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Cell Chemical Biology

The O-GlcNAc Transferase Intellectual Disability Mutation L254F Distorts the TPR Helix

Graphical Abstract

Highlights

- The intellectual disability L254F mutation in OGT affects activity

- The L254F mutation leads to shifts up to 12 Å in the OGT structure

- Thermal denaturing studies reveal reduction in TPR stability caused by L254F

- Simulations suggest the presence of alternate TPR_{L254F} conformations

Authors

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In Brief

Gundogdu et al. demonstrate how the intellectual disability-associated mutation in O-GlcNAc transferase (OGT), L254F, leads not only to large shifts in OGT structure and altered dynamics in solution, but also a non-specific reduction in activity, providing the first molecular characterization of such a mutation.
The O-GlcNAc Transferase Intellectual Disability Mutation L254F Distorts the TPR Helix

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INTRODUCTION

O-linked β-N-acetyl-β-D-glucosamine (O-GlcNAc) transferase (OGT) regulates protein O-GlcNAcylation, an essential post-translational modification that is abundant in the brain. Recently, OGT mutations have been associated with intellectual disability, although it is not understood how they affect OGT structure and function. Using a multidisciplinary approach we show that the L254F OGT mutation leads to conformational changes of the tetratricopeptide repeats and reduced activity, revealing the molecular mechanisms contributing to pathogenesis.

RESULTS AND DISCUSSION

The ID-associated mutation L254F (Vaidyanathan et al., 2017) located on TPR helix 7, distant from the active site (Figure 1A). In vitro OGT activity was tested with a short acceptor peptide derived from the RB2 protein (Pathak et al., 2015). In this assay, the L254F mutation had no effect on steady-state kinetics of OGT (peptide $K_{m} = 0.6$ mM, $V_{max} = 15$ nM/s for both enzymes; Figure S1A). Next, we measured OGT activity against de-O-GlcNAcylated HEK-293 cell lysate containing a multitude of OGT-target proteins in a sequence-specific fashion, in a manner similar to HSP70/90 organizing protein (Scheufler et al., 2000). These two models explain how OGT can modify thousands of intracellular proteins on specific sites while also serving as a scaffold in multi-protein complexes. Here, we demonstrate that the L254F mutation in OGT induces folding defects in the TPR superhelix, suggesting that changes in interactions with substrates and/or binding partners may underpin the ID disease phenotype.
control (Figures 1B and S1B). Thus, in our hands the ID OGT L254F mutation shows effects on in vitro activity.

A single TPR motif consists of an anti-parallel pair of α helices, named helix A and B, which are held together by interactions between conserved residues in the 34-amino-acid consensus sequence W4-L7-G8-Y11-A20-F24-A27-P32 (Jínek et al., 2004; Lamb et al., 1995). The W4-L7-G8-Y11 motif on helix A forms a hydrophobic pocket into which the bulky residue of the A20-F24-A27 motif on helix B is lodged. The OGT TPRs possess two additional features: helix A contains a ladder of conserved asparagines (N6) on TPRs 2–13.5; helix B contains a series of large aliphatic residues on TPRs 6–13.5 (Jínek et al., 2004; Lazarus et al., 2013; Rafie et al., 2017). While the N6 ladder is involved in substrate recognition (Jínek et al., 2004; Lazarus et al., 2013; Rafie et al., 2017), the series of large aliphatic residues (J30; where J represents Leu, Ile, or Val), of which Leu254 is part, interdigitate with aliphatic residues found at the first position within the TPR motifs (X1; where X represents Leu, Ala, or Pro). To investigate structural changes attributable to the L254F mutation, we determined the crystal structure of the mutant TPR domain (TPR_{L254F}). Recombinant protein was obtained from Escherichia coli using the construct boundaries previously employed to crystallize the wild-type OGT TPR domain (TPR_{WT}, Figure 1A) (Jínek et al., 2004). Diffraction data were collected to 1.75 Å (Table 1) and initial refinement starting from the TPR_{WT} structure required substantial rebuilding of the terminal TPRs, an early indication of considerable conformational changes. Indeed, the overall root-mean-square deviation (RMSD) on 343 Cα atoms of the refined TPRL254F structure versus that of TPR_{WT} was 1.6 Å.

In wild-type OGT, L254 occupies the interface between the helices of TPR7, with its side chain constricted in a pocket formed by the surrounding residues N223, L225, Y228, and R250 (Figure 1C). Mutation of L254 to the bulkier Phe appears to be accommodated in this pocket, however, by causing small changes in torsion angles of the residues lining the pocket and by displacing TPR helix 7B away from helix 7A by 1.5 Å (Figure 1C). We examined the effects of this change in the context of the full-length protein, modeled by merging the TPR_{WT}/TPR_{L254F} structures and an OGT structure containing the catalytic domain (residues 325–1,038; PDB: 4XIF [Pathak et al., 2015]) using the overlapping TPRs (residues 325–381) as superposition anchor (Figures 1A and 1D). This reveals a deviation from the wild-type TPR geometry that is propagated toward the N terminus (Figures 2D and 2E). Increasing shifts of the TPRs starting from the mutation site lead to a maximum shift of 12 Å for the N-terminal TPR (Figures 2D and 2E). Thus, the L254F mutation causes a distortion of the TPR helix.

To investigate possible effects of the L254F mutation on stability of the TPRs, we determined the melting temperatures of TPR_{WT} and TPR_{L254F} using differential scanning fluorimetry. A monophasic, sigmoidal melting curve was obtained for TPR_{WT} (Table 1) and initial refinement starting from the TPR_{WT} structure required substantial rebuilding of the terminal TPRs, an early indication of considerable conformational changes. Indeed, the overall root-mean-square deviation (RMSD) on 343 Cα atoms of the refined TPRL254F structure versus that of TPR_{WT} was 1.6 Å.

Figure 1A (Jínek et al., 2004). Diffraction data were collected to 1.75 Å (Table 1) and initial refinement starting from the TPR_{WT} structure required substantial rebuilding of the terminal TPRs, an early indication of considerable conformational changes. Indeed, the overall root-mean-square deviation (RMSD) on 343 Cα atoms of the refined TPRL254F structure versus that of TPR_{WT} was 1.6 Å.
with an inflection point ($T_m$) of 58°C (Figure 2A). The melting curve for TPR$_{L254F}$ was shifted, yielding a $T_m$ of 55°C, indicative of reduced thermal stability (Figure 2A). This is in agreement with previous characterization of OGT$_{L254F}$ in patient-derived cells, where the L254F mutation was found to reduce OGT half-life by 2-fold (Vaidyanathan et al., 2017). Thus it appears that the L254F mutation destabilizes OGT.

To explore the mechanisms underlying the reduction in thermal stability, we performed atomistic molecular dynamics simulations (2 μs) using a fragment of the TPR$_{WT/L254F}$ crystal structures, comprising TPRs 6, 7, and 8 (residues 189–294; hereafter sTPR$_{WT}$ and sTPR$_{L254F}$, respectively; Figure 1A). In these simulations we monitored the intra-TPR distance between the C$_{α}$ atoms of residues L$_{225}$ (the first residue of helix 7A) and L$_{254}$ (the last residue of helix 7B), the TPR6-7 B-A'-B' angle formed between the C$_{α}$ atoms of residues L$_{220}$, L$_{225}$, and L$_{254}$, and the $χ_1$ dihedral angle of residue 254 (Figure 2B). The intra-TPR distance and the angle B-A'-B' were stable in the sTPR$_{WT}$ simulations (Figures 2C, S2, and S3), while in the sTPR$_{L254F}$ simulations higher conformational plasticity was observed, showing a partition between two major conformations (Figures 2B and 2C). In sTPR$_{L254F}$ conformation 1 (sTPR$_{L254F-C1}$), the F$_{254}$ side chain occupies the same position as observed in the TPR$_{L254F}$ crystal structure, while in sTPR$_{L254F}$ conformation 2 (sTPR$_{L254F-C2}$), the F$_{254}$ phenyl moiety interacts with the side chains of N$_{223}$, L$_{225}$, and Y$_{228}$ and the backbone of the F$_{224}$ and R$_{250}$ (Figure 2B). In sTPR$_{L254F-C2}$ the F$_{254}$ side chain adopts a different conformation, with its aromatic ring positioned parallel to the N$_{223}$ backbone amide in a manner that enables a phenyl-amide interaction. This is accompanied by a 140° ± 17° shift in the F$_{254}$ $χ_1$ dihedral angle, which in turn distorts the TPR geometry, increasing the intra-TPR distance by 2.1 ± 0.1 Å and shifting the B-A'-B' angle by approximately 21° ± 7° (Figure 2B). Similar to the conformational rearrangement described for the TPR$_{L254F}$ crystal structure, these local changes propagate through subsequent TPR repeats and modify the overall geometry of the protein. Thus, the L254F mutation destabilizes the interface between TPRs 6 and 7.

The data presented here show that the L254F mutation causes a subtle structural distortion at the mutation site that propagates through the TPR superhelix, resulting in a substantial displacement of the N-terminal TPRs and a markedly increased structural plasticity compared with the TPR$_{WT}$. Although in vitro assays show global effects on the O-GlcNAc proteome, they are modest. However, given that OGT is essential for life from stem cells to vertebrates and resides on the X chromosome, it is likely that only relatively subtle mutations are tolerated in males. Moreover, it is possible that the ID-associated mutations result in misrepresentation of a distinct subset of the O-GlcNAc proteome in different cell lineages, or under certain stimuli. In light of this, it is interesting to note that all OGT ID mutations reported to date are at ages, or under certain stimuli. In light of this, it is interesting to note that all OGT ID mutations reported to date are at

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Related to Figures 1C and 1D. Values in parentheses represent the highest-resolution shell.

SIGNIFICANCE

O-GlcNAc was discovered more than three decades ago, and more than 1,000 proteins in the human proteome are known to be O-GlcNAc modified. However, there is still a substantial gap in our understanding of how O-GlcNAcylation regulates protein function and downstream cellular pathways. An inroad into this became possible with the recent discovery that patients with mutations in OGT suffer from ID, the clearest evidence yet linking O-GlcNAc to neuronal function, in addition to previous reports implying dysregulation of O-GlcNAc in neurodegeneration. Although several OGT mutations linked to ID have recently been reported, it is not understood how these mutations affect OGT at the molecular level. This article is the first to describe the substantial molecular consequences of such a mutation. The L254F mutation resides in the TPR helix, which facilitates OGT substrate recognition. Using X-ray crystallography we have uncovered that this mutation leads to shifts...
up to 12 Å in the TPR helix, which ties in with the observed reduction in activity. Furthermore, using differential scanning fluorimetry and molecular dynamics simulations we show that the TPR helix is significantly destabilized, leading to defects in substrate recognition. This is a major advance for the O-GlcNAc field as it provides a molecular understanding of this mutation and provides a platform for exploring effects on the O-GlcNAc proteome.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures and can be found with this article online at https://doi.org/10.1016/j.chembiol.2018.03.004.

**ACKNOWLEDGMENTS**

This work funded by a Wellcome Trust Investigator award (110061) to D.M.F.v.A and a Wellcome Trust ISSF award (WT097818MF) to U.Z. M.G. was supported by a University of Dundee Translational Medical Research Fund Ph.D. fellowship. A.G. was supported by a Wellcome Trust PhD studentship. Diffraction experiments were performed on beamline ID30A-1 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. We are grateful to Matthew Bowler at the ESRF for providing assistance in using the beamline ID30A-1.

**AUTHOR CONTRIBUTIONS**

D.M.F.v.A. and U.Z. conceived the study; M.G. performed all protein production, 
in vitro assays, and protein crystallography experiments; M.G. and A.G. performed the OGT activity assay against HEK-293 cell lysate; S.L. performed molecular dynamics simulations; A.T.F. performed molecular biology experiments; M.G., A.G., and S.L. analyzed the data; M.G. and D.M.F.v.A. interpreted the data and wrote the manuscript with input from all authors.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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REFERENCES


# STAR METHODS

## KEY RESOURCES TABLE

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## CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daan M. F. van Aalten (dmfvanaalten@dundee.ac.uk).
EXPERIMENTAL MODEL DETAILS

Cell Lines
Female HEK-293 cells were obtained from ATCC. Due to the use of HEK-293 lysates solely for biochemistry, the cell line was not further authenticated. The cells were tested negative for mycoplasma contamination (October 2017). HEK-293 cells were grown on 15 cm plates in DMEM (Life Technologies) supplemented with 2 mM L-glutamine (Sigma), 100 units/ml Penicillin and 100 µg/ml Streptomycin (Life Technologies), and 10% foetal calf serum (Labtech).

METHOD DETAILS

Molecular Cloning
The full-length codon optimised OGT was obtained from GenScript and subcloned as a BamHI–NotI fragment into pHEX-6P-1 (modified version of pGEX-6P-1 which contains a 6His tag instead of GST). The L254F mutation was introduced using a method similar to the QuikChange site-directed mutagenesis kit by Agilent but using KOD polymerase and DpnI from Fermentas. All inserts were confirmed by DNA sequencing. The TPR region of OGT (residues 26–410) was amplified from both the OGTWT and OGT_{L254F} expression constructs. These were cloned into pGEX-6P-1 as BamHI–NotI fragments and the inserts were confirmed by DNA sequencing. Forward and reverse primers used were CTGGGATCCGGCGGACGATGAAATATCAG and GATGCGGCCGCTTAGTCTTGCATTTCTTTCAGCGTATTAC, respectively.

OGT Expression and Purification
Full length OGT_{WT} and OGT_{L254F} were expressed in E. coli BL21-DE3 as N-terminal His fusion proteins as described previously (Willems et al., 2017). Briefly, transformed E. coli cells were grown in autoinduction medium at 37°C with agitation until OD_{600} reached 0.8, at which point the temperature was lowered to 18°C for overnight incubation. Cells were harvested by centrifugation at 4°C (35 min, 4,500 × g). Resulting cell pellet was resuspended in base buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5 mM TCEP (tris-[2-carboxyethyl]phosphine) supplemented with 25 mM imidazole 0.1 mg/ml DNase I and protease inhibitor cocktail (1 mM benzamidine, 0.2 mM PMSF, 5 mM leupeptin), and lysed via continuous flow cell disruptor (three passes at 15,000 PSI). Lysate was clarified by centrifugation (30,000 g for 1 h at 4°C) followed by incubation with 1 ml per litre of culture of Ni^{2+}–NTA agarose resin (GE Healthcare) for 2 h at 4°C. The resin was thoroughly washed and eluted with base buffer supplemented with 25 mM and 500 mM imidazole respectively. Eluted protein was dialyzed and cleaved from the His-tag overnight at 4°C in buffer A (0.1 M tris-HCl, pH 8.5, 25 mM NaCl) supplemented with PreScission protease (GE Healthcare), then passed through fresh Ni^{2+}–NTA agarose resin. Dialyzed protein was loaded onto 5 ml Hitrap Q Sepharose FF anion exchange resin (GE Healthcare) and eluted with a linear gradient up to 60% of buffer B (0.1 M tris-HCl, pH 8.5, 500 mM NaCl). Peak fractions were pooled, concentrated and further purified via size exclusion chromatography using 300-ml prepacked Superdex™ 200 column (GE Healthcare) equilibrated with base buffer. The peak fractions were concentrated to 10 mg/ml, mixed 1:1 with 50% glycerol, snap-frozen and stored at -80°C until use.

TPR Expression and Purification
The TPR region of OGT (residues 26–410), either wild type (TPR_{WT}) or bearing the L254F mutation (TPR_{L254F}), was expressed and purified as N-terminally GST fusion proteins as described previously (Jínek et al., 2004). Briefly, E. coli BL21-DE3 cells were transformed, grown and harvested as described for the full length OGT constructs. Resulting cell pellet was resuspended in base buffer (20 mM Na-HEPES, pH 7.5, 200 mM NaCl, 2 mM DTT supplemented with 0.1 mg/ml DNase I and protease inhibitor cocktail (1 mM benzamidine, 0.2 mM PMSF, 5 mM leupeptin), prior to being lysed and clarified as described above. Clarified lysate was then incubated with 1 ml per litre of culture of Glutathione Sepharose 4B resin (GE Healthcare) for 2 h at 4°C. The resin was thoroughly washed with base and the recombinant proteins were cleaved on-resin by addition of PreScission protease (GE Healthcare) and overnight incubation at 4°C. Cleaved protein was eluted, concentrated and further purified via size exclusion chromatography using 300-ml prepacked Superdex™ 200 column (GE Healthcare) equilibrated with base buffer. The peak fractions were concentrated to 30 mg/ml, snap-frozen in liquid nitrogen and stored at -80°C until use.

TPR Crystallisation and Structural Analysis
Crystallisation of TPR_{L254F} was performed at 22°C using MRC 96-well sitting drop crystallization plates (Molecular Dimensions) by combining 0.2 µl TPR_{L254F} (in 20 mM Na-HEPES-NaOH pH 7.5, 200 mM NaCl and 2 mM DTT) with 0.2 µl of reservoir solution (0.1 M Na-HEPES and 0.1 M MOPS-HCl pH 7.5, 0.04 M diethylene glycol, 0.04 M triethylene glycol, 0.04 M tetraethylene glycol, 0.04 M pentaethylene glycol, 20% v/v ethylene glycol and 10% w/v PEG 4000) (Morphues®, Molecular Dimensions (Gorrec, 2015)). Orthorhombic rod and disc shaped crystals appeared within 1-2 days. Prior to diffraction experiments, individual crystals were flash-frozen in liquid nitrogen without prior cryoprotection. Diffraction data were collected at the European Synchrotron Radiation Facility beamline ID30A-1. Data were processed with iMosflm (Battye et al., 2011) and scaled to 1.75 Å using SCALA (Winn et al., 2011). The structure was solved by molecular replacement using the structure for TPR_{WT} (PDB: 1W3BJ Jínek et al., 2004) as the search model. The resulting model was initially truncated at both N- and C-termini where the fit of the electron density and the model was poor, and manually rebuilt and refined using Coot (Winn et al., 2011) and REFMAC (Vagin et al., 2004), respectively. The editing and
refinement of the model was iterated until it was in complete agreement with the data. Scaling and model building statistics can be seen in Table 1.

**Thermal Denaturing Assay**

Thermal denaturation experiments were performed in triplicate, using constructs encompassing the TPR domain (residues 26-410). 50 μl solutions contained 5 μM protein and 1.1x SYPRO® Orange dye (Sigma) in base buffer of 25 mM HEPES-NaOH pH 7.5, 150 mM NaCl and 0.5 mM TCEP. CFX Connect™ Real-Time System (BIO-RAD) was used to measure fluorescence (λex = 530 nm, λem = 560 nm) while temperature was increased from 25 to 95°C at 1 degree per minute increments. The data were transformed, normalised and fitted to a four-parameter Boltzmann sigmoidal curve using GraphPad Prism 5.0.

**Molecular Dynamics Simulations**

Truncated OGT TPR wild type and L254F constructs comprising TPRs 6-8 (sTPRWT and sTPR<sub>L254F</sub>; residues 189-294, Figure 1A) were used in molecular dynamics simulations, similar to an approach previously used to simulate sections of the alpha-solenoid HEAT repeat protein importin-β (Kappel et al., 2010). Appropriate capping groups were added to N- and C-terminal ends of both sTPR<sub>WT</sub> and sTPR<sub>L254F</sub> constructs. The major axes of the sTPR constructs were aligned to the z-axis of a triclinic simulation box a triclinic box of 62.5 x 62.5 x 82.5 Å and solvated using explicit water molecules. Na<sup>+</sup> and Cl<sup>-</sup> ions were added in order to neutralise the system at the physiological NaCl concentration of 0.15 mM. The amber99SB-ildn force field (Lindorff-Larsen et al., 2010) and virtual sites for hydrogen atoms (Feenstra et al., 1999) were used. The TIP3P water model was used to model the solvent molecules and Joung and Cheatham III parameters (Joung and Cheatham, 2008) were used to model the counter ions. Simulations were carried out with the GROMACS molecular dynamics package, version 5.1.5 (Abraham et al., 2015). For each system, the geometry was minimized in four cycles that combined 3500 steps of steepest descent algorithm followed by 4500 of conjugate gradient. Thermalisation of the system was performed in 6 steps of 5 ns, where the temperature was gradually increased from 50 K to 298 K, while the protein was restrained with a force constant of 10 kJ mol<sup>-1</sup> Å<sup>2</sup>. Production runs consisted of four replicates of 500 ns simulations for each system (accounting for 2 μs of simulation time per system). Making use of virtual sites, the integration time-step was set to 4 fs. Temperature was kept constant by weakly coupling (t = 0.1 ps) protein and water and ions separately to a temperature bath of 298K with the velocity rescale thermostat of Bussi et al. (Bussi et al., 2007). The pressure was kept constant at 1 bar using semi-isotropic Berendsen coupling (Berendsen et al., 1984). Long-range electrostatic interactions were calculated using the smooth particle mesh Ewald method (Darden et al., 1993) beyond a short-range Coulomb cut-off of 10 Å. A 10-Å cut-off was also set for Lennard-Jones interactions. The LINCS algorithm (Hess et al., 1997) was used to restrain the bonds involving hydrogen and the SETTLE algorithm (Miyamoto and Kollman, 1992) was used to constrain bond lengths and angles of water molecules. Periodic boundary conditions were applied.

**In Vitro O-GlcNAcylation Assays**

Michaelis-Menten kinetics of OGT were measured as fluorimetric assay as described previously (Borodkin et al., 2014), with the exception of reduced reaction volume of 25 μl and usage of 384-well plate. As acceptor substrate, a 13 amino acid long sequence from retinoblastoma-like protein 2 (RB2; 419KENSAPAVTPVTA222; GlycoBioChem) was used. Reactions for Michaelis-Menten kinetics contained 0-768 μM acceptor peptide substrate, 200 μM UDP-GlcNAc and 50 nM OGT in 50 mM HEPES-NaOH pH 7.5, 0.1 mg/ml BSA and 10 μM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>. Reactions were stopped before 10% of the acceptor substrate was depleted by addition of 50 μl detection reagent (25 mM HEPES-NaOH pH 7.5, 10 mM NaCl, 15 μM xanthene based Zn(II) complex, 75 μM pyrocatechol violet and 50% methanol). The fluorescence was read using excitation and emission wavelengths of 485 nm and 530 nm, respectively. Data were background corrected and plotted using GraphPad Prism.

Additional O-GlcNAcylated assays were performed on de-O-GlcNAcylated HEK-293 lysate proteins. Cultured HEK-293 cells were washed twice with ice-cold PBS buffer (Life Technologies) prior to lysis. Cells were lysed by addition of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X100, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 50 mM NaF, 5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.27 M sucrose) supplemented with 1 μM benzamidine, 0.2 mM PMSF and 5 mM leupeptin. The lysate was transferred into microfuge tubes and clarified by centrifugation at 4°C (17,000 g for 15 min). The lysate was then treated with 120 μg CpOGA per mg of lysate protein and incubated for 90 minutes at 37°C. CpOGA and endogenous HEK-293 OGA were then neutralised by addition of 250 μM GlcNAcstatin-G, an OGA inhibitor. Reactions were then supplemented with OGT<sub>WT</sub> or OGT<sub>L254F</sub> (0.2 μM) in presence of 2 mM UDP-GlcNAc and incubated for an additional 2 h at 37°C. Proteins were resolved by SDS-PAGE (3-8% Tris-Acetate gels; Life Technologies), transferred onto nitrocellulose membrane (GE Healthcare), and probed using O-GlcNAc-RL2 (1:1,000 dilution; Abcam), HSP90 (1:5,000 dilution; Cell Signalling or Enzo Life Sciences) and OGT-H300 (1:1,000 dilution; Santa Cruz) primary antibodies and corresponding IRDye associated secondary antibodies (1:10,000 dilution; LI-COR). Resulting signal was quantified using a LI-COR Odyssey scanner and associated quantification software. Data were plotted using GraphPad Prism 5.0.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Michaelis-Menten kinetics of the OGT-catalysed reaction against the peptide substrate derived from RB2 was performed as three technical replicates and repeated two times (data presented in Figure S1A). The O-GlcNAc activity assay against deglycosylated HEK-293 cell lysate proteins was repeated six times (data presented in Figures 1B and S1B). The ThermoFluor assay was performed.
as seven technical replicates, and repeated two times (data presented in Figure 2A). For all activity and thermal stability assays, GraphPad Prism was used for calculation of statistics. Error bars represent the standard deviation of the mean in all presented data. The trajectories obtained by the molecular dynamics simulations were analysed with the MDAnalysis (RMSD and RMSF) (Gowers et al., 2016; Michaud-Agrawal et al., 2011) and MDtraj (distances and angles) (McGibbon et al., 2015) packages. The values reported in the results and discussion sections correspond to the mean values ± standard deviation of the mean.

**DATA AND SOFTWARE AVAILABILITY**

The crystallographic structure has been deposited in RCSB Protein Data Bank (http://www.rcsb.org/pdb) under ID code 6EOU.