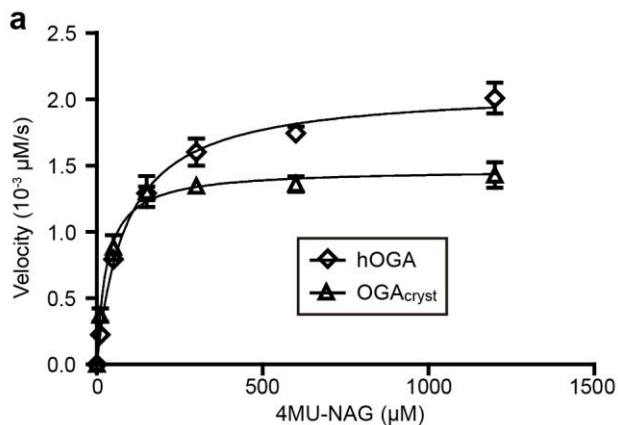


Supplementary Figure 1

Substrate-assisted mechanism of OGA.

OGA-catalyzed hydrolysis of O-GlcNAcylation via a substrate-assisted mechanism, adapted from a previous report (Dennis, R. J. *et al.*, *Nat. Struct. Mol. Biol.* **13**, 365–371, 2006). The roles of ancillary residues Lys98 and Tyr219 are inferred from the crystal structure of OGA_{cryst}-thiamet-G from this study.



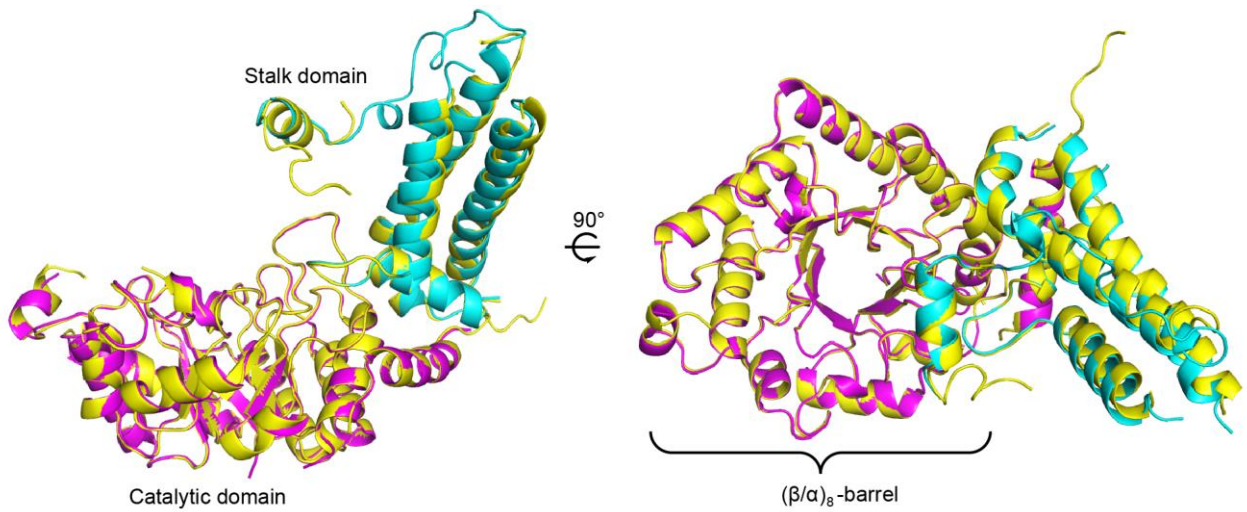
b

Kinetic constants	hOGA	OGA _{cryst}
K_m (μM 4MU-NAG)	87.57 ± 6.91	29.68 ± 3.37
k_{cat} (min^{-1})	62.35 ± 0.02	44.09 ± 0.02
k_{cat} / K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)	0.71 ± 0.06	1.48 ± 0.19

Supplementary Figure 2

Kinetic parameters of hOGA and OGA_{cryst} proteins.

(a) Michaelis–Menten plots of hOGA and OGA_{cryst} with varying concentrations of 4MU-NAG. Data were fitted using GraphPad Prism. Error bars represent s.d. values derived from three independent experiments. (b) Summary of the kinetic parameters of hOGA and OGA_{cryst}. The K_m and k_{cat} were determined from three independent experiments and displayed as average \pm s.d..



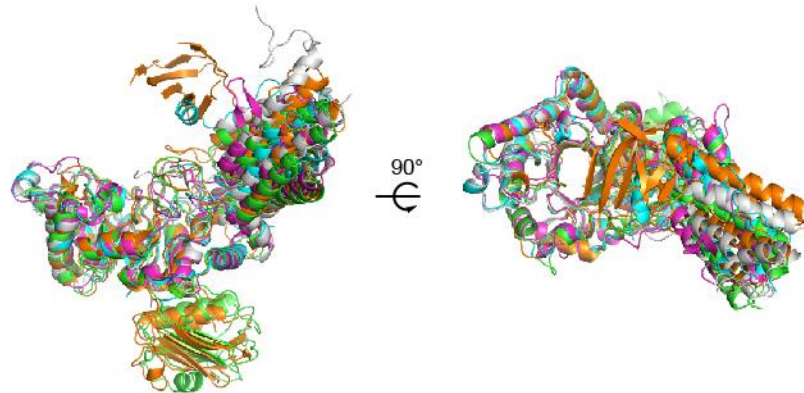
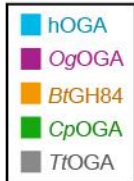
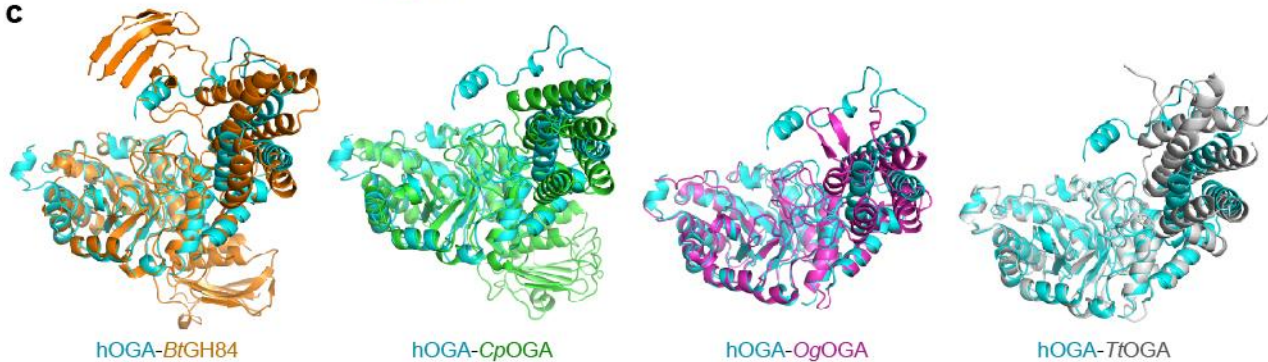
Supplementary Figure 3

Structural comparison of the two sister monomers of OGA_{cryst}.

Two perpendicular views of superimposed OGA α and OGA β reveal that the catalytic domains are folded into an identical (β/α)₈-barrel, while the stalk domains display a slight variation. OGA α is colored yellow; the catalytic domain and stalk domain of OGA β are colored magenta and cyan, respectively.

a

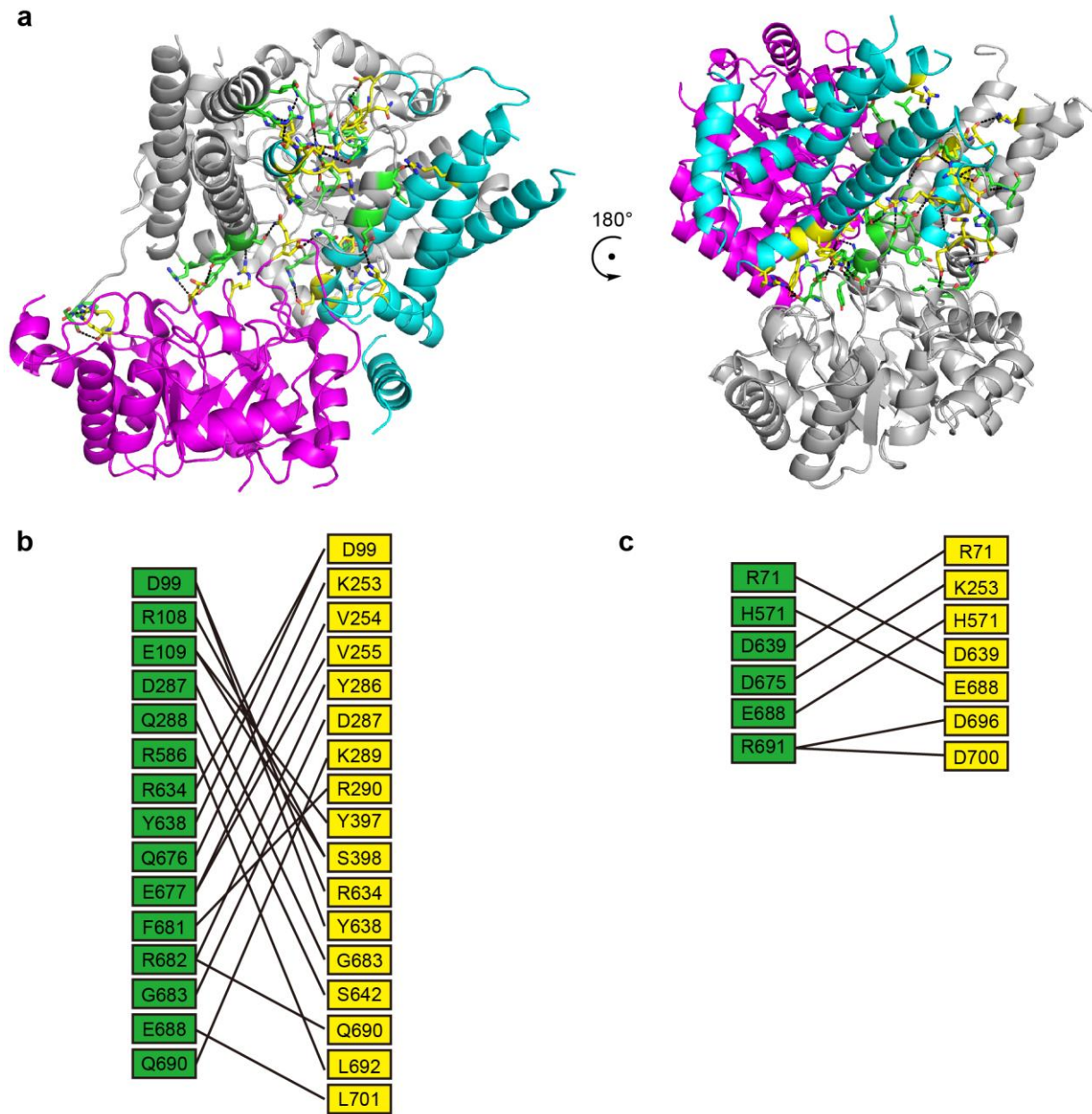
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Bacteroides_thetaiotaomic	
Clostridium_perfringens	
Thermobaculum_terrenum	
Homo_sapiens	437	IMSQGAALSCEPTTLTKEEKKQPDEEPMVMVVEKQEETDCHKNDNQILSEIVEAKMAEELKPMDDTKESI
Oceanicola_granulosus	
Bacteroides_thetaiotaomic	
Clostridium_perfringens	
Thermobaculum_terrenum	
Homo_sapiens	507	AESKSPEMSQEDCISDIAPMQTDEQTNKEQFVPGPNEKP ^Y Y ^T AEPVT.....
Oceanicola_granulosus	275	...DPDYAPERAI ^A AAVA... ^A WQPRFRL ^A F ^C DGAVP.....
Bacteroides_thetaiotaomic	416	...AKYDTWQT ^W KDAIRT ^I L ^F SAAEE.LECFAMHN...SDLGPNCHGYR
Clostridium_perfringens	454	...YDYDKA ^W NRAIDM ^L Y ^C DLAED.MKVFANHS...TRMDNKTWAKS
Thermobaculum_terrenum	282	...YNPQDS ^W RQAVST ^L L ^C EDNLSAMEKFPYQNTISCLPEPEP...
Homo_sapiens	555	...LED ^L Q ^L T ^A D ^L F ^Y LPYEHGPKGAC ^M LREFQWLRANSSVVS ^V N ^C K ^G K ^D SEKIE ^E WRSRAAK ^F E ^R MCGL
Oceanicola_granulosus	306	...SDLVAL ^L C ^D L ^F WQPFALGPETTR ^L LSALRAA...LT...VPRPDPSPAWRAALED ^L R ^D LKRR
Bacteroides_thetaiotaomic	458	...REESMD ^L Q ^P A ^E R ^F LKAFKEGK ^N YDKA...DFET...
Clostridium_perfringens	494	GREDAPE ^L RAK ^M DEL ^L WNKLS.SKEDASA...LIE ^E ...
Thermobaculum_terrenum	322	...LAY ^L T ^N L ^F KK.VQ...ED...FASFRFE...QGLRTL ^R E ^E IIS
Homo_sapiens	621	^Y MGM ^F ^T R ^L SNCA...NRT ^I L ^Y DMYS ^Y Y ^W DIK ^S IMS ^M VK ^S F ^V QW ^L GCR ^S HSS ^A QFLIGD ^O EPWAFR
Oceanicola_granulosus	363	^I NK ^L F ^T L ^M TEIE...NRD ^L F ^H TFHN ^Y I ^W EAEQEVGHLVAYCDWLDEAPP ^P GA ^V FPATDRIHNFYR
Bacteroides_thetaiotaomic	489	^L Q ^Y T ^F ER ^M KESADILLMN ^T ENK ^P LIVEITP ^W Y ^H QPKLTAEMGEEV...LKMVEGRNESYFLR
Clostridium_perfringens	528	^L YGE ^F AR ^M E ^E ACNNLKANLPEVA ^L ...EECSRO...LDEL...ITL ^A O ^G DKASLD...
Thermobaculum_terrenum	354	^M Q ^T T ^Y S ^R L ^S ...TQDSK ^F F ^W EIRP ^W I ^E EYK ^L WTD...YLD...QAM ^I T ^F S...NLFT
Homo_sapiens	683	GGLAG...EFOR ^L ...PIDGAND ^F FFQPP.....
Oceanicola_granulosus	425	RGF ^G V...AVQD ^L ...QRDRQGR ^Y HHGV.....
Bacteroides_thetaiotaomic	548	KYNHVKALQOQ ^M FYIDQTSNQN ^P YQPGVKTAT...RVIKPLIDRIFATVVV ^K FFN ^Q KFN ^A HLDA
Clostridium_perfringens	571	...MIVAQLNEDTEA ^Y ESAKEIAQNKLN ^T ALSSFAVI ^S EKVAQSF ^I Q ^E ALSFD ^L TL...
Thermobaculum_terrenum	399	GFPTADEEQAR ^R SLQKALQ ^C .RT ^V LREV...LKD ^A VDFR ^T RVCGD ^V VRN ^F LQ ^V VL ^R ST ^V SI...ELQA
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Oceanicola_granulosus	
Bacteroides_thetaiotaomic	608	TTD.....
Clostridium_perfringens	624	I.....
Thermobaculum_terrenum	460	E ^G K ^E W ^T A ^L P ^P G ^I V ^R D

b**c**

Supplementary Figure 4

The sequence and structure of the hOGA stalk domain are markedly different from those of its bacterial homologs.

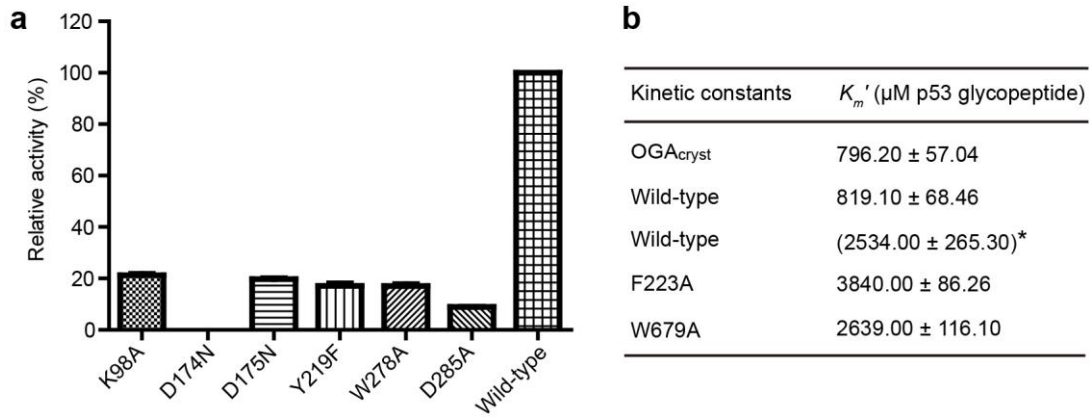
(a) Sequence alignment and (b-c) structural comparison of the stalk domains of OGA proteins from human (hOGA, cyan) and representative bacterial species: *Oceanicola granulosus* (OgOGA, PDB: 2XSA, magenta) (Schimpl, M. *et al.*, *Biochem. J.* **432**, 1–7, 2010), *Bacteroides thetaiotaomicron* (BtGH84, PDB: 2CHO, orange) (Dennis, R. J. *et al.*, *Nat. Struct. Mol. Biol.* **13**, 365–371, 2006), *Clostridium perfringens* (CpOGA, PDB: 2CBJ, green) (Rao, F. V. *et al.*, *EMBO J.* **25**, 1569–1578, 2006) and *Thermobaculum terrenum* (TtOGA, PDB: 5DIY, grey) (Ostrowski, A., *et al.*, *J. Biol. Chem.* **290**, 30291–30305, 2015). In the sequence alignment, variable and similar residues are shown in black and red, respectively.



Supplementary Figure 5

Extensive polar interactions stabilize the dimerization of OGA_{cryst}.

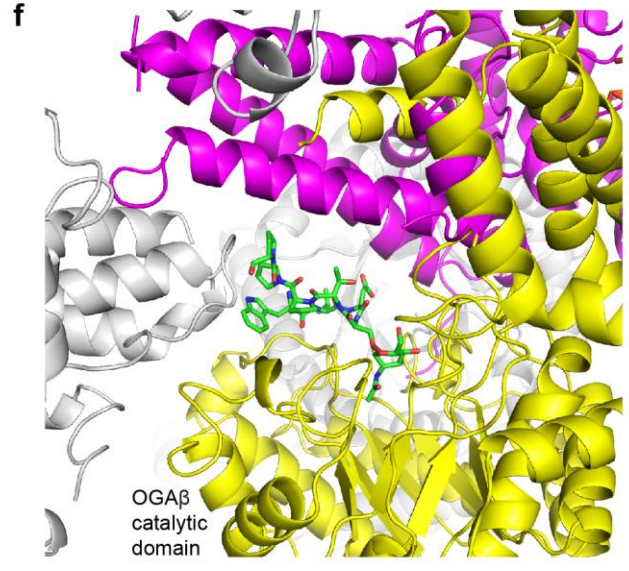
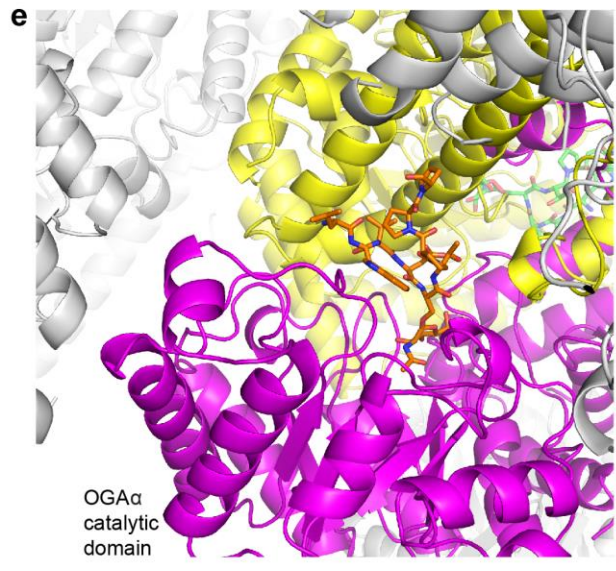
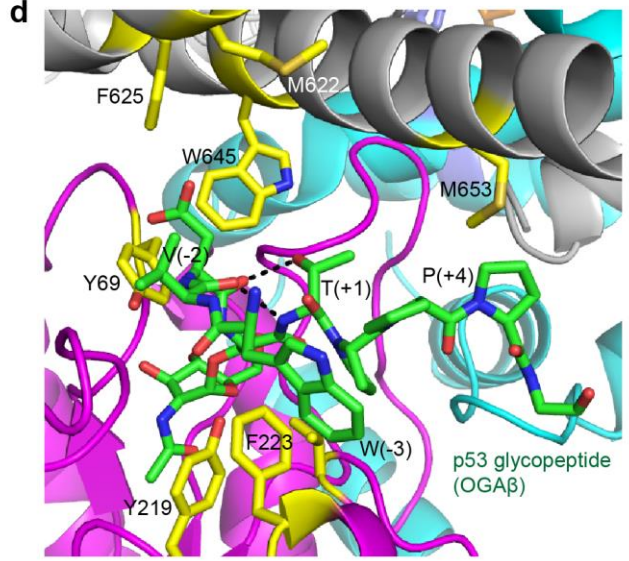
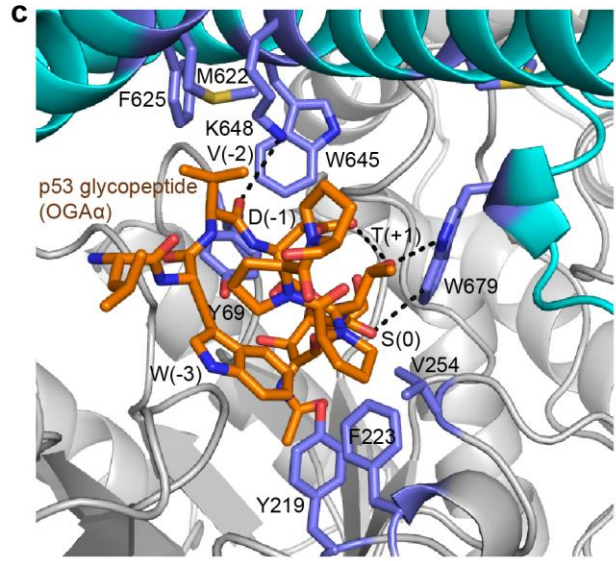
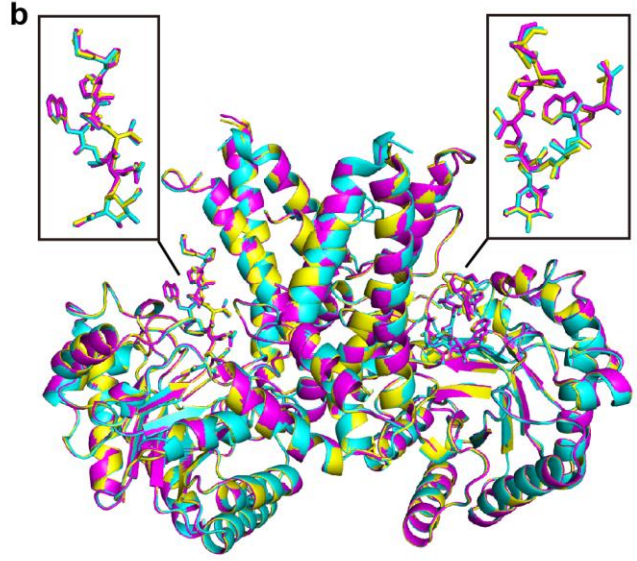
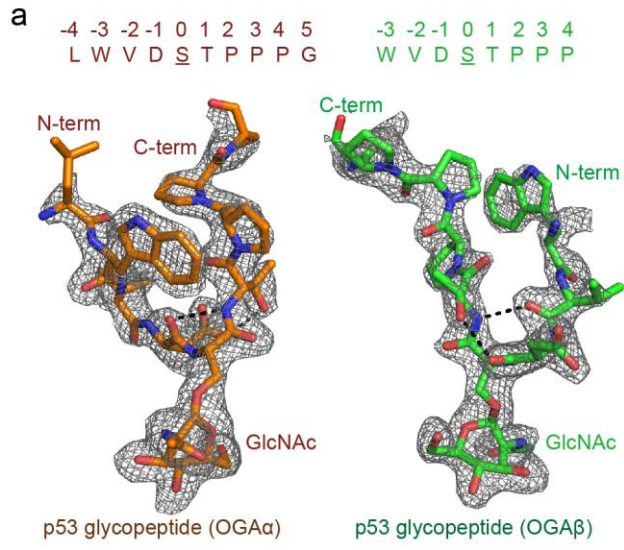
(a) Two different views of OGA_{cryst} with dimerization interface. OGA α is colored gray; the catalytic domain and stalk domain of OGA β are colored magenta and cyan, respectively. (b) Schematic representation of the hydrogen bonds detected at the dimerization interface. (c) Schematic representation of the salt bridges detected at the dimerization interface. Residues from OGA α and OGA β participating in the polar interactions are colored in yellow and green, respectively.



Supplementary Figure 6

Relative activities and substrate-binding evaluation of hOGA variants.

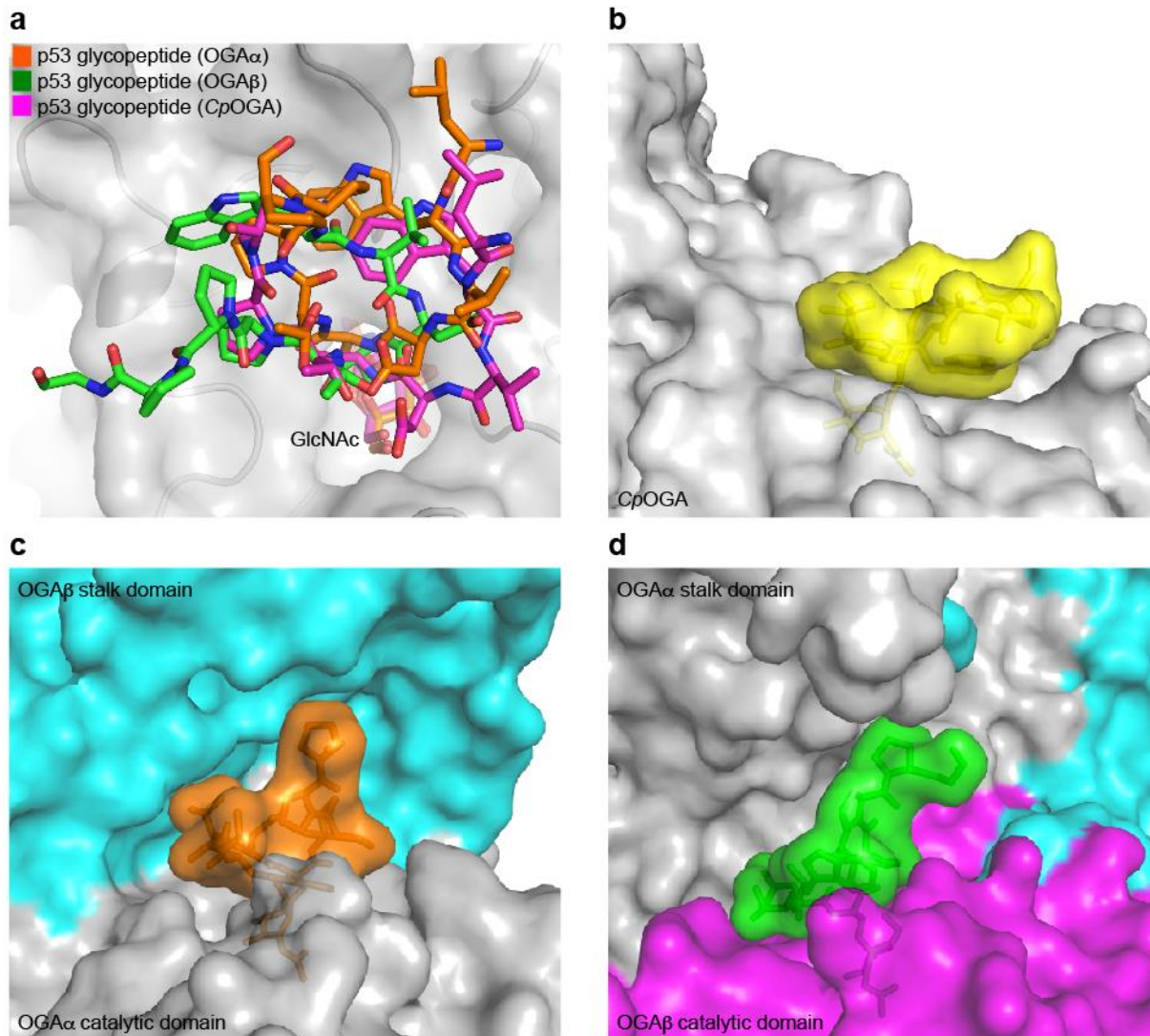
(a) The activities of hOGA mutants were measured using 4MU-NAG and normalized to the wild-type enzyme. The error bars represent the s.d. values derived from three independent experiments. (b) To evaluate the contribution of hydrophobic interactions with the p53 glycopeptide (Ac-QLWVDS(O-GlcNAc)TPPPG), the Michaelis constants of hOGA and mutants were determined using 4MU-NAG as the reporter substrate following multisubstrate enzyme kinetics as shown previously (Schimpl, M. *et al.*, *Biochem. J.* **432**, 1–7, 2010; Schimpl, M. *et al.*, *Chem. Biol.* **19**, 173–178, 2012; Xie, D. *et al.*, *Protein Science* **8**, 2460–2464, 1999). *The Michaelis constant of wild-type hOGA was measured towards a W(-3)A mutant of p53 glycopeptide (Ac-QLAVDS(O-GlcNAc)TPPPG) using a similar assay. The K_m' values were determined from three independent experiments and displayed as average \pm s.d..



Supplementary Figure 7

Binding conformation of p53 glycopeptides in the OGA_{cryst}-p53 complex.

(a) *F_o-F_c* difference map of p53 glycopeptides from the OGA_{cryst}-p53 complex (contoured at 3 σ). The peptides from OGA α and OGA β are shown in orange and green sticks, respectively. Intramolecular hydrogen bonds are displayed as dotted lines. (b) Superposition of OGA_{cryst}-p53 complex structures collected from three independent soaking experiments (inserts: enlarged p53 glycopeptides from each monomer. The p53 glycopeptides in the right insert have been rotated for better clarity). (c-d) Close-up views of the binding conformations of p53 glycopeptides in (c) OGA α and (d) OGA β . Residues on the inner surface of the substrate-binding cleft that are in close vicinity of the peptide are highlighted in (c) blue sticks and (d) yellow sticks, respectively. Hydrogen bonds are displayed in dashed lines. (e-f) Crystal packing potentially contributes to stabilize p53 glycopeptide in OGA β but not in OGA α . OGA α and OGA β are shown in magenta and yellow, respectively. The symmetric molecule is shown in grey.



Supplementary Figure 8

The p53 glycopeptide adopts distinct binding conformations in the structures of human and bacterial OGAs.

(a) Superposition of the p53 glycopeptide (stick representation) in the CpOGA–p53 complex (PDB: 2YDR) (Schimpl, M. *et al.*, *Chem. Biol.* **19**, 173–178, 2012) with those in the OGA_{cryst}–p53 complex. (b) Surface representation of the CpOGA–p53 complex, highlighting the p53 glycopeptide (yellow) bound on top of the active site (PDB: 2YDR). (c) Surface representation of the p53 glycopeptide (orange) bound in the substrate-binding cleft that comprises the catalytic domain of OGA α (grey) and the stalk domain of OGA β (cyan). (d) Surface representation of the p53 glycopeptide (green) bound in the substrate-binding cleft that comprises the stalk domain of OGA α (grey) and the catalytic domain of OGA β (magenta). In b–d panels, stick presentation of p53 glycopeptides is displayed as semitransparent.

a

Homo_sapiens 1 MVQKESQATLEERESELSNPAAASAGASLEFPAPAPGEDNPAGAGGAAVAGAAGGARFRFLCGVVECFYGL
Rattus_norvegicus 1 MVQKESQAALERESEERNANPASVGSASLEFPAPAPGEDNPAGAGGAAGTGAAGGARFRFLCGVVECFYGL
Mus_musculus 1 MVQKESQAALERESEERNANPAASAGASLEQSVAPAPGEDNPAGAGGAAVAGAAGGARFRFLCGVVECFYGL
Caenorhabditis_elegans 1MECTESFDRRKAVQNSKYICGVVECFYGL
Drosophila_melanogaster 1MADEAGSQADGKRFRFLCGVVECFYGL
Ostrinia_furnacalis 1MTESSPDEFSRNRKDFICGVVECFYGL

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Rattus_norvegicus 71 RPWVMEQRKELFRRLQKWEIN...YIYAPKDDMKHRMFWREMYSVEEAEOLMTLTLAAREYEIEFIFYAI
Mus_musculus 71 RPWVMEQRKELFRRLQKWEIN...YIYAPKDDMKHRMFWREMYSVEEAEOLMTLTLAAREYEIEFIFYAI
Caenorhabditis_elegans 29 RPWVMEQRKELFRRLQKWEIN...YIYAPKDDMKHRMFWREMYSVEEAEOLMTLTLAAREYEIEFIFYAI
Drosophila_melanogaster 26 RPWVMEQRKELFRRLQKWEIN...YIYAPKDDMKHRMFWREMYSVEEAEOLMTLTLAAREYEIEFIFYAI
Ostrinia_furnacalis 27 RPWVMEQRKELFRRLQKWEIN...YIYAPKDDMKHRMFWREMYSVEEAEOLMTLTLAAREYEIEFIFYAI

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Rattus_norvegicus 138 SPGCDITFSNPKFVSTLTKKRLQVSVFCGRSFAFLFDDIDHNMCAADKEVFSFAFAHQVSVITNIIYQYLG
Mus_musculus 138 SPGCDITFSNPKFVSTLTKKRLQVSVFCGRSFAFLFDDIDHNMCAADKEVFSFAFAHQVSVITNIIYQYLG
Caenorhabditis_elegans 96 SPGCDITFSNPKFVSTLTKKRLQVSVFCGRSFAFLFDDIDHNMCAADKEVFSFAFAHQVSVITNIIYQYLG
Drosophila_melanogaster 96 SPGCDITFSNPKFVSTLTKKRLQVSVFCGRSFAFLFDDIDHNMCAADKEVFSFAFAHQVSVITNIIYQYLG
Ostrinia_furnacalis 94 SPGCDITFSNPKFVSTLTKKRLQVSVFCGRSFAFLFDDIDHNMCAADKEVFSFAFAHQVSVITNIIYQYLG

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Rattus_norvegicus 208 EPEFLFCPTCYCGTFCYVNVQSPYLRTVGEKLLPGLTEVIVTGTGPKVSKETPVESTIEVSKTIKRAPVI
Mus_musculus 208 EPEFLFCPTCYCGTFCYVNVQSPYLRTVGEKLLPGLTEVIVTGTGPKVSKETPVESTIEVSKTIKRAPVI
Caenorhabditis_elegans 166 TK.TFMRCPTCYCGTFCYVNVQSPYLRTVGEKLLPGLTEVIVTGTGPKVSKETPVESTIEVSKTIKRAPVI
Drosophila_melanogaster 166 SP.FRLFCPTCYCGTFCYVNVQSPYLRTVGEKLLPGLTEVIVTGTGPKVSKETPVESTIEVSKTIKRAPVI
Ostrinia_furnacalis 164 CP.FRLFCPTCYCGTFCYVNVQSPYLRTVGEKLLPGLTEVIVTGTGPKVSKETPVESTIEVSKTIKRAPVI

Homo_sapiens 278 WDNHANDYDKRRIFLCPYKGRSTELIPLRKGVLITNPNCEFEANVVAIHTLTLWYKSNMNGVRKDVMTD
Rattus_norvegicus 278 WDNHANDYDKRRIFLCPYKGRSTELIPLRKGVLITNPNCEFEANVVAIHTLTLWYKSNMNGVRKDVMTD
Mus_musculus 278 WDNHANDYDKRRIFLCPYKGRSTELIPLRKGVLITNPNCEFEANVVAIHTLTLWYKSNMNGVRKDVMTD
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Drosophila_melanogaster 235 WDNHANDYDKRRIFLCPYKGRSTELIPLRKGVLITNPNCEFEANVVAIHTLTLWYKSNMNGVRKDVMTD
Ostrinia_furnacalis 233 WDNHANDYDKRRIFLCPYKGRSTELIPLRKGVLITNPNCEFEANVVAIHTLTLWYKSNMNGVRKDVMTD

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Drosophila_melanogaster 298 .NS.SLSADIKLETEN...DDD...LPAEFLSKNVYHFRLATKNAETWIEQEBGVPHQYSRQVAHS
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Rattus_norvegicus 406 GA.KTSVVDGTFVLV...AAPS LNAT...TVVT.TVYQEPFIMSQGA
Mus_musculus 406 GA.KTSVVDGTFVLV...AAPS LNAT...TVVT.TVYQEPFIMSQGA
Caenorhabditis_elegans 364 PPQL.TADVAGYVDRRCVLLDLPETHG...VIRPEVPMVAEII
Drosophila_melanogaster 357 PQVQM.VMPIIPIIPVINTCMVLTITTTST...SRTVPTVNTIQLQALADVCVVT...
Ostrinia_furnacalis 360 PPIVIPPVPIIPIIPVINTCMVLTITTTST...SRTVPTVNTIQLQALADVCVVT...LIIATGI

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Drosophila_melanogaster 412 ...SLTPISNPVMNSLVSPTKVIITNDIINPPTTAASNIELKKIIPHSVVPVPMETKSVSEASVELAL
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Mus_musculus 467 ...MVVEKQEEAEHKNQI...SEIVEAKMAEEKPM...
Caenorhabditis_elegans 427 ...LAA...EYSPMTE...
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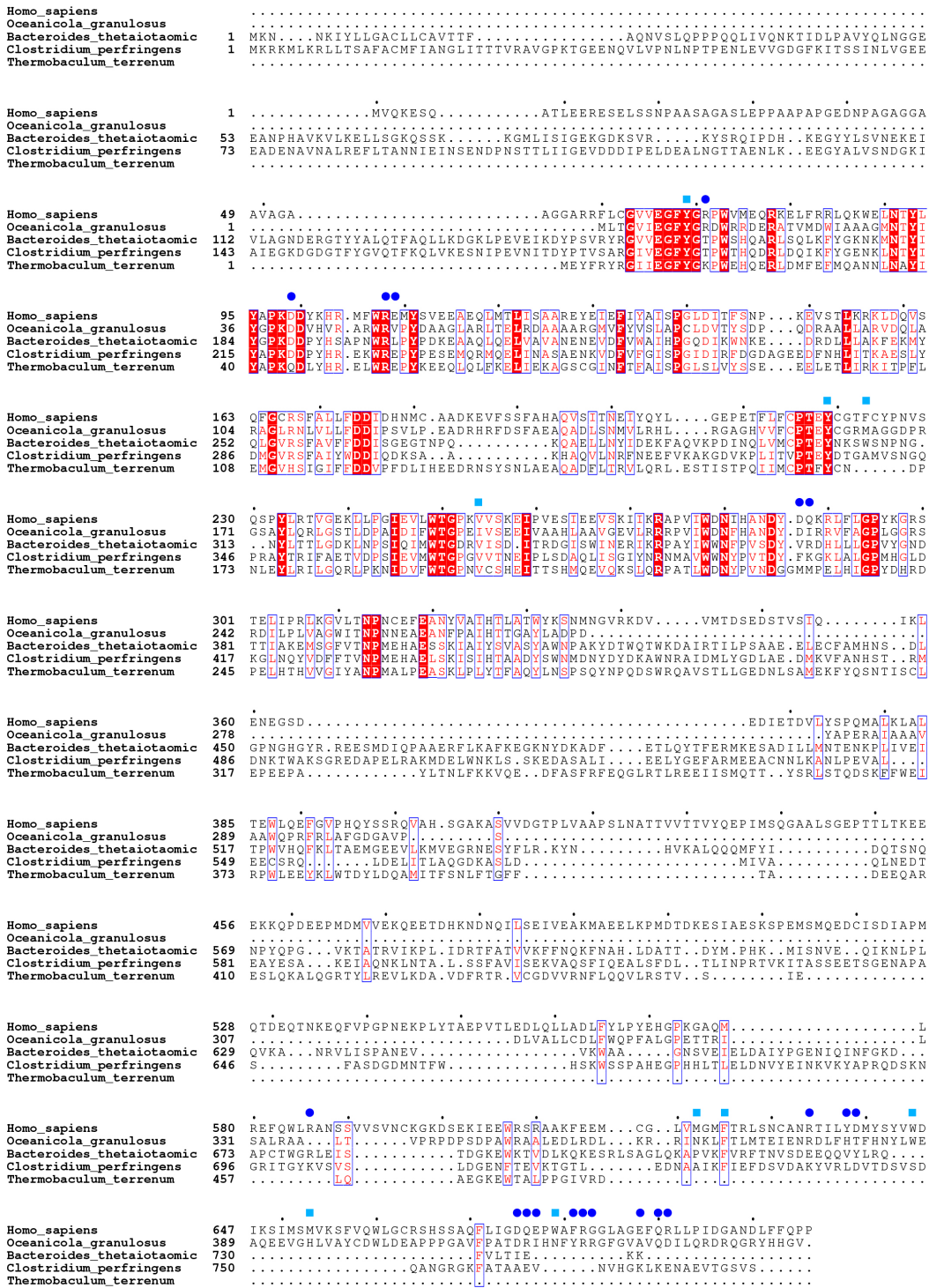
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Mus_musculus 505 ...SMA...PDVAPMOTDEQ...TQKEQFVPGP
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Ostrinia_furnacalis 559 PQTLSPERGS LFAPPEPEPGIMEAAEGDVCVNDGMSENGSMQVEPS.NSPLSTDMVEMTEPEVPEVDET

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b



Supplementary Note 1

Sequence alignment of OGA proteins to show that residues on the dimerization interface and the substrate-binding cleft are highly conserved in eukaryotes but not in prokaryotes.

(a) Sequence alignment of OGA proteins from representative eukaryotic species: *Homo sapiens* (uniprot: O60502), *Rattus norvegicus* (uniprot: Q8VIJ5), *Mus musculus* (uniprot: Q9EQQ9), *Caenorhabditis elegans* (uniprot: A8WF17), *Drosophila melanogaster* (uniprot: Q9VDC9), and *Ostrinia furnacalis* (uniprot: H9NID4). (b)

Sequence alignment of OGA proteins from human and representative prokaryotic species: *Oceanicola granulosus* (uniprot: Q2CEE3), *Bacteroides thetaiotaomicron* (uniprot: Q89Z12), *Clostridium perfringens* (uniprot: Q0TR53), and *Thermobaculum terrenum* (uniprot: D1CDN2). Variable residues are shown in black with white background, similar residues are shown in red with white background, and conserved residues are shown in white with red background. Residues involved in the dimerization of OGA_{cryst} are marked with dark blue dots. Hydrophobic residues on the substrate-binding cleft in close vicinity of the p53 glycopeptide are labeled by light blue squares.

Supplementary Table 1 A list of polar interactions between OGA α and OGA β within 4 Å

OGAβ	Length (Å)	OGAα
Hydrogen bonds		
D99 [O]	3.0	Y638 [OH]
D99 [O]	3.0	R634 [NH1]
R108 [O]	3.4	S398 [OG]
E109 [OE]	2.8	Y397 [N]
E109 [OE2]	3.1	S398 [N]
D287 [OD2]	2.9	G683 [N]
Q288 [NE2]	2.8	S642 [OG]
R586 [NE]	2.7	L692 [O]
R634 [NH1]	2.9	D99 [O]
Y638 [OH]	3.0	D99 [O]
Q676 [O]	2.6	K253 [NZ]
E677 [OE1]	3.2	V255 [N]
E677 [OE2]	3.0	V254 [N]
F681 [O]	3.8	R290 [NH1]
R682 [O]	3.8	Q690 [NE2]
R682 [NH1]	2.9	Y286 [O]
G683 [N]	2.8	D287 [OD2]
E688 [OE2]	3.2	L701 [N]
Q690 [OE1]	2.8	K289 [NZ]
Salt bridges		
R71 [NH2]	3.2	D639 [OD1]
H571 [NE2]	2.7	E688 [OE1]
D639 [OD1]	3.0	R71 [NH2]
D675 [OD2]	2.8	K253 [NZ]
D675 [OD1]	3.8	K253 [NZ]
E688 [OE1]	2.7	H571 [NE2]
R691 [NE]	3.9	D696 [OD1]
R691 [NE]	4.0	D700 [OD2]
R691 [NH1]	2.5	D700 [OD1]
R691 [NH1]	2.9	D700 [OD2]

Supplementary Table 2 Summary of primers used to make OGA mutants in this study

Primer	Sequence (5'-3')
K98A_fwd	ATACATACTTGTATGCCCCAGCAGATGACTACAAACATAGGATG
K98A_rev	CATCCTATGTTTGTAGTCATCTGCTGGGGCATACAAGTATGTAT
D174N_fwd	TGGGTGCAGATCATTTGCTTTGCTTTTTAATGATATAGACCATAATA
D174N_rev	TATTATGGTCTATATCATTAAAAAGCAAAGCAAATGATCTGCACCCA
D175N_fwd	GATCATTTGCTTTGCTTTTTGATAATATAGACCATAATATGTGTGCAGC
D175N_rev	CACATATTATGGTCTATATTATCAAAAAGCAAAGCAAATGATCTGCAC
Y219F_fwd	CTTCTGTCCCACAGAATTCTGTGGCACTTTCTGT
Y219F_rev	ACAGAAAGTGCCACAGAATTCTGTGGGACAGAAG
F223A_fwd	CCACAGAATACTGTGGCACTGCCTGTTATCCAAATGTGTCTC
F223A_rev	GAGACACATTTGGATAACAGGCAGTGCCACAGTATTCTGTGG
W278A_fwd	TTAAGAGAGCTCCAGTAATCGCGGATAACATTCATGCTAATG
W278A_rev	CATTAGCATGAATGTTATCCGCGATTACTGGAGCTCTCTTAA
D285A_fwd	CTGGGATAACATTCATGCTAATGCTTATGATCAGAAGAGACTGTTTC
D285A_rev	GAAACAGTCTCTTCTGATCATAAGCATTAGCATGAATGTTATCCCAG
W679A_fwd	GGAGACCAAGAACCCGCGGCCTTTAGAGGTGG
W679A_rev	CCACCTCTAAAGGCCGCGGGTTCTTGGTCTCC