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Evidence of a compensatory regulation of colonic *O*-GlcNAc transferase and *O*-GlcNAcase expression in response to disruption of *O*-GlcNAc homeostasis.

Amélie DECOURCELLE¹, Ingrid LOISON¹, Steffi BALDINI², Dominique LEPRINCE¹ and Vanessa DEHENNAUT^{1,*}.

(1) *Université de Lille, CNRS, Institut Pasteur de Lille, UMR8161, M3T: Mechanisms of tumorigenesis and Targeted Therapies, F-59000 Lille, France.*

(2) *Université de Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F-59000 Lille, France.*

*** To whom correspondence should be addressed:**

e-mail: vanessa.dehennaut@ibl.cnrs.fr

Highlights:

- In the colon cancer cell line HCT116, OGT and OGA expression are sensitive to flux modulations through the hexosamine biosynthetic pathway.
- Manipulating OGT or OGA expression or activity in this cell line induces a compensatory regulation of their expression mostly at the transcriptional level for OGA and mainly at the post-transcriptional level for OGT.
- The compensatory regulation of OGT and OGA expression in response to changes in *O*-GlcNAc homeostasis also occurs *in vivo* in mouse colon.

ABSTRACT

O-GlcNAcylation is a post-translational modification of thousands of intracellular proteins that dynamically regulates many fundamental cellular processes. Cellular *O*-GlcNAcylation levels are regulated by a unique couple of enzymes: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which adds and removes the GlcNAc residue, respectively. Maintenance of *O*-GlcNAc homeostasis is essential to ensure optimal cellular function and disruption of this homeostasis has been linked to the etiology of several human diseases including cancer. The mechanisms through which the cell maintains *O*-GlcNAc homeostasis are not fully understood but several studies have suggested that a reciprocal regulation of OGT and OGA expression could be one of them. In this study, we investigated the putative regulation of OGT and OGA expression in response to disruption in *O*-GlcNAc homeostasis in colon. We provide *in vitro* and *in vivo* evidences that in colon cells, modulation of *O*-GlcNAcylation levels leads to a compensatory regulation of OGT and OGA expression in an attempt to restore basal *O*-GlcNAcylation levels. Our results also suggests that the regulation of colonic OGA expression in response to changes in *O*-GlcNAc homeostasis occurs mostly at the transcriptional level whereas OGT regulation seems to rely mainly on post-transcriptional mechanisms.

Key words: *O*-GlcNAc transferase, *O*-GlcNAcase, *O*-GlcNAc homeostasis, colon

1. INTRODUCTION

O-linked β -N-acetylglucosaminylation (*O*-GlcNAcylation) is a reversible post translational modification of cytosolic, nuclear, and mitochondrial proteins that consists in the covalent linkage of a unique residue of N-acetylglucosamine (GlcNAc) to serine and threonine residues of targeted proteins. *O*-GlcNAcylation levels are regulated by a single pair of enzymes: *O*-GlcNAc Transferase (OGT) that catalyzes the transfer of GlcNAc from UDP-GlcNAc onto the protein and *O*-GlcNAcase (OGA) that hydrolyzes the residue. UDP-GlcNAc

is synthesized through the hexosamine biosynthesis pathway (HBP) at the crossroad of glucose, amino acids, fatty acids and nucleotides metabolisms (**Figure 1A**). Therefore, *O*-GlcNAcylation is highly sensitive to nutrient availability and is considered as a nutritional sensor that dynamically regulates numerous fundamental cellular processes among which transcription, signal transduction, metabolism and chromatin remodeling [1,2]. Maintenance of *O*-GlcNAc homeostasis (the balance between *O*-GlcNAc addition and removal) appears to be critical to ensure proper cellular functions since disruption of this homeostasis has been linked to the etiology of several human diseases including diabetes, neurodegenerative disorders and cancer [1,3,4]. The mechanisms through which the cell maintains *O*-GlcNAc homeostasis are not fully understood but several studies have suggested that a reciprocal regulation of OGT and OGA expression could be one of them in numerous cell types. For instance, increasing overall *O*-GlcNAcylation levels through the pharmacological inhibition of OGA has been showed to promote a compensatory transcription of *Oga* in SH-SY5Y neuroblastoma cells, HeLa cervical cells and K562 leukemia cells [5]. Conversely, inhibition of OGT with OSMI-1 or Ac4-5S-GlcNAc decreases OGA levels in CHO cells [6]. A compensatory up-regulation of OGT has also been evidenced in the hepatic cell line HEPG2 and in Neuro-2a neuroblastoma cells in response to glucose deprivation [7–9]. By manipulating OGT or OGA activity or expression in a variety of cell types including mouse hepatocytes, CV-1 monkey kidney cells, HEK 293T cells and primary PDAC cells, Qian et al., demonstrated that OGT and OGA regulate each other at the transcriptional level [10]. Mechanistically the authors demonstrated that OGA promotes *Ogt* transcription through cooperation with the histone acetyltransferase p300 and transcription factor C/EBP β [10]. In response to changes in *O*-GlcNAc levels, OGT expression is also post-transcriptionally regulated via a mechanism of intron retention involving a conserved intronic splicing silencer

(ISS): lowering *O*-GlcNAc levels induces efficient splicing and expression of OGT whereas increasing *O*-GlcNAcylation leads to intron four retention and loss of OGT mRNA [11].

In this study, we investigated the putative regulation of OGT and OGA expression in response to disruption in *O*-GlcNAc homeostasis in colon. We provide *in vitro* and *in vivo* evidences that in colon cells, modulation of *O*-GlcNAcylation levels leads to a compensatory regulation of OGT and OGA expression in an attempt to restore basal *O*-GlcNAcylation levels. These results strengthen the hypothesis that a reciprocal regulation of OGT and OGA contributes to the maintenance of *O*-GlcNAc homeostasis and that this phenomenon occurs in most cell types.

2. MATERIAL AND METHODS

2.1 Cell culture

HCT116 cells purchased from ATCC were maintained in Mc Coy's 5A (modified) medium with Glutamax (Thermofischer Scientific) supplemented with 10% fetal calf serum and 1 % ZellShield™ (Biovalley). Cells were cultured at 37°C in water-saturated 5% CO₂ atmosphere.

For glucose deprivation experiments, cells were first maintained in DMEM + Glutamax medium (Thermofisher Scientific) containing 4,5 g/l glucose for 24h. Cells were then rinsed two times with PBS and placed in DMEM + Glutamax containing 1g/l glucose for 48h.

For glucosamine supplementation experiments, cells were first maintained in low glucose medium for 48h as described above and then treated with 5 mM Glucosamine (Sigma-Aldrich) for 24h.

***O*-GlcNAc cycling enzymes inhibitors**

The OGA inhibitor, Thiamet G (Sigma-Aldrich), was dissolved in phosphate buffered saline (PBS) at 1 mM as stock solution and used at a final concentration of 1 μ M. The OGT inhibitor, OSMI-1 (Sigma-Aldrich) was dissolved in DMSO at 25 mM as stock solution and used at a final concentration of 50 μ M. Inhibitors were added to the cell culture medium for 24h.

2.2 Small Interfering RNA

HCT116 cells were reverse-transfected with Lipofectamine RNAiMax (Invitrogen) according to manufacturer's instructions using 5 nM small interfering RNA targeting OGT (siGENOME human OGT siRNA D-019111-01 (si OGT#1) and D-019111-02 (si OGT#2), Dharmacon), OGA (siGENOME human OGA siRNA D-012805-01 (si OGA#1) and D-012805-02 (si OGT#2), Dharmacon) or a scrambled control sequence (si Ctrl; siGENOME RISC free control siRNA, Dharmacon) as previously described [12]. 72h later, cells were harvested for RNA/protein extraction.

2.3 Quantitative RT-PCR

RNA was isolated using Nucleospin® RNA mini spin kit (Macherey-Nagel) according to the manufacturer's instructions. 1 μ g of total RNA was reverse transcribed using random primers and MultiScribe™ reverse transcriptase (Applied Biosystems). Real-time PCR analysis was performed by Power SYBR Green (Applied Biosystems) in a MX3005P fluorescence temperature cycler (Stratagene) according to the manufacturer's instructions. Results were normalized with respect to RPLP0 RNA used as internal control. The primers used for the RT-qPCR analyses are summarized in supplementary table S1.

2.4 Western blotting and antibodies.

For total proteins extraction, cells were lysed in RiPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and proteases inhibitors added at the time of preparation). Protein concentration was determined using the Micro BCA Protein Assay Kit (ThermoFisher Scientific). Equal amount of proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE healthcare). After 1h of blocking in PBSM (PBS with 5% milk), the membranes were incubating overnight at 4°C with specific primary antibodies in PBSTM (PBSM with 0.1% Tween) and washed three times with PBSN (PBS with 0.1% NP-40). The membranes were next incubated for 1h at room temperature with secondary antibodies coupled to peroxidase (Amersham) in PBSM, washed three times in PBSN and revealed by chemiluminescence.

Mouse monoclonal anti-O-GlcNAc (RL2) was purchased from Life technologies (MA1072). Rabbit polyclonal anti-OGT (DM17) was purchased from Sigma-Aldrich (#O6264). Antibodies for OGA (345) were gracious gifts from the laboratory of Gerald Hart (Johns Hopkins University, Baltimore, Maryland, USA). Mouse monoclonal anti-tubulin (sc-23948), mouse monoclonal anti-GAPDH (sc-32223) and goat anti-chicken IgY-HRP (sc-2428) were purchased from Santa Cruz biotechnologies. Anti-mouse IgG-HRP was purchased from GE healthcare (NA931V). Donkey anti-rabbit IgG-HRP was purchased from Millipore (AP182P).

2.5 *In vivo* experiments

Eight-week-old male C57BL/6J mice were purchased from Charles River laboratories (Saint-Germain sur l'Arbresle) and adapted to the environment for 1 week before study. Mice were maintained in a 12h light/dark cycle with water and standard diet (65% carbohydrate, 11% fat, 24% protein). Mice were intraperitoneally injected daily with Thiamet-G (20 mg/kg/d in PBS) or with PBS during a 15 days period as previously described [12]. Procedures were carried out according to the French guidelines for the care of experimental animals. Colon were collected

and washed in PBS. Samples were immediately flash frozen in liquid nitrogen, reduced to powder with hand mortar and pestle and kept at -80°C for further ARN/protein extraction.

2.6 Statistics

Experiments were performed at least thrice independently. Statistical analyses were performed by unpaired Student's t test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

3. RESULTS

3.1. In the colon cancer cell line HCT116, OGT and OGA expression are sensitive to flux modulations through the hexosamine biosynthetic pathway.

To test whether OGT and OGA expression are regulated in response to disruption in *O*-GlcNAc homeostasis in colon, we examined their expression both at the mRNA and protein level in the colon cancer cell line HCT116 under several different conditions that alter global *O*-GlcNAcylation levels (**Figure 1A**). First, we induced flux modulation through the HBP either by glucose deprivation or glucosamine supplementation. As expected, *O*-GlcNAcylation levels decreased in glucose-depleted cells (**Figure 1B**) whereas glucosamine treatment increased them (**Figure 1C**). OGT protein and mRNA expression raised after glucose depletion while both OGA protein and mRNA expression significantly decreased (**Figure 1B**). Conversely, glucosamine-mediated increase in *O*-GlcNAcylation concomitantly lowered OGT and augmented OGA proteins (**Figure 1C**). It is to note that in these two experimental conditions, OGA protein levels follow the levels of transcripts. This is not the case for OGT, for which the variations in transcript levels are weak or even non-existent while the protein expression varies markedly. This suggests that OGA expression is mainly

regulated at the transcriptional level in response to flux modulations through the HBP whereas OGT expression is regulated mainly at the post-transcriptional level.

3.2 Manipulating OGT or OGA expression or activity in this cell line induces a compensatory regulation of their expression.

To further test the influence of changes in *O*-GlcNAc homeostasis on the regulation of OGT and OGA expression in HCT116 cells, we then modulated the *O*-GlcNAcylation levels upwards or downwards by inhibiting the expression of OGA or OGT by RNA interference, respectively and examined the levels of transcripts and proteins as described above (**Figure 2**). As shown in **figure 2A**, knocking-down OGT with 2 different siRNA induced a drastic loss of OGA protein expression that is highly correlated with a decrease in its transcripts. Conversely, the knock-down of OGA also brought about a decrease in OGT mRNA and protein but at a lesser extent. Next, we decreased *O*-GlcNAcylation levels by treating cells with OSMI-1, a specific OGT inhibitor [6] (**Figure 2B**). In agreement with our OGT siRNA results, we observed a sharp drop in OGA mRNA and protein in OGT inhibitor-treated cells. Furthermore, our results show that pharmacological inhibition of OGT also leads to a compensatory induction of its own expression. Inversely, increasing *O*-GlcNAcylation levels through the pharmacological inhibition of OGA with the potent inhibitor Thiamet G [14] led to a concomitant increase of OGA and a decrease of OGT (**Figure 2C**). As we observed in the conditions of modulations of the HBP flux (**Figure 1B** and **1C**), in response to OGT and OGA pharmacological inhibition, variations in OGT protein expression are poorly correlated to variations in the expression of its transcripts while OGA transcripts and proteins vary in the same range. This result strengthens the hypothesis that, in colon, regulation of OGT expression in response to changes in *O*-GlcNAc homeostasis might occur mostly through post-transcriptional mechanisms while OGA regulation seems to mainly occur at the transcriptional level.

3.3 The compensatory regulation of OGT and OGA expression in response to changes in *O*-GlcNAc homeostasis also occurs *in vivo* in mouse colon.

Taken together, our results demonstrate that in HCT116 cells, modulation of *O*-GlcNAcylation levels leads to a compensatory regulation of OGT and OGA expression in an attempt to restore basal *O*-GlcNAcylation levels. Finally, we wanted to confirm our *in vitro* results, *in vivo* in mice. We had previously demonstrated that a daily intraperitoneal injection of Thiamet G for 15 days was sufficient to induce an increase in colonic *O*-GlcNAcylation levels in mice [12] (**Figure 3**). In this model, we now demonstrated that Thiamet G led to a concomitant increase of OGA and a decrease of OGT both at the protein (**Figure 3A**) and mRNA levels (**Figure 3B**) thus confirming our *in vitro* observations.

4. DISCUSSION

More and more studies sustain the hypothesis that global *O*-GlcNAcylation levels must be retained to preserve normal cellular functions and that this maintenance of *O*-GlcNAc homeostasis is achieved in part through a reciprocal regulation of OGT and OGA expression [15]. Aberrant *O*-GlcNAcylation levels, resulting from disruption of *O*-GlcNAc homeostasis, have been linked to the development of several pathologies among which colorectal cancer. Indeed, we and others observed increased contents of *O*-GlcNAcylation in human colon cancer samples in comparison with normal tissues [12,16–18]. Contrary, reducing *O*-GlcNAcylation levels by silencing OGT reduces proliferation, adhesion, migration and anchorage-independent cell growth of colon cancer cell lines [16,19], defining aberrant *O*-GlcNAcylation levels as a new hallmark of colorectal cancer. Thus, understanding the mechanisms by which *O*-GlcNAc homeostasis is maintained or lost in the colon is of considerable interest. In this study, we provide evidences that in colon cells, modulation of *O*-GlcNAcylation levels leads to a compensatory regulation of OGT and OGA expression in an

attempt to restore basal *O*-GlcNAcylation levels (**Figure 4**). Our results also suggests that the regulation of colonic OGA expression in response to changes in *O*-GlcNAc homeostasis occurs mostly at the transcriptional level as it has been previously shown in a variety of cell types [5,10]. In contrast, for OGT, the regulation of its expression seems to be slightly related to a modulation of its transcription but rather seems to rely mainly on post-transcriptional mechanisms. These results are contrary to those obtained by Qian et al. showing that overexpression of OGA in primary hepatocytes produced a concomitant increase in OGT transcripts [10] and those from Taylor et al., which demonstrated that glucose deprivation in the hepatocyte cell line HEPG2 cells also induces a compensatory up-regulation of OGT at the mRNA level [7,8]. However, our results are in agreement with those obtained by Zhang et al. who showed that unlike OGA, OGT transcript levels varied very slightly following OGA inhibition with Thiamet G in SH-SY5Y neuroblastoma cells, HeLa cervical cells and K562 leukemia cells [5]. This suggests that the modulation of OGT expression in response to disruption in *O*-GlcNAc homeostasis should preferably be done either by transcriptional or post-transcriptional mechanisms, depending on the cell type. Regarding the post-transcriptional mechanisms that could be involved, it has been showed that OGT can be *O*-GlcNAcyated [20,21]. The role of OGT *O*-GlcNAcylation is still not known but it can be assumed that this could constitute a self-regulatory mechanism of the enzyme: *O*-GlcNAcylation could regulate the stability/degradation of OGT as it has already been shown for many other proteins such as β -catenin [12], Fatty Acid Synthase [22] or cyclin D1 [23].

To conclude, this study strengthens the hypothesis that a reciprocal regulation of OGT and OGA contributes to the maintenance of *O*-GlcNAc homeostasis in most cell types including colon cells. In the next future, determining precisely how *O*-GlcNAcylation regulates the expression of OGT and OGA in colon cells and how *O*-GlcNAc homeostasis is lost in these cells will be crucial to understanding the mechanisms of colorectal carcinogenesis.

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7. LEGENDS

Figure 1: OGT and OGA expression are sensitive to flux modulations through the Hexosamine Biosynthetic Pathway.

(A) Scheme depicting the Hexosamine Biosynthetic Pathway and *O*-GlcNAcylation processes. The different molecules and inhibitors used in this study to modulate *O*-GlcNAcylation levels up or down are also indicated. GFAT1/2: Glutamine Fructose-6-Phosphate Amido Transferase 1/2 (rate limiting enzyme of the HBP), GK: Glucosamine Kinase, OGT: *O*-GlcNAc Transferase, OGA: *O*-GlcNAcase. (B) HCT116 cells were cultured either in high glucose (4,5 g/l) or in low-glucose (1g/l) containing medium for 48h. (C) After having been cultured in a low glucose medium for 48 hours, HCT116 cells were treated with 5mM glucosamine for 24 hours. (B-C) *Left*: Treatment efficiency was verified by Western Blot (WB) analyses of *O*-GlcNAcylation levels. OGT and OGA expression were also assessed. Tubulin was used as a loading control. Data shown are representative of three independent experiments. *Right*: Cells were harvested for total RNAs extraction. The mRNA expression levels of OGT and OGA were assessed by quantitative RT-PCR. Values were normalized to RPLP0. Data shown are the average \pm SD of three independent experiments. NS: Non Significant, *P<0.05, **P<0.01, ***P<0.001 Student's t-test.

Figure 2: Manipulating OGT or OGA expression or activity in HCT116 cells induces a compensatory regulation of their expression.

(A) HCT116 cells were transfected with non-target control siRNA (si Ctrl) or with either two different siRNAs targeting OGT (siOGT #1 and #2) or OGA (siOGA #1 and #2) for 72h. *left*: *O*-GlcNAcylation levels, OGT and OGA expression were assessed by Western Blot (WB) analyses. GAPDH was used as a loading control. Data shown are representative of three independent experiments. *Right*: Cells were also harvested for total RNAs extraction. The mRNA expression levels of OGT and OGA were assessed by quantitative RT-PCR. Values

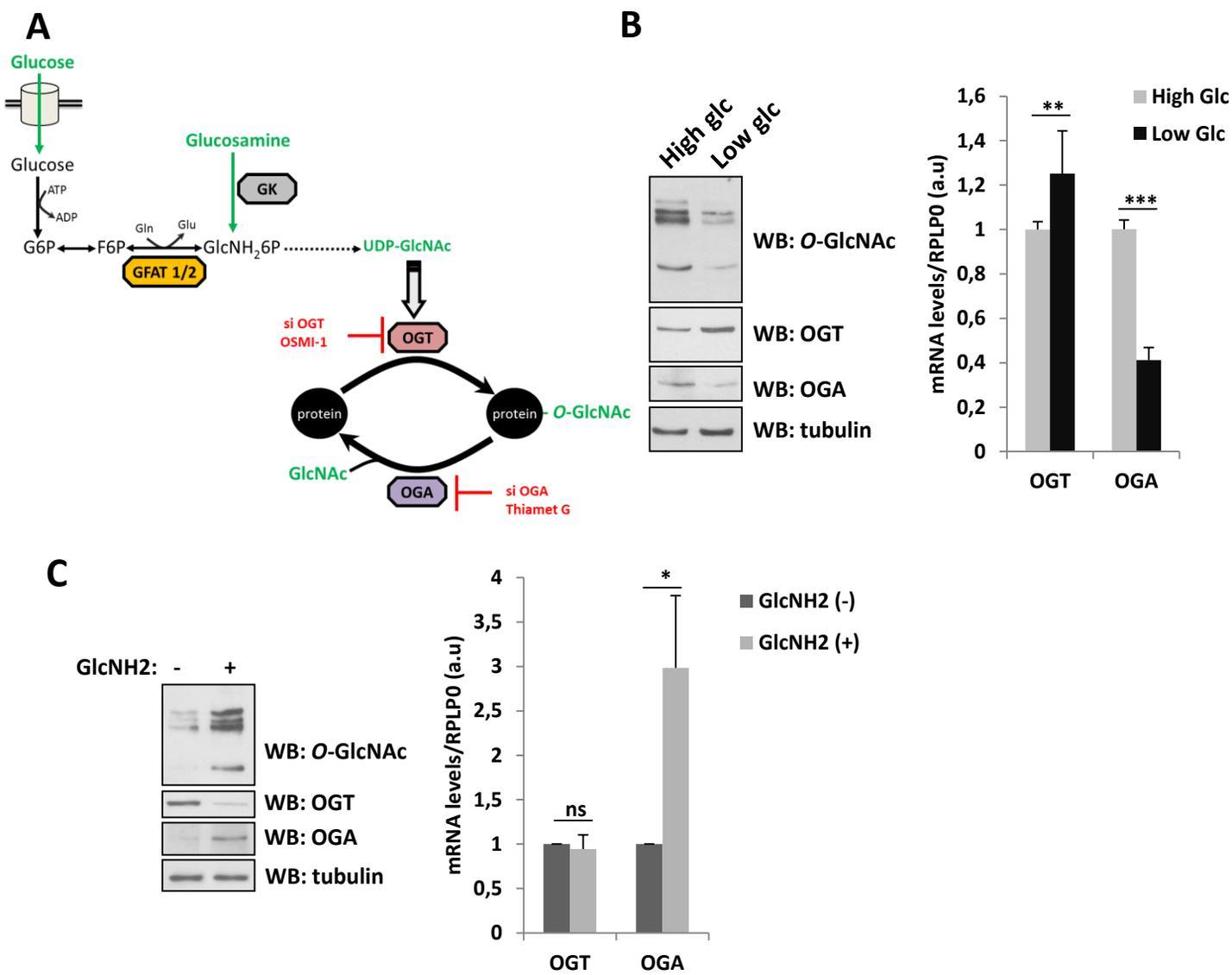
were normalized to RPLP0. Data shown are the average \pm SD of three independent experiments. NS: Non Significant, *P<0.05, **P<0.01, ***P<0.001 Student's t-test. **(B-C)**: HCT116 cells were treated either with the OGT inhibitor OSMI-1 **(B)** or with the OGA inhibitor Thiamet G **(C)** for 24h or the corresponding solvent as a negative control. *Left*: Treatment efficiency was ensured by Western Blot (WB) analyses of *O*-GlcNAcylation levels. OGT and OGA expression were also assessed. GAPDH was used as a loading control. Data shown are representative of three independent experiments. *Right*: Cells were harvested for total RNAs extraction. The mRNA expression levels of OGT and OGA were assessed by quantitative RT-PCR. Values were normalized to RPLP0. Data shown are the average \pm SD of three independent experiments. NS: Non Significant, *P<0.05, **P<0.01, ***P<0.001 Student's t-test.

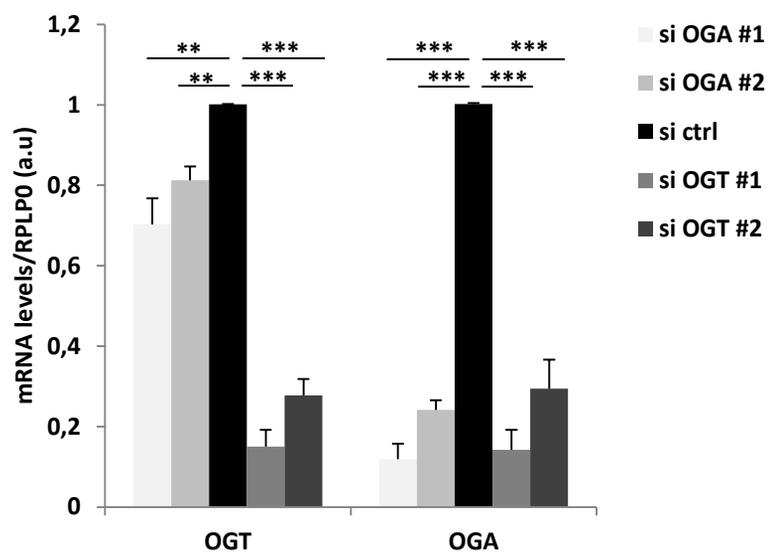
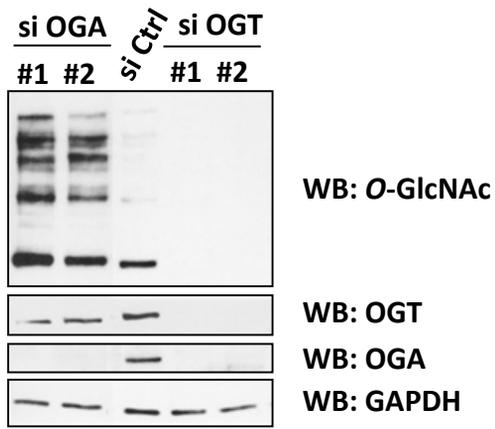
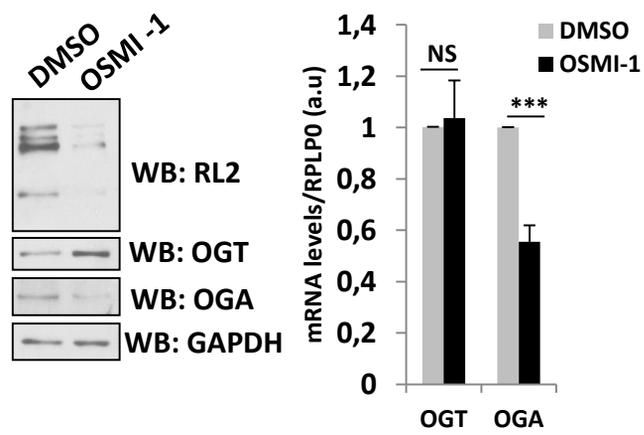
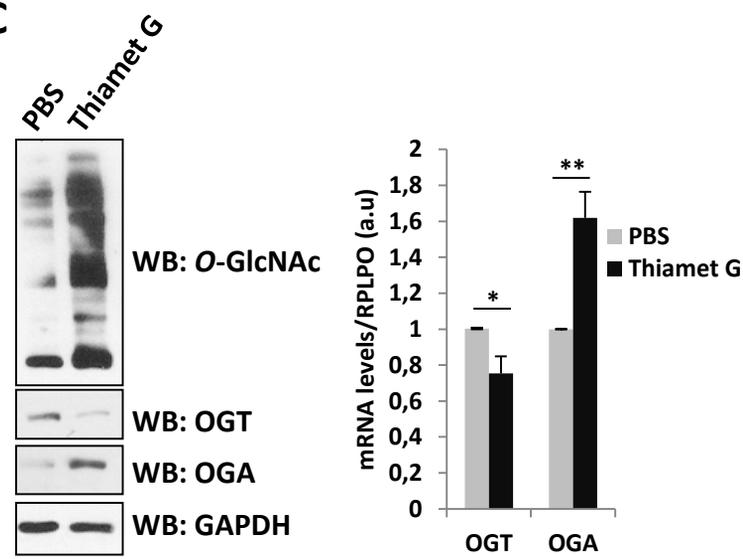
Figure 3: The compensatory regulation of OGT and OGA expression in response to changes in *O*-GlcNAc homeostasis occurs *in vivo* in the colon of mice.

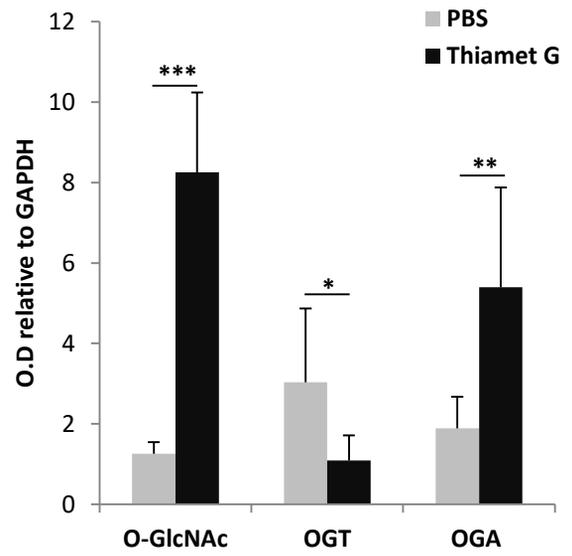
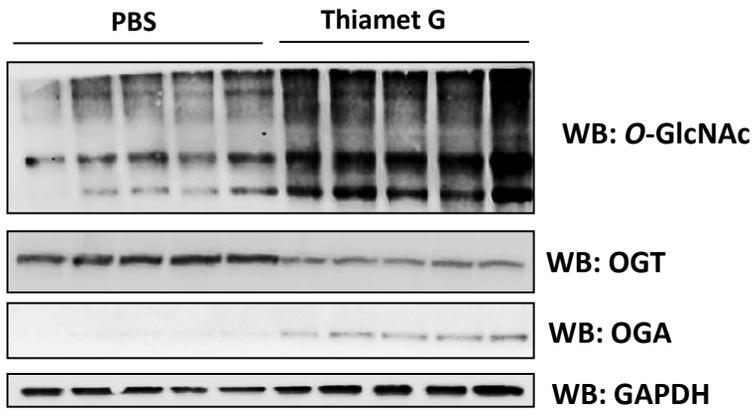
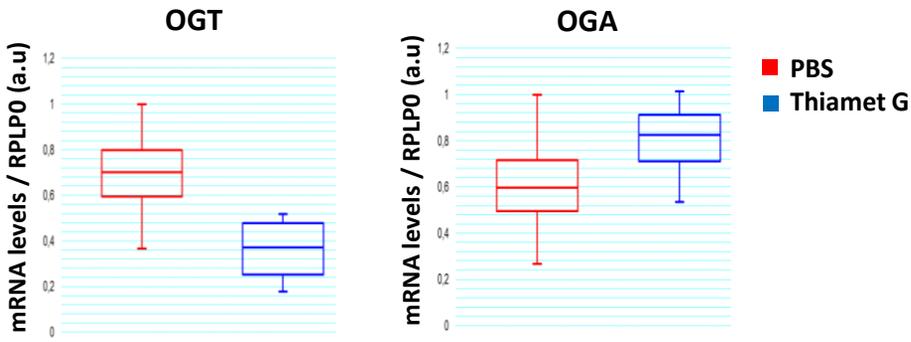
(A) A part of the colon homogenates from five mice treated with Thiamet G for two weeks or with PBS as a negative control were analyzed by Western Blot (WB) according to their *O*-GlcNAcylation, OGT and OGA contents. GAPDH was used as a loading control. The average ratios \pm S.D of *O*-GlcNAcylation/GAPDH, OGT/GAPDH and OGA/GAPDH are represented as histograms (*P<0.05, **P<0.01, ***P<0.001 Student's t-test). **(B)** Another part of the colon homogenates were harvested for total RNAs extraction. The mRNA expression levels of OGT and OGA were assessed by quantitative RT-PCR. Values were normalized to RPLP0. Results are represented as box plots. The box area corresponds to the first and third quartile. The median is shown as a horizontal line in the box. The maximum and minimum of the values are indicated by the whiskers above and below the box.

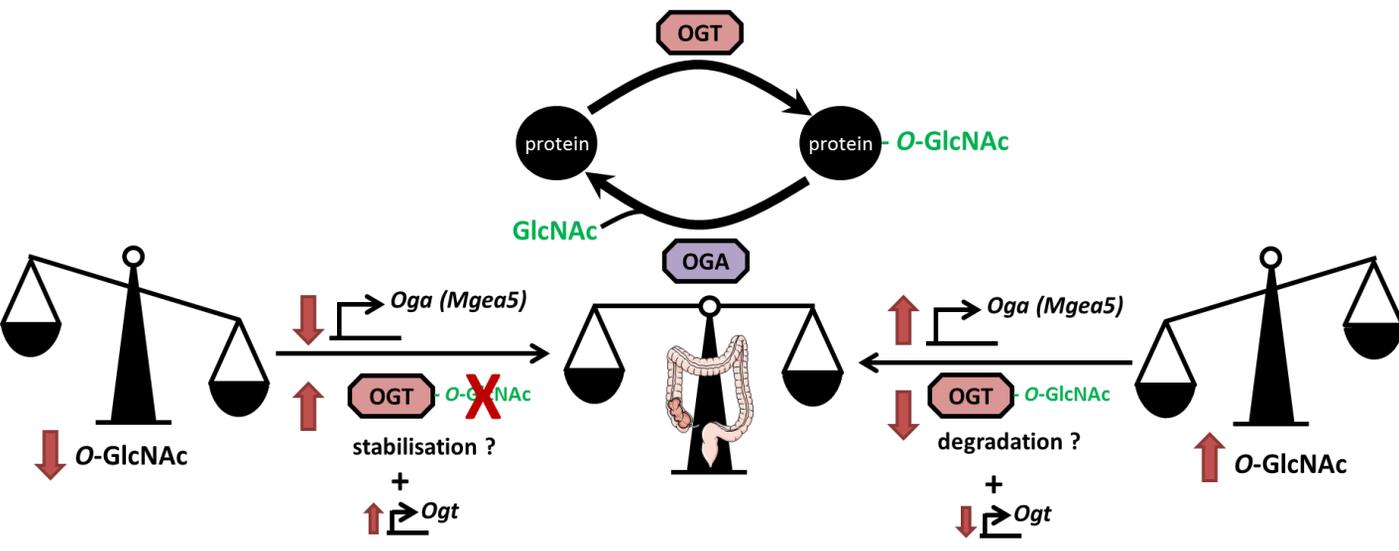
Figure 4: Scheme depicting the main results of the study.

O-GlcNAc homeostasis must be retained to preserve normal colonic cells functions. Indeed, aberrant *O*-GlcNAcylation levels, resulting from disruption of this *O*-GlcNAc homeostasis, have been linked to the development of several pathologies among which colorectal cancer. In this study, we provide evidences that in colon cells, modulation of *O*-GlcNAcylation levels leads to a compensatory regulation of OGT and OGA expression in an attempt to restore basal *O*-GlcNAcylation levels. The regulation of colonic OGA expression in response to changes in *O*-GlcNAc homeostasis occurs mostly at the transcriptional level whereas OGT regulation seems to rely mainly on post-transcriptional mechanisms.



A**B****C**

A**B**



Decourcelle et al., Figure 4