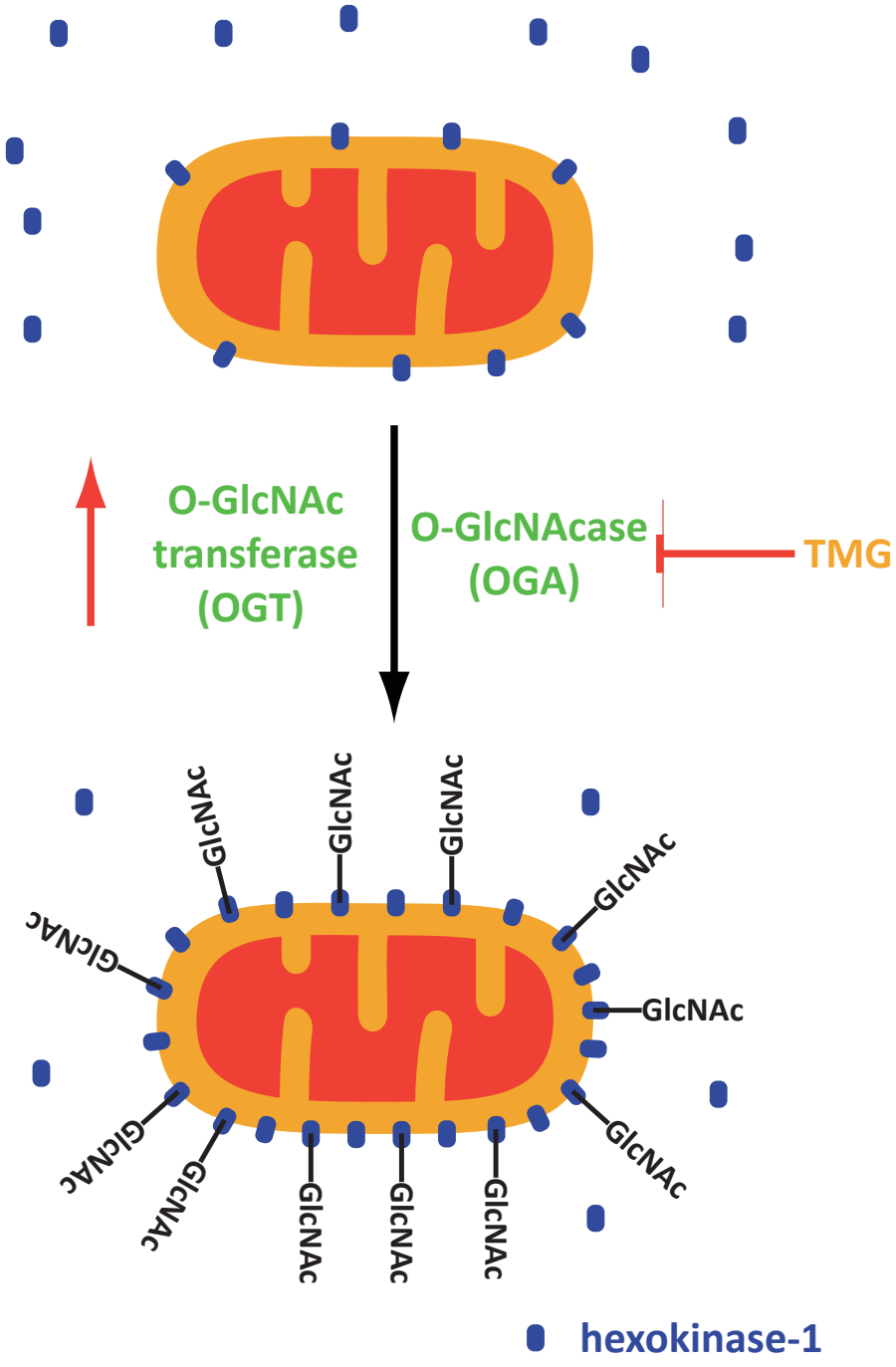

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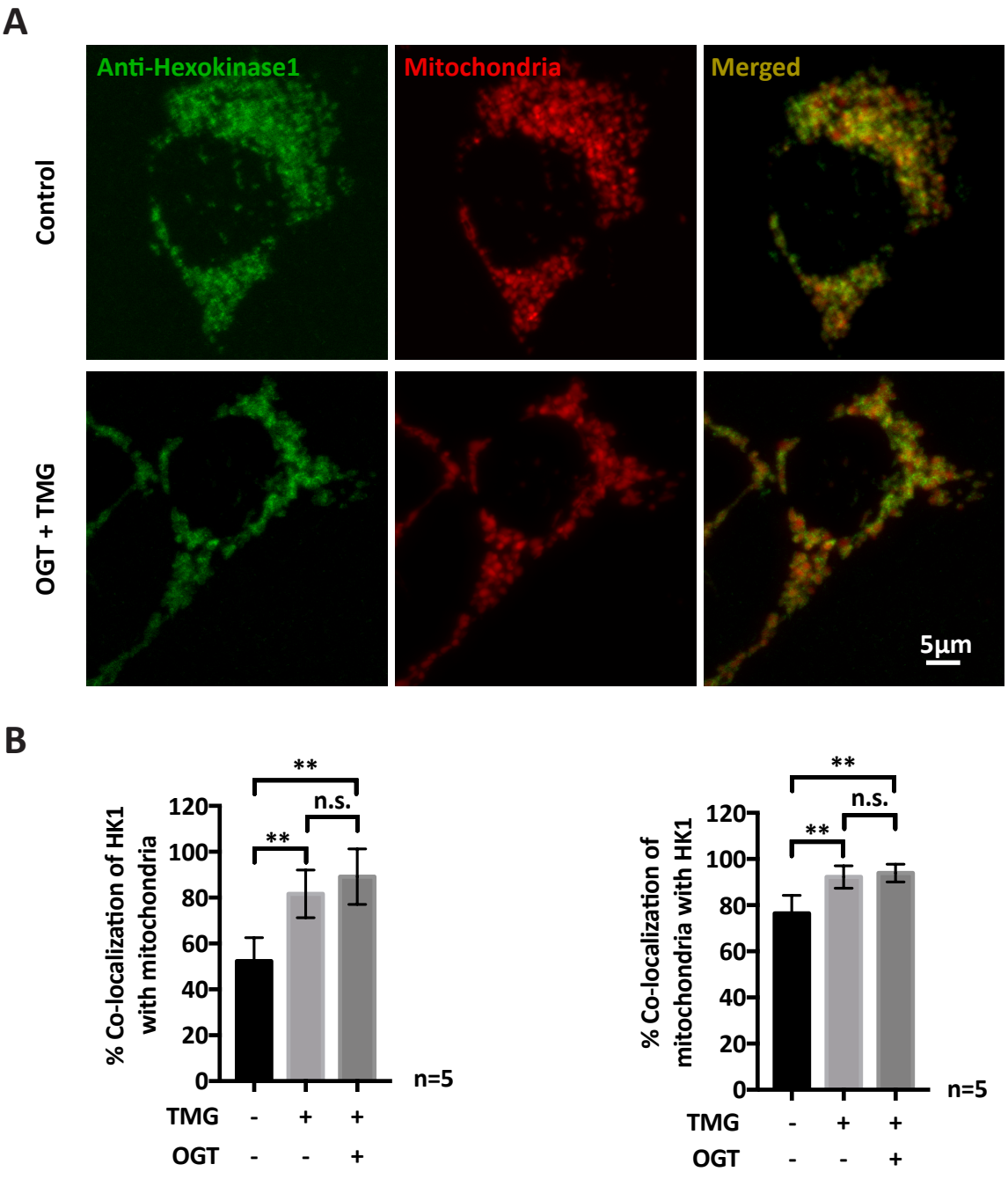

Figure 3. Enhanced O-GlcNAcylation Recruits Endogenous Hexokinase-1 on Mitochondria

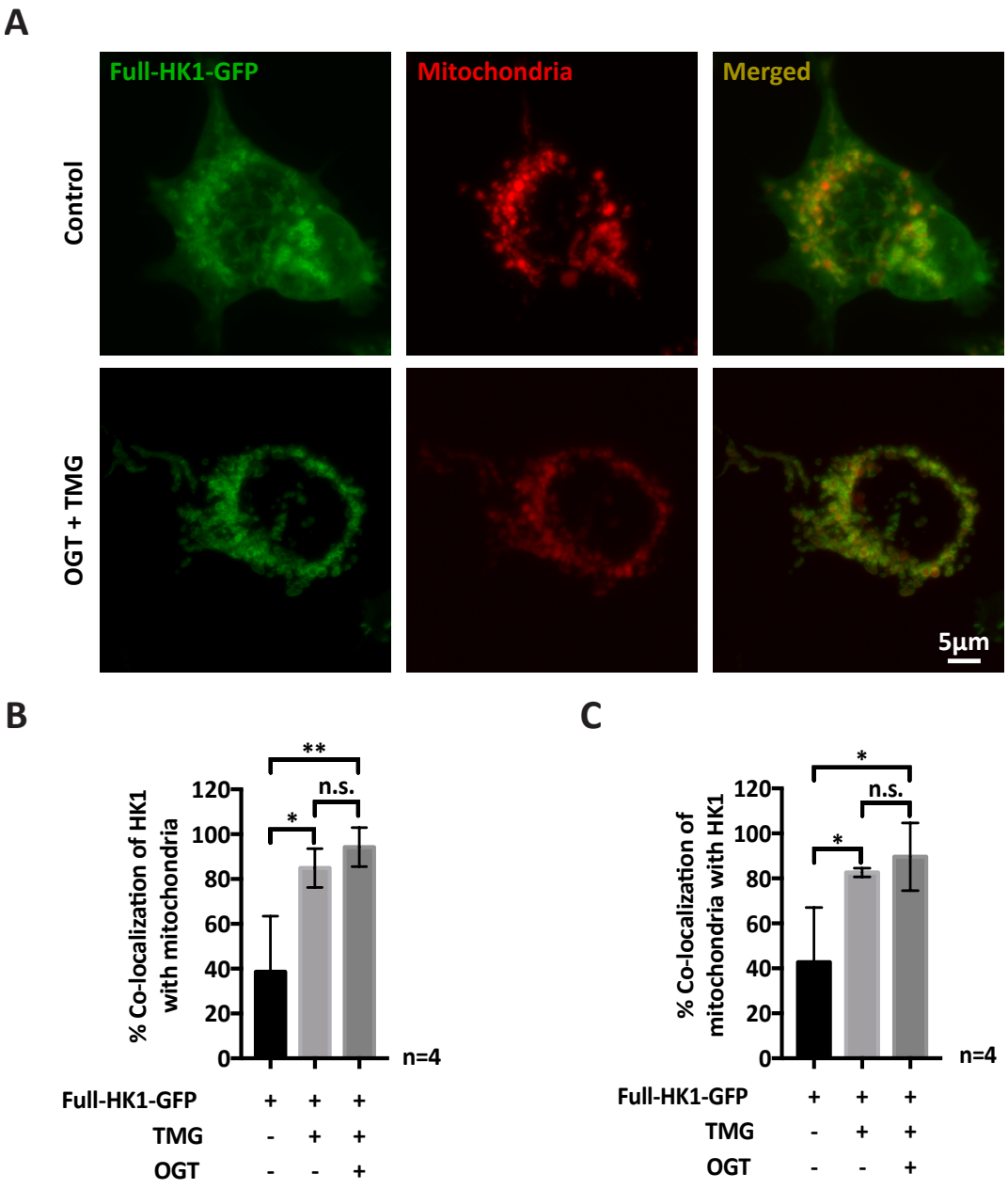

Figure 4. Enhanced O-GlcNAcylation Recruits HK1-GFP on Mitochondria.

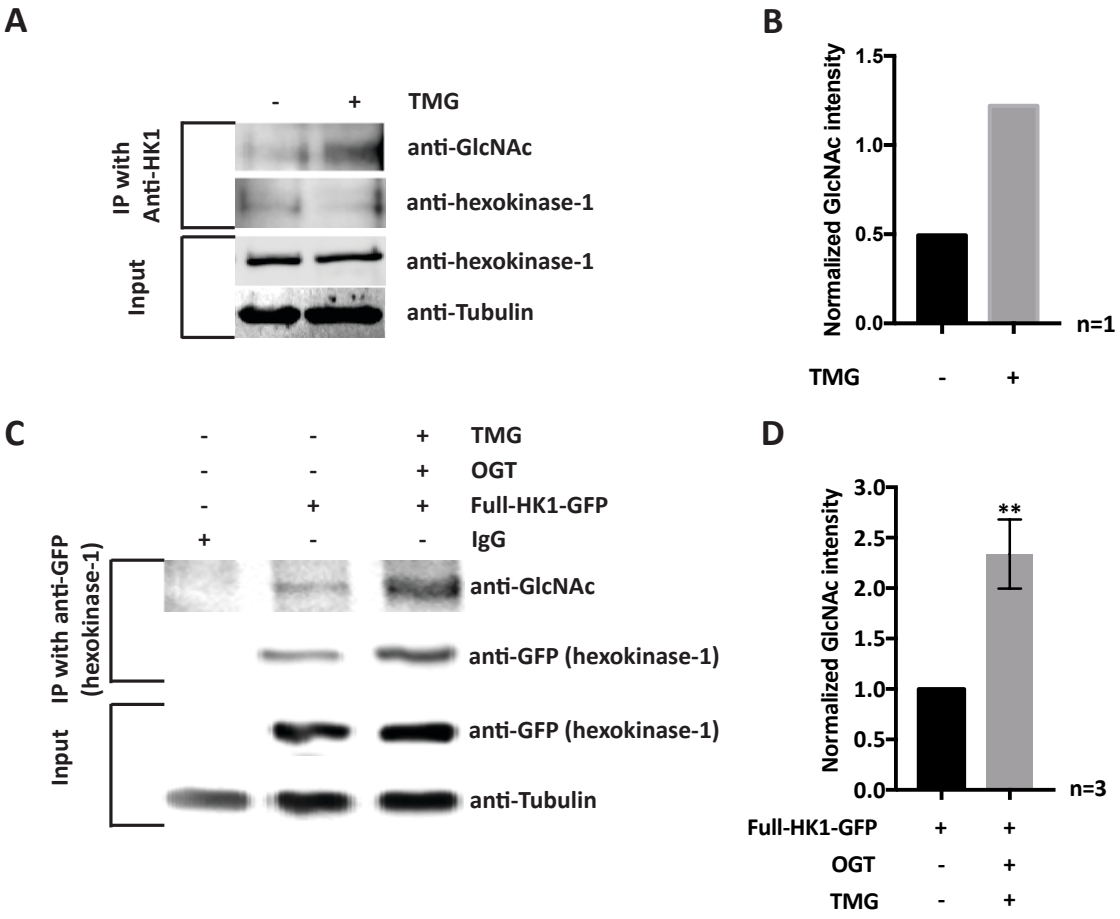

Figure 5. Hexokinase-1 is a Direct Substrate of OGT.

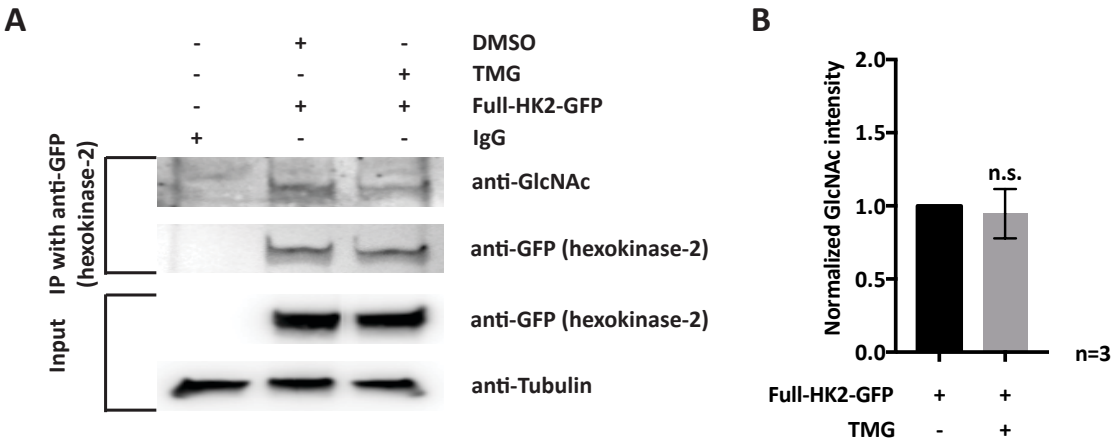

Figure 6. Hexokinase-2 O-GlcNAcylation is not Dynamic.

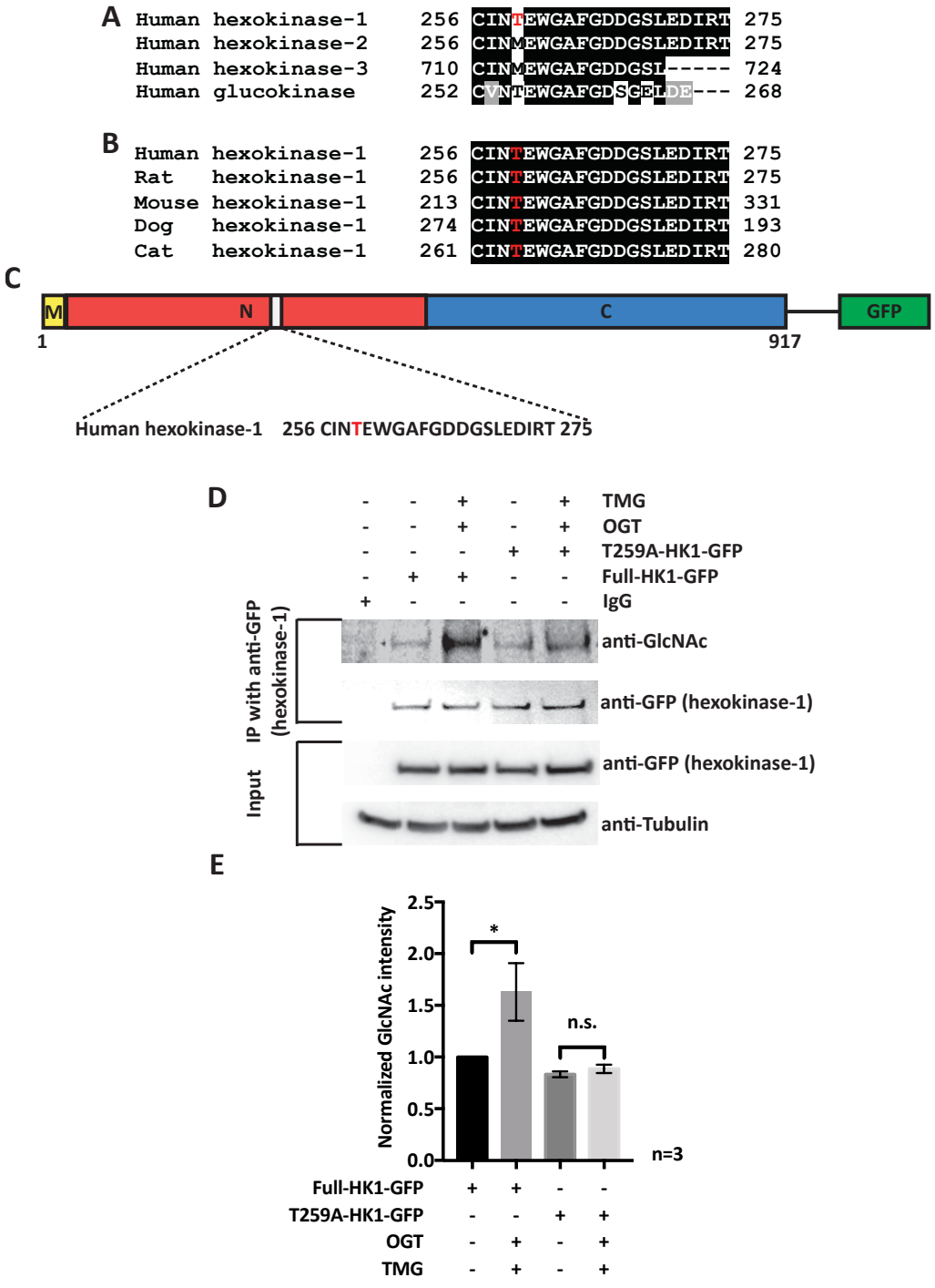

Figure 7. Mapping and Mutating the O-GlcNAcylation Site of Hexokinase-1.

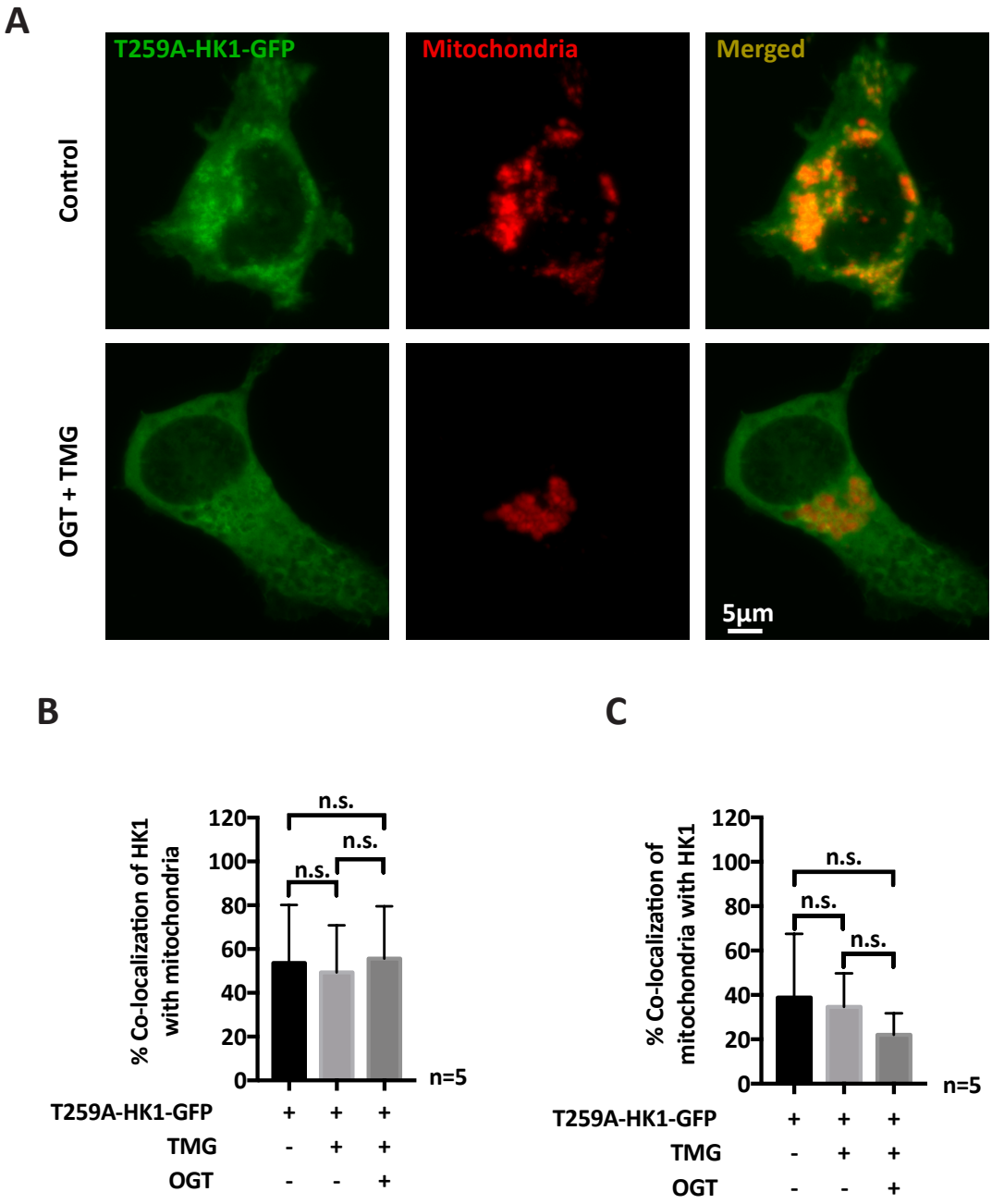

Figure 8. Enhanced O-GlcNAcylation Cannot Recruit T259A-HK1 on Mitochondria.

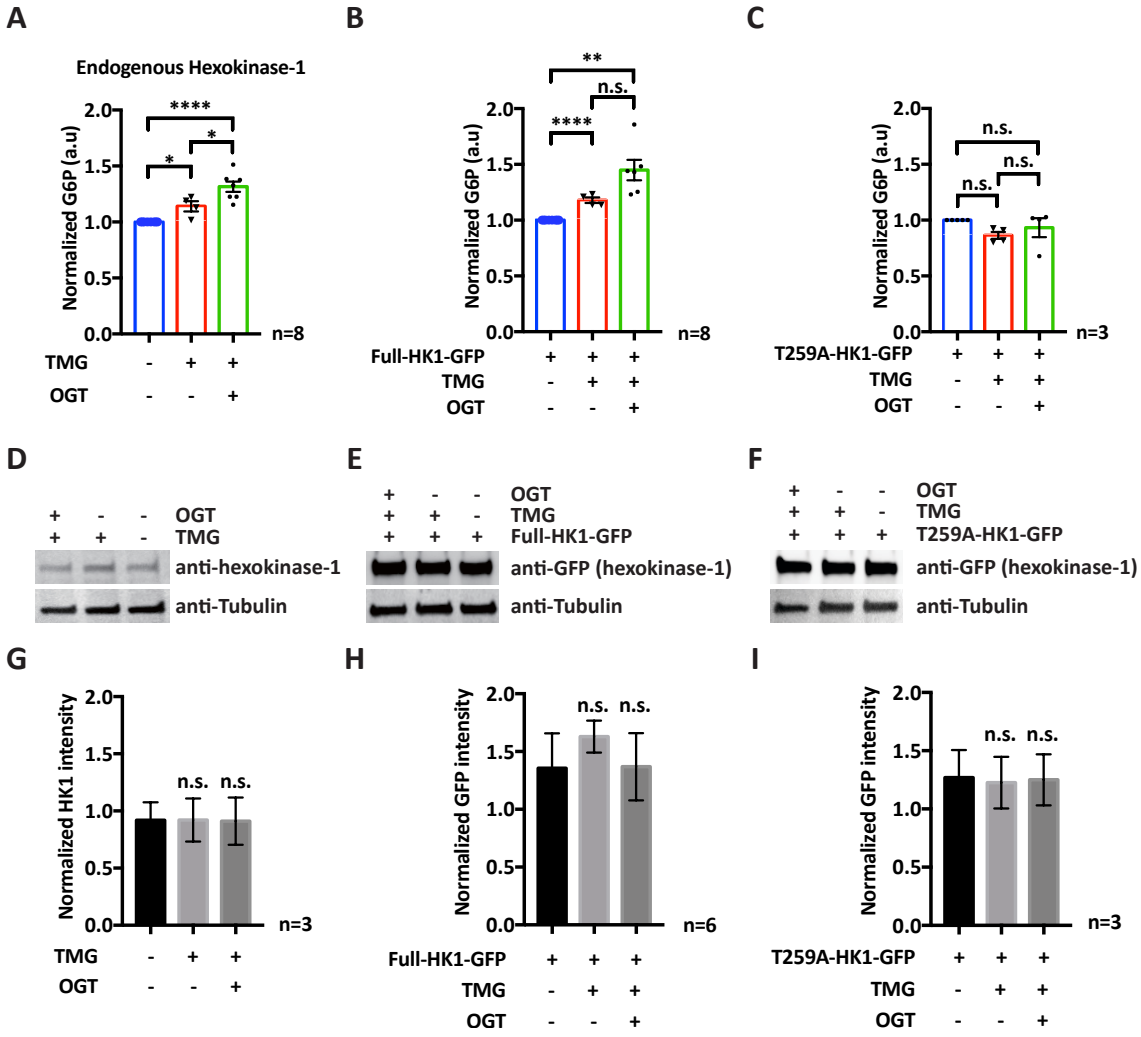

Figure 9. O-GlcNAcylation of Hexokinase-1 Increases its Enzymatic Activity.

METHODS

Cell Culture and Transfection

HEK293T cells were cultured in DMEM supplemented with L-glutamine, penicillin/streptomycin (Life Technologies), and 10% FBS (Atlanta Premium). Plasmid DNA transfections were performed with the calcium phosphate method. When TMG (Calbiochem, San Diego, CA, USA) was used, it was applied at 10 μM for 12–20 hr before cell lysis.

Cortical neurons were isolated from E15-18 rat (Envigo) embryos as previously described (Nie and Sahin, 2012) and plated 1.25-1.5x10^6 cells/well density in a 6-well plate (Corning™ Costar™), coated with 20 mg/ml poly-L-Lysine (Sigma) and 3.5 mg/ml laminin (Life Technologies) and maintained in NB medium supplemented with B27 (Life Technologies), L-glutamine, and penicillin/streptomycin, unless modified as specified. For particular treatments of neuronal cultures, see the Neuron Treatments.

Immunocytochemistry and Protein Analyses

HEK293T cells were fixed by 4% PFA, incubated (15min, room temperature) and washed three times with phosphate-buffered saline (PBS), incubated (30min, room temperature) in blocking buffer (PBS with 0.5% Saponin and 1% BSA), and incubated (18h, 4°C) with primary antibodies. After washing three times in PBS (15 min/each), slices were incubated with secondary antibodies (1h, room temperature), washed three times in PBS (15 min/each), and imaged. Details of used and diluted antibodies, see Immunoreagents.

Plasmid Constructs

The following DNA constructs were used in this study: Mito-DsRed (Clontech, Mountain View, Ca), nucleocytoplasmic OGT (Repeats et al., 1997), Full-HK1-GFP, Full-HK2-GFP were gifts from Hossein Ardehali (Addgene plasmid # 21917; http://n2t.net/addgene:21917; RRID: Addgene_21917; Sun et al., 2008). HK1 O-GlcNAcylation site mutants (T259A) were generated using Geneblock (Integrated DNA Technologies, San Jose, California).

Immunoreagents

For immunocytochemistry of HEK293T cells, the following antibodies were used: anti-HK1 at 1:200 (C35C4, Cell Signaling), anti-GFP at 1:500 (Thermo Fisher Scientific). The following fluorescently tagged secondary antibodies were used: Alexa-488 conjugated anti-rabbit at 1:500 (Molecular Probes, Invitrogen). The following primary antibodies were used for probing blots: anti-HK1 at 1:1000 (C35C4, Cell Signaling), anti-hMilton (TRAK1) at 1:2000 (Sigma-Aldrich), anti-VDAC1 at 1:1000 (20B12AF2, abcam), anti-ATP5B at 1:2000 (Sigma-Aldrich), anti-GAPDH at 1:400 (Calbiochem), anti-O-GlcNAc at 1:2000 (RL2, abcam), anti-Tubulin at 1:2000 (DM1A, Sigma-Aldrich), anti-GFP at 1:4000 (Thermo Fisher Scientific). HRP-conjugated anti-mouse, rabbit at 1:5000 (Jackson ImmunoResearch Laboratories, Inc.). Secondary antibodies were used for enhanced chemiluminescent detection with Super Signal West Dura (Pierce Biotechnology, Thermo Scientific). For quantitative Western blots, CyDye (Cy2, 3 and 5) conjugated anti-mouse and rabbit secondary antibodies at 1:5000 (Thermo

Fisher Scientific) were used and scanned by an Azure C600 Image Scanner (Azure Biosystems, Inc. Dublin, CA).

Neuron Treatments

Glucose: Neuronal cultures were established with regular NB medium containing 25 mM glucose, unless otherwise indicated.

TMG: TMG (Calbiochem, San Diego, CA, USA), OGA inhibitor, was applied at 1μM for 15 hr prior to cell lysis.

O-GlcNAcylation Measurements

HEK293T cells were plated at $6x10^5$ cells/well density in a 6-well plate (Corning™ Costar™) and transfected with the indicated plasmid constructs the next day. Three days after reaching confluency, cells were washed once with ice-cold phosphate-buffered saline (PBS) containing 8 mM TMG and lysed in 500μL buffer containing: 2% Nonidet P-40 (Calbiochem, San Diego, CA, USA), 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 40 mM GlcNAc, 8 mM TMG, (Calbiochem, San Diego, CA), 2 mM DTT (GBiosciences), 0.1 mg/ml PMSF and protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) at 1:1000. Lysates were centrifuged for 10 mins at 13,000 xg at 4°C and the supernatants were collected. For immunoprecipitations of Full-hexokinase1-GFP, GlcNAc site mutants or endogenous hexokinase-1, 2 μg anti-GFP or 5 μg anti-hexokinase-1 antibody were used as indicated, incubated for 2 hrs at 4℃ with 500 μL of whole-cell lysates, then 1 more hour with protein-G-Mag-Sepharose beads. Beads were washed three times with lysis buffer and resuspended with 1x Laemmli buffer. 80%–90% of immunoprecipitates were separated by SDS-PAGE and transferred

to nitrocellulose membranes. For GlcNAcylation measurements, blots were first incubated with blocking buffer containing 3% bovine serum albumin (w/v) in Tris-buffered saline 0.1% Tween-20 (Sigma-Aldrich, Saint Louis, MO) and then probed with anti-GlcNAc antibody overnight (Whelan et al., 2008). For quantitative immunodetection of the GlcNAc signal, Cy5 conjugated secondary antibody was used at 1:5000. The same blot was re-probed with anti-GFP or anti-HK1 antibody after washing thoroughly and blocking with 5% non-fat dry milk in PBS for 1-2 hrs at room temperature. For quantitative immunodetection, Cy2 conjugated secondary antibody was used at 1:5000. Blots were scanned by an Azure C600 Image Scanner (Azure Biosystems, Inc. Dublin, CA). Image acquisition settings were changed to keep the fluorescent signal within the linear range. The ImageJ gel analyzer function was used to analyze the fluorescence intensity of each band.

Measurement of Glucose-6-Phosphate Levels

Cells were removed from plastic 6-well-plates (Corning™ Costar™) with a plastic cell lifter (Corning™). Cell lysates were prepared in 500μL ice-cold phosphate-buffered saline (PBS) using 25G 5/8 and 30G 1/2 syringe needle. Lysates were centrifuged for 5 mins at 13,000 xg at 4°C and the supernatants were collected. The supernatant was filtered through Amicon® Ultra-0.5mL Centrifugal Filters, Ultracel-10K (Millipore Sigma) by centrifuged for 15 mins at 14,000 xg at 4°C. BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA) was used to measure the concentrated samples' total protein concentration. The deproteinized samples were used to measure G6P levels by following the protocol provided in the G6P Assay Kit (High Sensitivity) from Abcam (Cambridge, MA). Colorimetric

measurements were made at room temperature using Spark 20M (Tecan Life Science, Switzerland).

Mass Spectrometry Analysis

Full-HK1-GFP were immunoprecipitated from HEK293T cells as described above. Cells were treated with 5μM TMG overnight. ‘‘SimpleBlue Safestain’’ (Invitrogen, Carlsbad, CA) stained gel band corresponding to full-HK1-GFP (as well as the control lane) were excised and minced. Samples were analyzed by mass spectrometry as described previously (Trinidad et al., 2012).

Image Acquisition and Quantification

Images were acquired on a Zeiss LSM 780 confocal microscope at room temperature with a 100 x / 1.4 Oil DIC Plan-APOCHROMAT objective. Images were captured as Z-stack project. Laser power was set to < 20% for each channel to minimize damage. Manders' coefficients (MANDERS et al., 1993) was analyzed by using ImageJ’s (developed by Wayne Rasband) COLOC2 (developed by Johannes Schindelin). Calculations from ImageJ were extracted, and statistical analysis was performed with GraphPad Prism version 7.0 for Mac OS (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical Analysis

Throughout the paper, data was expressed as mean ± SEM. Statistical analysis was performed with GraphPad Prism v7.0 for MacOS. The Mann-Whitney U test was used to determine the significance of differences between two conditions. Multiple conditions were compared by Kruskal-Wallis nonparametric ANOVA test, which was followed by Dunn’s multiple comparisons test or by one-

way ANOVA with post hoc Tukey's test as appropriate to determine significance of differences between each condition and the control condition. *$p<0.05$ was considered significant

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