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Regulatory *O*-GlcNAcylation sites on FoxO1 are yet to be identified

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ABSTRACT

O-GlcNAcylation is a reversible post-translational modification that regulates cytosolic and nuclear proteins. We and others previously demonstrated that FoxO1 is O-GlcNAcylated in different cell types, resulting in an increase in its transcriptional activity. Four O-GlcNAcylation sites were identified in human FOXO1 but directed mutagenesis of each site individually had modest (T317) or no effect (S550, T648, S654) on its O-GlcNAcylation status and transcriptional activity. Moreover, the consequences of mutating all four sites had not been investigated. In the present work, we mutated these sites in the mouse Foxo1 and found that mutation of all four sites did not decrease Foxo1 O-GlcNAcylation status and transcriptional activity, and would even tend to increase them. In an attempt to identify other O-GlcNAcylation sites, we immunoprecipitated wild-type O-GlcNAcylated Foxo1 and analysed the tryptic digest peptides by mass spectrometry using High-energy Collisional Dissociation. We identified T646 as a new O-GlcNAcylation site on Foxo1. However, site directed mutagenesis of this site individually or together with all four previously identified residues did not impair Foxo1 O-GlcNAcylation and transcriptional activity. These results suggest that residues important for the control of Foxo1 activity by O-GlcNAcylation still remain to be identified.

1. Introduction

O-GlcNAcylation corresponds to the addition of N-acetyl glucosamine (GlcNAc) on serine or threonine residues of cytosolic, nuclear and mitochondrial proteins. Analogous to phosphorylation, this reversible post-translational modification regulates protein phosphorylation, sub-cellular localisation, stability and activity. O-GlcNAcylation is controlled by two enzymes, O-linked β-N-Acetyl glucosamine transferase (OGT) and O-linked β-N-Acetyl-glucosaminidase (OGA), which catalyse the addition and removal of O-GlcNAc, respectively [1]. The substrate of OGT, UDP-GlcNAc, is provided by the hexosamine biosynthetic pathway (HBP). A fraction (2–3%) of the glucose entering the cell is used in the HBP for the synthesis of UDP-GlcNAc. Therefore, O-GlcNAcylation regulates protein functions according to glucose availability, and a growing amount of evidence indicates that O-GlcNAcylation may participate in the glucotoxicity phenomenon associated with diabetes [2,3]. FoxO1 is a transcription factor that regulates genes involved in the control of cell proliferation and cell death, as well as regulation of oxidative stress and metabolism [4]. Our laboratory previously provided the first evidence that FoxO1 activity is regulated by O-GlcNAcylation in human hepatocytes and kidney

Abbreviations: FOXO1, human forkhead box other 1 protein; Foxo1, mice forkhead box other 1 protein; FoxO1, chordate forkhead box other 1 protein; GFP, green fluorescent protein; GlcNAc, N-acetyl-glucosamine; HBP, hexosamine biosynthetic pathway; HCD, high-energy collisional dissociation; LC-MS/MS, Liquid chromatography tandem mass spectrometry; NButGT, 1,2-dideoxy-2′-propyl-alpha-D-glucopyranoso-[2,1-D]-delta 2′-thiazoline; OGA, O-linked β–N-Acetyl-glucosaminidase; OGT, O-GlcNAc transferase; PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate; YFP, yellow fluorescent protein.

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derived cells [5,6]. We showed that O-GlcNAcylation stimulates the transcriptional activity of FoxO1 independently of any change in its phosphorylation by Akt or subcellular localization [5]. Shortly thereafter, these results were confirmed by others in liver [7], and then extended to other cell types, including endothelial and [8] and β-pancreatic cells [9]. Using electron transfer dissociation tandem mass spectrometry (ETD-MS/MS) analysis, Housley et al. identified 4 residues that are O-GlcNAcylated (T317, S550, T648, S654) [7]. However, only mutagenesis of threonine 317 into alanine resulted in a modest reduction in FOXO1 transcriptional activity, whereas mutation of the other residues had no effect. In agreement with a modest effect of this mutation on FOXO1 transcriptional activity, FOXO1 global O-GlcNAcylation was only marginally reduced by this mutation [7]. Surprisingly, simultaneous mutagenesis of all 4 identified residues was not performed in this study [7]. In the present work, to gain further insight on the importance of identified FoxO1 O-GlcNAcylation sites, we studied the effect of simultaneous mutation of all four sites. Moreover, using HCD MS/MS analysis, we identified an additional threonine as a new O-GlcNAcylation site and evaluated the consequences of its mutation on FoxO1 O-GlcNAcylation and transcriptional activity.

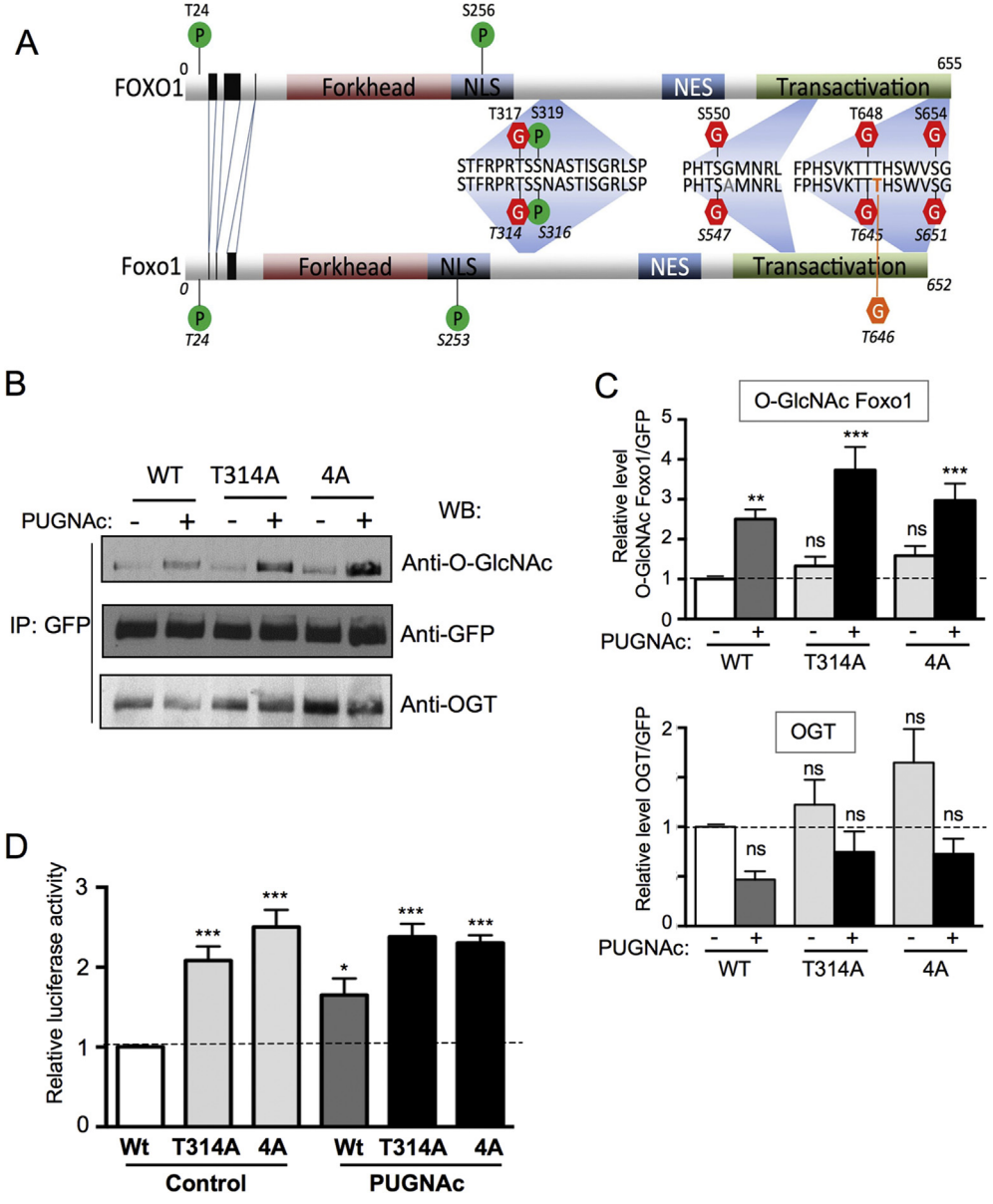

Fig. 1. O-GlcNAcylation levels and transcriptional activities of FoxO1 mutants. (A) Comparison of human (FOXO1) and mouse (Foxo1) sequences surrounding phosphorylation and O-GlcNAcylation sites. Akt phosphorylation sites are highlighted in green, the four O-GlcNAcylation sites identified by Housley et al. are highlighted in red, and the new O-GlcNAcylation site identified in the present study is highlighted in orange. (B) HEK293 cells transfected with YFP-tagged wild-type or mutated Foxo1 cDNAs were treated for 24 h with the hexosaminidases inhibitor PUGNAc. After cell lysis, Foxo1 was immunoprecipitated using anti-GFP antibody and immunoprecipitates were analysed by western-blotting (WB) using anti-O-GlcNAc (CTD 110.6), anti-GFP, and anti-OGT antibodies. A typical western-blot is shown. (C) Densitometric analysis of O-GlcNAc and OGT bands relative to the corresponding GFP bands. Results are the mean ± SEM of 6-8 experiments. (D) HEK293 cells were co-transfected with TK-IRS3-luciferase reporter gene, *Renilla* luciferase, and YFP-tagged wild-type or mutated Foxo1 cDNAs. Transfected cells were treated or not with PUGNAc for 24 h. Results are the mean ± SEM of 5 experiments and are expressed as fold-increase in luciferase activity compared to untreated wild-type Foxo1 transfected cells. *, **, ***, $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively; ns, not significative, when compared to wild-type transfected control using Dunnett's test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

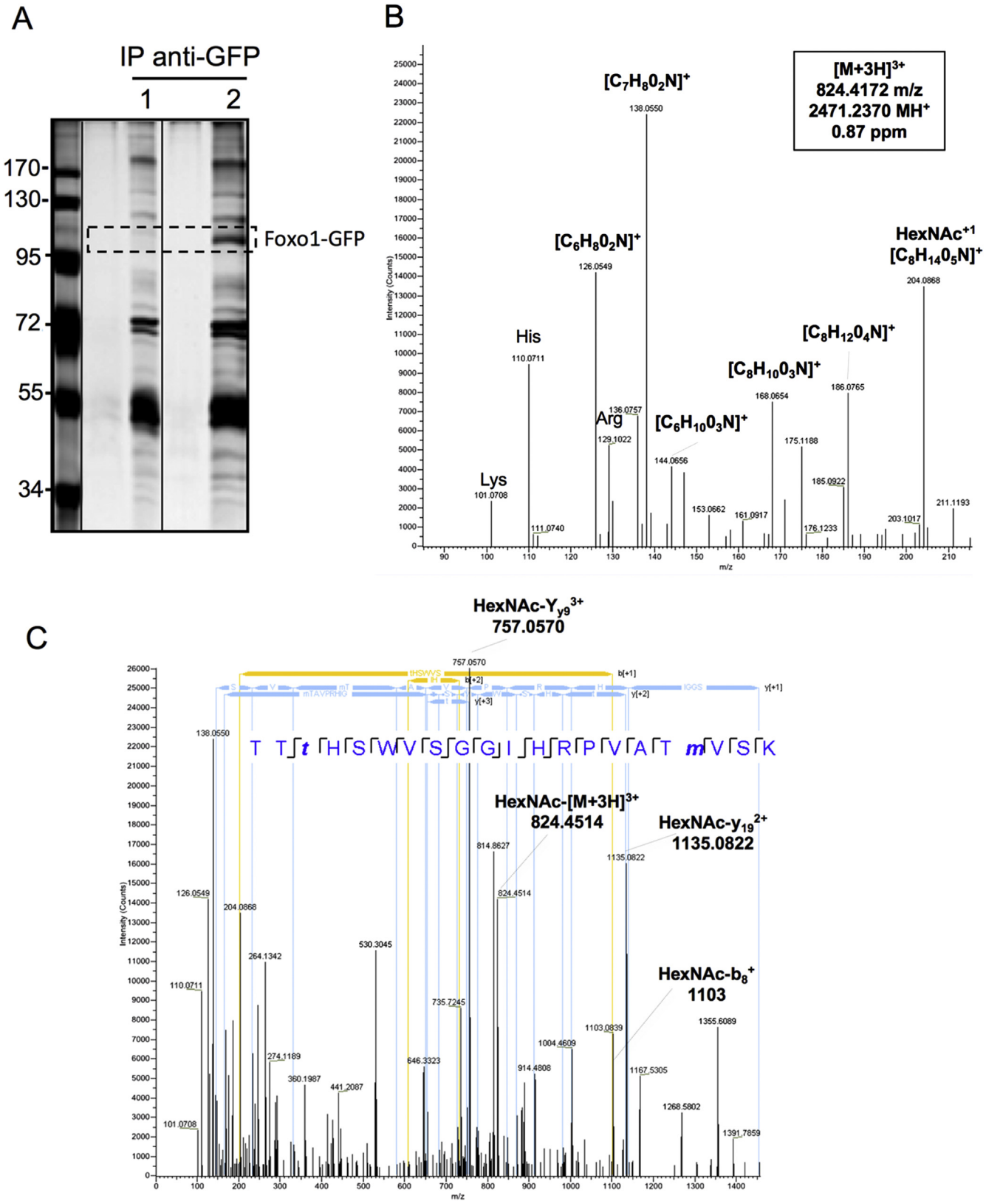

Fig. 2. Production, purification and analysis of Foxo1-GFP by HCD-LC-MS/MS (A) HEK293 cells transfected with either a vector encoding GFP (lane 1) or Foxo1 fused to GFP (lane 2) were cultured for 24 h in presence of the OGA inhibitor NButGT, in order to increase O-GlcNAc level in Foxo1. Cell homogenates were pre-cleared by incubation with protein G sepharose beads. Foxo1-GFP was then immunopurified as described in the Methods section. Protein complexes were resolved by SDS-PAGE and visualized by silver staining. (B, C) HCD MS/MS spectrum of O-GlcNAc modified peptide. (B) Zoom over the low m/z range of HCD spectrum highlighting immonium ions (Lys, His and Arg), HexNAc oxonium ions (*m/z* 204.09) and the distinctive series of ion pattern of HexNAc Fragments (*m/z* 186, *m/z* 168, *m/z* 144, *m/z* 138 and *m/z* 126). (C) Full HCD annotated spectrum of the O-GlcNAc modified peptide.

2. Material and methods

2.1. Chemicals and antibodies

All chemicals were obtained from Sigma, except PUGNAc, which was from Toronto Research Chemicals Inc. NButGT was a kind gift from D. Vocadlo, Simon Fraser University, Canada. Antibodies used in this work were as follows: anti-GFP (Roche Applied Science), anti-O-GlcNAc (CTD 110.6, Covance; RL2, Pierce Biotechnololgy) and anti-OGT, Cell Signalling Technology).

2.2. Cell culture, transfection and treatments

HEK293 cells maintained in DMEM containing 25 mM glucose and 10% foetal calf serum (FCS) were transfected using FuGENE 6 (Roche).

Twenty-four hours after transfection, cells were treated with 100 μM PUGNAc. Cells were then extracted 24 h later for immunoprecipitation or luciferase assays as described previously [5,9].

In some immunoprecipitation experiments, cells were also treated for 24 h in presence of 100 μM PUGNAc and 5 mM glucosamine.

2.3. Immunoprecipitation

2×10^6 cells transfected with YFP-tagged wild-type or mutated Foxo1 were lysed in buffer containing 50 mM Tris–HCl (pH 8), 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP40, 50 mM NaF, 10 mM di-sodium β-glycerophosphate, 1 mM Na_3VO_4, 1 mM streptozotocin and a protease inhibitor cocktail (pepstatin, antipain, leupeptin, aprotinin and AEBSF, 1 μg/ml each). Lysates were centrifuged during 15 min at 10,000 g to remove insoluble material [10]. An aliquot (20 μl) of the clarified extract was used to quantify wild-type and mutant FoxO1-YFP expression in each experimental condition by measuring fluorescence emission at 530 nm after excitation at 485 nm [11]. After normalization for fluorescence activity, cell extracts were incubated with anti-GFP antibody and protein G-Sepharose beads for 3 h at 4 °C. After 4 washes, proteins were eluted from the beads in Laemmli buffer, separated by SDS-PAGE and revealed by western-blotting using Enhanced Chemoluminescence (ECL, Amersham) [12].

2.4. Dual-luciferase assay

The reporter gene 3xIRS-Luc (firefly luciferase coding sequence under the control of an artificial promoter composed of three "Insulin-responsive-sequences") was used [13]. Cells seeded at 0.8×10^5 cells/well in 12-well-plates were transfected 24 h later with 1 μg of 3xIRS-Luc plasmid combined with 2 ng of Renilla luciferase cDNA to normalise for transfection efficiency. After treatment, cells were lysed and luciferase activities were measured with a Centro LB 910 luminometer (Berthold, Thoiry, France) using the DUAL Luciferase Assay kit (Promega, Charbonnieres, France).

2.5. Identification of a new O-GlcNAcylation site on Foxo1

40-50% confluent HEK293 cells were transfected with wild-type Foxo1-GFP or GFP alone. The day before harvest, cells were incubated with NButGT at a final concentration of 100 μM. Cells were homogenized in lysis buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulphate (w/v), and protease inhibitors. Cell extracts were centrifuged at 20,000 *g* for 10 min at 4 °C. The supernatant was first pre-cleared with protein-G-sepharose for 1 h at 4 °C. Foxo1 was then immunoprecipitated with an anti-GFP antibody overnight at 4 °C. Protein-G-sepharose beads were added for 1 h. The beads were gently centrifuged for 1 min at 2500 *g* and washed as follows: lysis buffer; lysis buffer supplemented with 500 mM NaCl; lysis buffer/TNE (10 mM Tris/HCl, 150 mM NaCl, and 1 mM EDTA; pH 7.4; v/v); and finally TNE alone. Protein complexes were resolved by SDS-PAGE and gels were silver stained. The band corresponding to Foxo1 was excised, reduced, alkylated and submitted to tryptic digestion [14]. After digestion, the samples were desalted and dried down. The peptide mixture was dissolved in 4% (v/v) Acetonitrile (ACN), and 0.1% (v/v) Formic Acid (FA) and analysed by LC-MS/MS. The chromatography was performed on an Ultimate 3000 Rapid Separation LC (Dionex). Fractions were loaded onto the enrichment column (C18 PepMap100, 100 μm internal diameter (id), 100 Å pore size, 5 μm particle size, Dionex) using 4% ACN, 0.1% FA. After this step, the analytical column (C18 PepMap100, 75 μm id, 100 Å pore size, 2 μm particle size) was switched in-line, the nano pump delivered a 60 min linear gradient from 4% ACN, 0.1% FA to 37% ACN, 0.1% FA at 300 nL/min flow rate. Mass spectrometry was performed on an LTQ-Orbitrap Velos (Thermo Scientific). Data dependent analysis selected the ten most highly abundant ions for subsequent HCD fragmentation. Precursor scans and HCD products ions were measured in the Orbitrap at a resolution of 30,000 and 7500 respectively. Raw LC-MS/MS were analysed using Proteome-Discoverer 1.4.1.14 using Sequest and against the Uniprot Mouse database implemented with the Foxo-GFP sequence.

2.6. Construction of FoxO1 mutants

Site-directed mutagenesis of Foxo sequence cloned in pEYFP-N1 was performed as described previously [15] using primers listed in Supplementary Table 1.

2.7. Statistical analysis

Statistical analysis was performed by ANOVA followed by Dunnett's post-test using PRISM software.

3. Results

To evaluate the impact of O-GlcNAcylation sites on Foxo1 activity, we used the mouse Foxo1 cDNA fused to YFP. The human and mouse sequences are highly homologous (more than 95% homology), with conservation of the three Akt phosphorylation and the four previously identified O-GlcNAcylation sites (see Fig. 1A). HEK-293 cells transfected with these constructs were treated with PUGNAc (an inhibitor of OGA) for 24 h. Cells were lysed, the expression of Foxo1 was evaluated by measuring the fluorescence of the extracts at 530 nm after excitation at 485 nm. After normalization for fluorescence, equivalent amounts of wild-type and mutant Foxo1-YFP were immunoprecipitated with anti-GFP antibody. Immunoblotting with anti-O-GlcNAc antibody revealed that mutation of T314 (equivalent to T317 in human) or of all four O-GlcNAcylation sites (T314, S547, T645, S651) into alanine did not decrease Foxo1 O-GlcNAcylation (Fig. 1B, C) and rather appeared to increase it. Interestingly, the amount of OGT co-immunoprecipitated with Foxo1-YFP also tended to be higher with mutant Foxo1 compared to wild-type, although the differences were not statistically significant (Fig. 1B, C). In presence of PUGNAc, less OGT was recovered, reflecting the well-known inhibitory effect of this drug on OGT expression [16,17].

The transcriptional activity of the mutant forms of Foxo1 was then evaluated using TK IRS3-luciferase reporter gene [9]. We observed that the basal transcriptional activity was not inhibited

and was even increased with Foxo1-T314A and Foxo-4A mutants compared to wild-type Foxo1 (Fig. 1D).

In order to determine whether the presence of other hitherto undetected sites can explain the persistence of O-GlcNAcylation on Foxo1-4A mutant, wild-type Foxo1 was transfected in HEK293 cells. After 24 h treatment with the inhibitor of OGA NButGT, Foxo1 was immunoprecipitated with anti-GFP antibody and resolved by SDS-PAGE (Fig. 2A). The immunoprecipitated band was excised, in-gel digested with trypsin and analysed by LC-MS/MS. We identified threonine 646 (adjacent to the third O-GlcNAcylation site identified by Housley et al., Fig. 1A) as a new O-GlcNAcylation site on FoxO1 (Fig. 2B, C).

Foxo1-YFP and Foxo1-4A-YFP were then mutated on T646 and transfected in HEK-293 cells. Transfected cells treated for 24 h with PUGNAc were lysed, and after normalization of the extracts for YFP fluorescence, Foxo1 was immunoprecipitated using an anti-GFP antibody. O-GlcNAcylation of wild-type and mutant Foxo1 was evaluated by western-blotting using anti-O-GlcNAc antibody. We observed that Foxo1-T646A and Foxo1-4A-T646A (denominated Foxo1-5A) mutants were O-GlcNAcylated in HEK-293 cells at levels similar or slightly higher than wild-type Foxo1 (Fig. 3A, B). Again, the amount co-immunoprecipitated OGT tended to be higher with mutant than wild-type Foxo1 (Fig. 3A, B).

The transcriptional activity of these new mutants, evaluated using IRS3-luciferase reporter gene, was not inhibited and was even higher for T646A mutant compared to wild-type Foxo1 (Fig. 3C).

Whereas PUGNAc induces protein O-GlcNAcylation through inhibition of OGA, glucosamine stimulates protein O-GlcNAcylation by increasing the pool of UDP-GlcNAc, the substrate of OGT. We also evaluated the effect of glucosamine on O-GlcNAcylation of Foxo1 mutants. As shown in Fig. 4, glucosamine treatment, in absence or presence of PUGNAc, stimulated the O-GlcNAcylation of all Foxo1 mutants at levels similar than to those observed with wild-type Foxo1. Similar results were obtained when the western-blots were probed with another anti-O-GlcNAc antibody (RL2, Supplementary Fig. S1).

4. Discussion

FoxO1 belongs to a family of transcription factors that modulate important cellular functions, by regulating the expression of genes involved in apoptosis, cell cycle, oxidative stress, cell differentiation and glucose metabolism. These factors are regulated by several post-translational modifications, including phosphorylation and O-GlcNAcylation. Upon phosphorylation by Akt on T24, S256 and S316, FoxO1 associates with 14.3.3 protein, which excludes it from the nucleus and sequestrates it in the cytosol, resulting in inhibition of its transcriptional activity [18]. Although O-GlcNAcylation often occurs on serine or threonine phosphorylated residues (so-called Yin-Yang mechanism), we previously showed that this does not occur on FoxO1 Akt phosphorylation sites, as mutation of FoxO1 on all three residues did not impair its O-GlcNAcylation and

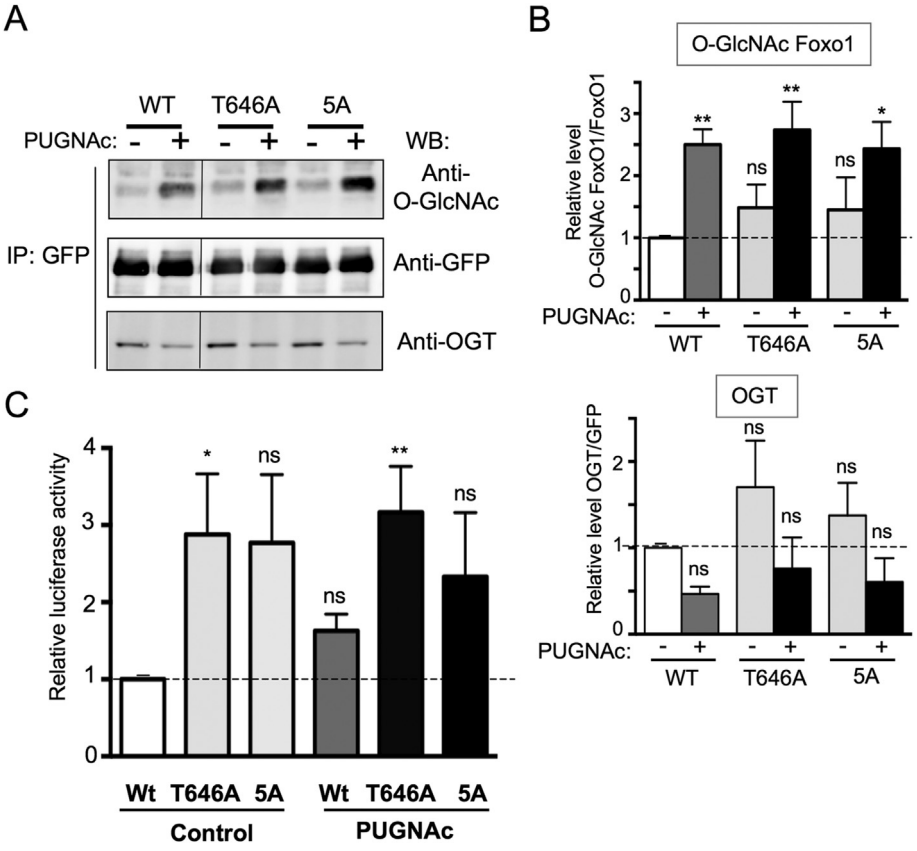

Fig. 3. O-GlcNAcylation and transcriptional activity of Foxo1 mutated on the newly identified O-GlcNAcylation site. HEK293 cells transfected with YFP-tagged wild-type or mutated Foxo1 cDNAs were treated for 24 h with PUGNAc. After cell lysis, Foxo1 was immunoprecipitated using anti-GFP antibody and analysed by western-blotting (WB) using anti-O-GlcNAc (CTD 110.6), anti-GFP and anti-OGT antibodies. (A) A typical western-blot is shown. (B) Densitometric analysis of O-GlcNAc and OGT bands relative to the corresponding GFP bands. Results are the mean ± SEM of 4-8 experiments (C) HEK293 cells were co-transfected with TK-IRS3-luciferase reporter gene, *Renilla* luciferase, and YFP-tagged wild-type or mutated Foxo1 cDNAs. Transfected cells were treated or not with PUGNAc for 24 h. Results are the mean ± SEM of 3-8 experiments and are expressed as fold-increase in luciferase activity compared to untreated wild-type Foxo1 transfected cells. *, **, $p < 0.05$, $p < 0.01$ respectively; ns, not significative, when compared to wild-type transfected control cells using Dunnett's test.

subsequent increase in its transcriptional activity [5]. Identification of the O-GlcNAcylated residues on FoxO1 is therefore of considerable importance in order to understand its mechanism of activation by this post-translational modification. Although Housley et al. identified four O-GlcNAcylated residues (T317, S550, T648, S654) by electron transfer dissociation tandem mass spectrometry (ETD-MS/MS), only mutagenesis of threonine 317 into alanine resulted in a modest (about 25%) reduction in FOXO1 transcriptional activity, whereas mutation of the three other residues had no effect [7]. Moreover, FOXO1 global O-GlcNAcylation, evaluated by western-blotting using CTD110.6 anti-O-GlcNAc antibody, was only marginally reduced by this mutation. Indeed, by densitometric analysis using Image J software, we evaluated that O-GlcNAcylation of the FOXO1-T317A mutant was reduced by only 27% compared to wild-type FOXO1 on the western-blot provided by Housley et al. Moreover, only one western-blot showing O-GlcNAcylation of this mutant was presented in this study, and therefore no statistical analysis was performed to evaluate the significance of this result [7]. In addition, the effects of simultaneous mutagenesis of all four identified residues on FOXO1 O-GlcNAcylation and transcriptional activity were not investigated in this study [7]. We therefore felt that the sites important for regulation of FOXO1 activity by O-GlcNAcylation were not firmly established.

In order to determine whether mutation of all four identified O-GlcNAcylation sites resulted in more robust effects, we generated the corresponding single (T314A) and quadruplet mutant (Foxo1-4A) in the highly conserved mouse Foxo1 cDNA sequence (see Fig. 1A). We observed that O-GlcNAcylation and transcriptional activity of Foxo1

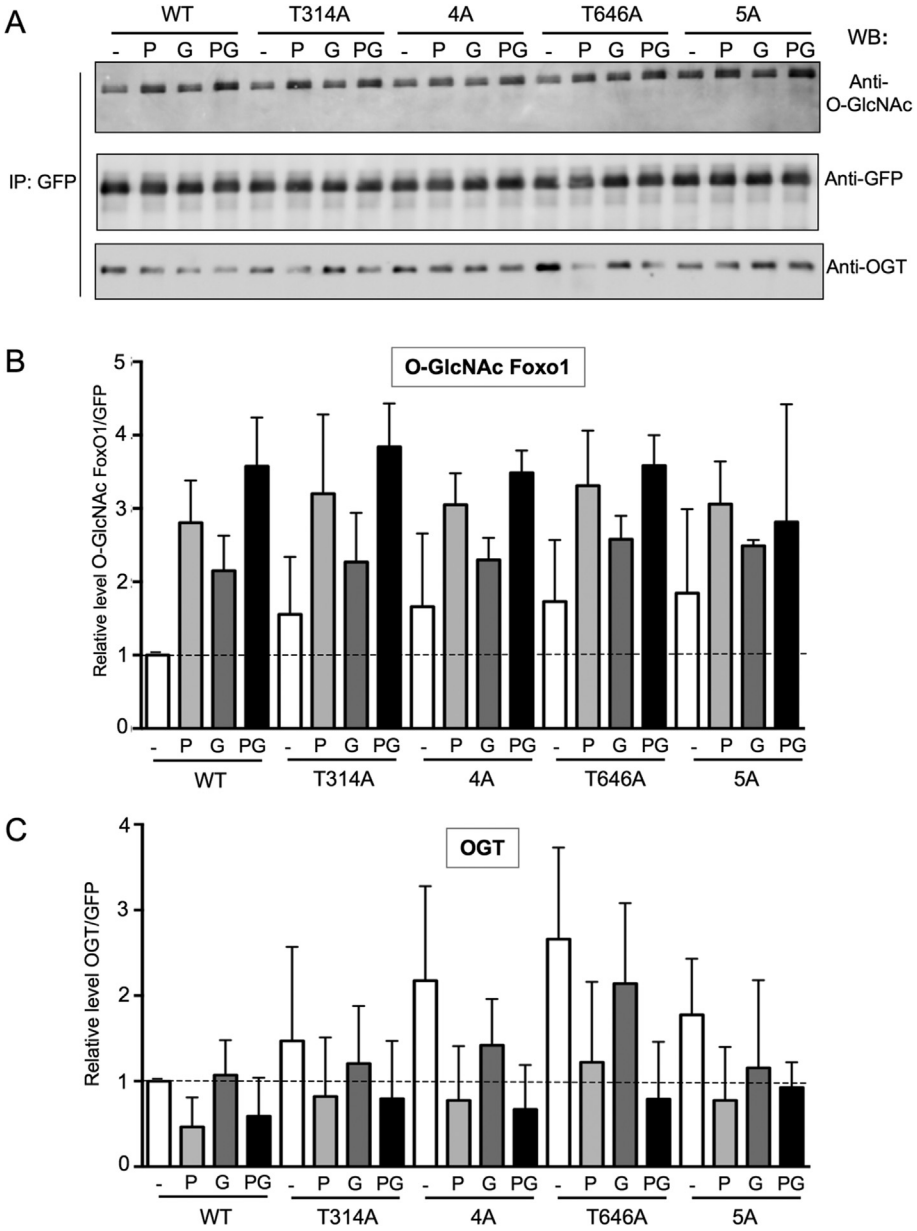

Fig. 4. Effect of PUGNAc and glucosamine on O-GlcNAcylation of the different Foxo1 mutants. HEK293 cells transfected with YFP-tagged wild-type or mutated Foxo1 cDNAs were treated for 24 h with PUGNAc (P), glucosamine (G) or PUGNAc + Glucosamine together (PG). After cell lysis, Foxo1 was immunoprecipitated using anti-GFP antibody and analysed by western-blotting (WB) using anti-O-GlcNAc (CTD 110.6), anti-GFP and anti-OGT antibodies. (A) A typical western-blot is shown. (B, C) Densitometric analysis of O-GlcNAc and OGT bands relative to the corresponding GFP bands. Results are the mean ± SEM of 2 independent experiments.

mutants were not reduced by these mutations and even had the tendency to be increased, compared to their wild-type counterpart.

However, Housley et al. used FLAG-tagged FoxO1 whereas we used YFP-tagged FoxO1. We therefore performed control experiments to verify that YFP itself was not modified by O-GlcNAc and had no effect on 3xIRSLuc luciferase activity. As shown in Supplementary Fig. S2-A, basal and PUGNAc stimulated O-GlcNAcylation was readily detected on Foxo1-YFP, whereas no O-GlcNAc signal could be detected on YFP alone, in agreement with our previous results using the CFP (Cyan Fluorescent Protein) variant [5]. Moreover, YFP itself had no activity on the 3xIRS-Luc reporter gene (Supplementary Fig. S2-B), confirming that the differences between Housley et al. mutants and ours were not related to the presence of the YFP tag.

Thus, our results strongly suggested that sites important for Foxo1 O-GlcNAcylation remain undiscovered. We therefore embarked on identifying additional O-GlcNAcylation sites on Foxo1 using HCD tandem mass spectrometry and found that threonine 646, adjacent to the third O-GlcNAcylation site identified by Housley et al. (T645 in Foxo1), is O-GlcNAcylated. However, mutation of this residue into alanine in wild-type Foxo1 or Foxo1-4A did not reduce O-GlcNAcylation level or transcriptional activity compared to wild-type Foxo1.

Together, our results suggest that additional crucial Foxo1 O-GlcNAcylation sites are yet to be identified. Alternatively, it is possible that when the identified O-GlcNAcylation sites are mutated on Foxo1, OGT glycosylates other adjacent sites. Indeed, promiscuity of OGT for adjacent sites has been described previously in numerous proteins [19], and persistence of high global O-GlcNAc levels after mutagenesis of O-GlcNAcylation sites have also been observed in other proteins [20].

We reproducibly observed that mutagenesis of identified O-GlcNAcylation sites resulted in an increase rather than a decrease in Foxo1 O-GlcNAcylation. Although not observed in all experiments, we also found in several occasions that the amount of OGT co-immunoprecipitated with Foxo1 was higher with mutant than with wild-type Foxo1 (see for instance Fig. 1B and C). This rather puzzling observation suggests that in the absence of its “favourite” O-GlcNAcylation sites, OGT may interact longer with Foxo1, resulting in higher O-GlcNAcylation level.

This work indicates that neither the four previously nor the newly identified O-GlcNAcylation sites on Foxo1 are major regulators of its transcriptional activity. Alternatively, it is possible that the identified residues are not only targets for O-GlcNAcylation but also for phosphorylation. If phosphorylation of one or several of these residues inhibits Foxo1 transcriptional activity, O-GlcNAcylation of these residues may increase Foxo1 activity through inhibition of their phosphorylation [21]. In this case, mutation of these amino acids into alanine should have similar stimulatory effect on Foxo1 transcriptional activity than their O-GlcNAcylation, explaining the increased transcriptional activity observed with Foxo1 mutants. It is also possible that depending on the specific residues that are modified, O-GlcNAcylation may have both positive and negative effects on Foxo1 transcriptional activity, as previously observed for other transcription factors such as Sp1 [22–26] and NFκB [20,27–29].

Clearly, additional work will be necessary to firmly determine which residues are important for regulation of FoxO1 by O-GlcNAcylation and to fully understand the molecular mechanisms by which this modification increases Foxo1 transcriptional activity.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.114.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.114.

References

[1] T. Lefebvre, V. Dehennaut, C. Guinez, S. Olivier, L. Drougat, A.M. Mir, M. Mortuaire, A.S. Vercoutter-Edouart, J.C. Michalski, Dysregulation of the nutrient/stress sensor O-GlcNAcylation is involved in the etiology of cardiovascular disorders, type-2 diabetes and Alzheimer's disease, Biochim. Biophys. Acta 1800 (2009) 67–79.
[2] T. Issad, M. Kuo, O-GlcNAc modification of transcription factors, glucose sensing and glucotoxicity, Trends Endocrinol. Metab. 19 (2008) 380–389.
[3] T. Issad, E. Masson, P. Pagesy, O-GlcNAc modification, insulin signaling and diabetic complications, Diabetes Metab. 36 (2010) 423–435.
[4] E.L. Greer, A. Brunet, FOXO transcription factors at the interface between longevity and tumor suppression, Oncogene 24 (2005) 7410–7425.
[5] M. Kuo, V. Zilberfarb, N. Gangneux, N. Christeff, T. Issad, O-glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene, FEBS Lett. 582 (2008) 829–834.
[6] M. Kuo, V. Zilberfarb, N. Gangneux, N. Christeff, T. Issad, O-GlcNAc modification of FoxO1 increases its transcriptional activity: a role in the glucotoxicity phenomenon? Biochimie 90 (2008) 679–685.
[7] M.P. Housley, J.T. Rodgers, N.D. Udeshi, T.J. Kelly, J. Shabanowitz, D.F. Hunt, P. Puigserver, G.W. Hart, O-GlcNAc regulates FoxO activation in response to glucose, J. Biol. Chem. 283 (2008) 16283–16292.
[8] V. Cifarelli, S. Lee, D.H. Kim, T. Zhang, A. Kamagate, S. Slusher, S. Bertera, P. Luppi, M. Trucco, H.H. Dong, FOXO1 mediates the autocrine effect of endothelin-1 on endothelial cell survival, Mol. Endocrinol. 26 (2012) 1213–1224.
[9] Y. Fardini, E. Masson, O. Boudah, R. Ben Jouira, C. Cosson, C. Pierre-Eugene, M.S. Kuo, T. Issad, O-GlcNAcylation of FoxO1 in pancreatic beta cells promotes Akt inhibition through an IGFBP1-mediated autocrine mechanism, Faseb J. 28 (2014) 1010–1021.
[10] T. Issad, M. Combettes, P. Ferre, Isoproterenol inhibits insulin-stimulated tyrosine phosphorylation of the insulin receptor without increasing its serine/threonine phosphorylation, Eur. J. Biochem. 234 (1995) 108–115.
[11] C. Blanquart, J. Achi, T. Issad, Characterization of IRA/IRB hybrid insulin receptors using bioluminescence resonance energy transfer, Biochem. Pharmacol. 76 (2008) 873–883.
[12] J.F. Liu, T. Issad, E. Chevet, D. Ledoux, J. Courty, J.P. Caruelle, D. Barritault, M. Crepin, B. Bertin, Fibroblast growth factor-2 has opposite effects on human breast cancer MCF-7 cell growth depending on the activation level of the mitogen-activated protein kinase pathway, Eur. J. Biochem. 258 (1998) 271–276.
[13] S. Guo, G. Rena, S. Cichy, X. He, P. Cohen, T. Unterman, Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence, J. Biol. Chem. 274 (1999) 17184–17192.
[14] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.

[15] D. Lacasa, N. Boute, T. Issad, Interaction of the insulin receptor with the receptor-like protein tyrosine phosphatases PTPalpha and PTPepsilon in living cells, Mol. Pharmacol. 67 (2005) 1206–1213.
[16] C. Slawson, N.E. Zachara, K. Vosseller, W.D. Cheung, M.D. Lane, G.W. Hart, Perturbations in O-linked beta-N-acetylglucosamine protein modification cause severe defects in mitotic progression and cytokinesis, J. Biol. Chem. 280 (2005) 32944–32956.
[17] Z. Zhang, E.P. Tan, N.J. VandenHull, K.R. Peterson, C. Slawson, O-GlcNAcase expression is sensitive to changes in O-GlcNAc homeostasis, Front. Endocrinol. (Lausanne) 5 (2014) 206.
[18] Y. Zhao, Y. Wang, W.G. Zhu, Applications of post-translational modifications of FoxO family proteins in biological functions, J. Mol. Cell. Biol. 3 (2011) 276–282.
[19] P. Zhao, R. Viner, C.F. Teo, G.J. Boons, D. Horn, L. Wells, Combining high-energy C-trap dissociation and electron transfer dissociation for protein O-GlcNAc modification site assignment, J. Proteome Res. 10 (2011) 4088–4104.
[20] W.H. Yang, S.Y. Park, H.W. Nam, H. Kim do, J.G. Kang, E.S. Kang, Y.S. Kim, H.C. Lee, K.S. Kim, J.W. Cho, NFkappaB activation is associated with its O-GlcNAcylation state under hyperglycemic conditions, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 17345–17350.
[21] S. Olivier-Van Stichelen, V. Dehennaut, A. Buzy, J.L. Zachayus, C. Guinez, A.M. Mir, I. El Yazidi-Belkoura, M.C. Copin, D. Boureme, D. Loyaux, P. Ferrara, T. Lefebvre, O-GlcNAcylation stabilizes beta-catenin through direct competition with phosphorylation at threonine 41, Faseb J. 28 (2014) 3325–3338.
[22] X.L. Du, D. Edelstein, L. Rossetti, I.G. Fantus, H. Goldberg, F. Ziyadeh, J. Wu, M. Brownlee, Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12222–12226.
[23] X. Yang, K. Su, M.D. Roos, Q. Chang, A.J. Paterson, J.E. Kudlow, O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 6611–6616.
[24] J.E. Kudlow, Post-translational modification by O-GlcNAc: another way to change protein function, J. Cell. Biochem. 98 (2006) 1062–1075.
[25] H.J. Goldberg, C.I. Whiteside, G.W. Hart, I.G. Fantus, Posttranslational, reversible O-glycosylation is stimulated by high glucose and mediates plasminogen activator inhibitor-1 gene expression and Sp1 transcriptional activity in glomerular mesangial cells, Endocrinology 147 (2006) 222–231.
[26] S.S. Chung, J.H. Kim, H.S. Park, H.H. Choi, K.W. Lee, Y.M. Cho, H.K. Lee, K.S. Park, Activation of PPARgamma negatively regulates O-GlcNAcylation of Sp1, Biochem. Biophys. Res. Commun. 372 (2008) 713–718.
[27] D.F. Allison, J.J. Wamsley, M. Kumar, D. Li, L.G. Gray, G.W. Hart, D.R. Jones, M.W. Mayo, Modification of RelA by O-linked N-acetylglucosamine links glucose metabolism to NF-kappaB acetylation and transcription, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 16888–16893.
[28] D. Xing, K. Gong, W. Feng, S.E. Nozell, Y.F. Chen, J.C. Chatham, S. Oparil, O-GlcNAc modification of NFkappaB p65 inhibits TNF-alpha-induced inflammatory mediator expression in rat aortic smooth muscle cells, PLoS One 6 (2011) e24021.
[29] L. Baudoin, T. Issad, O-GlcNAcylation and inflammation: a vast territory to explore, Front. Endocrinol. (Lausanne) 5 (2015) 235.