Mechanism of glycogen synthase inactivation and interaction with glycogenin

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Abstract

The macromolecule glycogen is the major glucose reserve in eukaryotes and defects of glycogen metabolism and structure lead to glycogen storage diseases and neurodegeneration. Glycogenesis begins with self-glucosylation of glycogenin (GN), which recruits glycogen synthase (GS). GS is activated by glucose-6-phosphate (G6P) and inactivated by phosphorylation, but how these opposing processes are coupled is unclear. We provide the first structure of phosphorylated human GS-GN complex revealing an autoinhibited GS tetramer flanked by two GN dimers. Phosphorylated N- and C-terminal tails from two GS protomers converge to form dynamic “spike” regions, which are buttressed against GS regulatory helices. This keeps GS in a constrained “tense” conformation that is inactive and more resistant to G6P activation. Mutagenesis that weaken the interaction between the regulatory helix and phosphorylated tails leads to a moderate increase in basal/unstimulated GS activity, supporting the idea that phosphorylation contributes to GS inactivation by constraining GS inter-subunit movement. We propose that multivalent phosphorylation supports GS autoinhibition through interactions from a dynamic “spike” region, thus allowing a “tuneable rheostat” for regulating GS activity. Our structures of human GS-GN provide new insights into the regulation of glycogen synthesis, facilitating future studies of glycogen storage diseases.

Introduction

Glycogen is a branched polymer of glucose that functions as a primary energy store in eukaryotes. In its mature form, the glycogen particle can comprise up to ~50,000 glucose units that can be rapidly utilized when glucose levels are low. Glycogen is stored predominantly in the muscle and liver cells, and to a lesser extent in other organs and tissues including kidney, brain, fat and heart¹.

Glycogen is synthesized through the cooperative action of three enzymes: glycogenin (GN), glycogen synthase (GS) and glycogen branching enzyme (GBE)². GN initiates the process via auto-glucosylation of a conserved tyrosine residue, producing a primer glucose chain of 8-12 residues connected by α -1,4-linkages³. This glycogen initiating particle is further extended by GS after its recruitment by the GN C-terminus (GN³⁴) allowing the addition of glucose residues using α -1,4-linkages^{4,5}. GBE introduces α -1,6-linkages every 6-8 residues to the growing glycogen molecule, thus creating the final globular structure containing GN at the centre^{2,6}. Glycogen exists as a population of molecules with varying sizes (10-290 nm) in different tissues and species, although the importance of this variability is not well understood^{1,7}.

Glycogen synthesis and breakdown are tightly regulated processes, and thus dysregulation of enzymes involved in glycogen metabolism contributes to glycogen storage diseases (GSDs), diabetes, neuroinflammation, neurodegeneration and muscle tissue damage^{1,8}. Excessive and/or abnormal glycogen is a common characteristic in most GSDs. Pompe disease (GSDII) is caused by deficiency of acid- α -glucosidase, resulting in accumulation of lysosomal glycogen and consequent lysosomal destruction and dysfunction⁹. Lafora disease is a fatal neurodegenerative condition, of which the hallmark is Lafora bodies, which are hyperphosphorylated and poorly branched, insoluble glycogen deposits¹⁰. Furthermore, loss of GS-GN interaction results in muscle weakness and cardiomyopathy¹¹.

Studies using mouse models have found inhibition of glycogen synthesis, and in particular by reducing GS activity, to be beneficial for multiple GSDs^{12,13}. To date there is no structure of the GS-GN complex and no structure of human GS. Since inhibition of GS activity is potentially beneficial for GSD patients, obtaining a human GS-GN structure and understanding how GS is regulated is instrumental in developing new therapeutics.

GN is found in two isoforms, GN1 and GN2, encoded by the *GYG1* and *GYG2* genes respectively. While *GYG1* is widely expressed, *GYG2* is restricted to the liver, pancreas and

heart^{14,15}. GN belongs to the GT8 family of glycosyltransferases, containing a glycosyl transferase A (GT-A) fold with a single Rossmann fold domain at the N-terminus, which is essential for binding of the glucose donor uridine diphosphate glucose (UDP-G)¹⁶⁻¹⁸. The C-terminus comprises a highly conserved region of ~34 residues (GN³⁴) which is the minimal targeting region for binding GS^{5,19}. Other interaction interfaces have been suggested²⁰, however, further investigation into the full-length complex is required to determine any additional interaction interfaces. The area between the N-terminal catalytic domain and C-terminal GS binding motif is a linker region that is variable in sequence and in length (**Fig. 1a and Supplementary Fig. 1**).

GS is also found as two isoforms, GS1 and GS2, encoded by the *GYS1* and *GYS2* genes respectively. These are differentially expressed, with *GYS1* being expressed predominantly in skeletal muscle and most other cell types where glycogen is present, while *GYS2* is expressed exclusively in the liver²¹⁻²³. Eukaryotic GS belongs to the GT3 family of glycosyltransferases with a GT-B architecture comprising an N-terminal and a C-terminal Rossmann fold domain, with an interdomain cleft that contains the active site^{16,24}. GS is the rate limiting enzyme in glycogen biosynthesis and as such its activity is tightly regulated²⁵. GS is inactivated by covalent phosphorylation at numerous N- and C-terminal sites (**Fig. 1a**), and is allosterically activated by glucose-6-phosphate (G6P) binding and/or dephosphorylation^{2,26,27}. Human GS (HsGS) phosphorylation sites lie at the N-terminus (sites 2 and 2a) and C-terminus (sites 3a, 3b, 3c, 4, 5, 1a, 1b), and phosphorylation occurs in a hierarchical fashion, whereby the phosphorylation of a specific site is the recognition motif for subsequent phosphorylation^{28,29} (**Fig. 1a and Supplementary Fig. 2**). How the metazoan GS is inhibited is not clear and while coupling of G6P binding and phosphorylation has been described for the yeast GS paralogues³⁰ no structural information of the phosphorylated version of the enzyme exists.

The complex interplay between allosteric activation and inhibitory phosphorylation is not yet fully understood, at least in part because of the lack of structural data for the full GS-GN complex. Although a binary GS-GN complex was co-purified over 30 years ago³, we have yet to confirm the stoichiometry of this complex and identify precisely how the two proteins cooperate to make glycogen.

Here, we report the structural and functional analysis of the full-length human GS-GN complex and the cryo-EM structure of phosphorylated HsGS. The structure reveals phosphoregulatory elements form a flexible inter-subunit “spike” region emanating from two GS protomers which help to keep GS in an inactive conformation through interactions of phosphorylated S641 (site

3a) with arginine residues from GS regulatory helices (arginine cradle). Moreover, low resolution maps of GN bound to GS reveal two flexible GN dimers coordinating a GS tetramer, providing new insights into the stoichiometry and the conformational plasticity of this enzyme complex. Collectively, these results shed light on the regulation of glycogen biosynthesis and the inner workings of how GS and GN cooperate to synthesize glycogen.

Results

The GS-GN complex forms an equimolar 4:4 complex

To characterize the synthesis of glycogen by the GS-GN complex, we expressed and purified human full length GS1 and GN1 in insect cells. Consistent with previous reports, co-expression of GS with GN resulted in improved production yields over the expression of GS alone^{31,32}. Purification of the wild-type (WT) complex resulted in a highly glucosylated sample, as shown by a smear on an SDS-PAGE gel corresponding to glucosylated GN detected by Coomassie and periodic acid-Schiff (PAS) staining (**Fig. 1c**). Mutation of the GN auto-glucosylating tyrosine 195^{33,34} to a phenylalanine (Y195F), resulted in a non-glucosylated GN species, as shown by a single band for GN migrating at the expected size (~37.5 kDa) on an SDS-PAGE gel and absence of glucosylated species after PAS staining (**Fig. 1b and 1c**).

To determine the stoichiometry of the GS-GN complex, we first performed mass photometry analysis of GS-GN and GS-GN(Y195F) mutant complexes, which enables mass measurements of single molecules in solution. Mass photometry measurements of the GS-GN(Y195F) complex showed a predominant molecular weight of 473 +/- 43 kDa, which is suggestive of a 4:4 stoichiometry (calculated mass of 485 kDa) (**Fig. 1d**). Analysis of the GS-GN(WT) sample identified a species with an average molecular weight of 534 +/- 110 kDa and the measured peak was broader than the non-glucosylated species (**Fig. 1d**). The increase in molecular weight and distribution compared to the Y195F complex can be attributed to the glucosylation associated with the WT complex.

Fig. 1 Structural analysis of the full-length GS-GN complex

a Domain architecture of human GS (top) and human GN (bottom). Known *in vivo* phosphorylation sites of GS are shown in red and are labelled with residue number and classical nomenclature (in bold). GN tyrosine 195 that becomes auto-glucosylated and mutated to a phenylalanine (Y195F) in this study is indicated. Not to scale. **b** Cartoon representation of GN WT and Y195F. **c** SDS-PAGE analysis of GS-GN WT and Y195F complexes (left) and periodic acid-Schiff (PAS) staining of both complexes (right). **d** Mass photometry of GS-GN(Y195F) (top) and WT complex (bottom). Expected stoichiometry for each peak is indicated. **e** Selected 2D class averages after negative-stain electron microscopy (nsEM) analysis of GS-GN(Y195F) (top) and WT (bottom), revealing a GS tetramer and two GN dimers. **f** nsEM final map (C1 symmetry, ~22 Å) is shown in transparent surface, with fitted human GN crystal structure (PDB ID 3T7O) and human GS cryo-EM structure (reported here).

To understand how GS and GN interact within the GS-GN complex, and to reveal the overall shape of the complex we performed negative stain electron microscopy (nsEM) of both the WT and Y195F complexes. 2D class averages show two GN dimers, one on either side of a GS tetramer, for both complexes (**Fig. 1e**). Final 3D maps for both complexes are consistent with the 2D classes, and the density can accommodate a GS tetramer and two GN dimers (**Fig. 1f**). This nsEM confirms a 4:4 stoichiometry and is consistent with previous findings showing that glucose chains are not essential for the GS-GN interaction and that GS can

interact with four GN C-terminal peptides simultaneously^{5,19}. Surprisingly, GN dimers do not engage GS dimers in an identical fashion, with one GN tilted slightly towards one of the GS protomers (**Fig. 1f**). Collectively, these results confirm that the GS-GN complex has a 4:4 stoichiometry where two GN dimers can engage a single GS tetramer. This provides the first glimpse of the GS-GN complex structure and explains how GS can extend glucose chains from two GN dimers simultaneously.

The structure of human GS reveals a dynamic “spike” region

Previous attempts to crystallise full length GS in complex with full length GN were unsuccessful¹⁹ and led us to pursue structural analysis using cryo-electron microscopy (cryo-EM). NsEM indicated that the position of each GN dimer is different suggesting flexibility of GN in the complex. Cryo-EM analysis of the GS-GN(Y195F) complex confirmed this GN flexibility, evident from the lack of GN signal in 2D class averages (**Supplementary Fig. 4a**) and subsequent 3D maps. Although we could detect GN presence after data processing without the application of symmetry averaging (**Supplementary Fig. 5a and 5b**), it was not possible to trace the connecting residues between the GN globular domain and the C-terminal GN³⁴ region that binds GS. To gain a higher resolution structure for the human GS, we applied D2 symmetry and achieved a global resolution of 4 Å (**Fig. 2a, Supplementary Fig. 4, Supplementary Table 1**). The reconstruction revealed a tetrameric arrangement of HsGS in agreement with the crystal structures of *C. elegans* (CeGS) and yeast (yGS) (**Supplementary Fig. 6a**). Density for the C-terminal GS interacting region of GN is sufficient for a model of the human GN C-terminus to be placed and this is consistent with the CeGS-GN³⁴ crystal structure (**Fig. 2a and Supplementary Fig. 6b**). Structural analysis of the HsGS-GN(WT) complex revealed a 6 Å map of GS and comparing this to the GS structure from HsGS-GN(Y195F) complex reveals no differences at this resolution (**Supplementary Fig. 5c and 5d**).

Notably, the EM density maps show density for an inter-subunit apparatus containing the GS phosphoregulatory regions. This occupies the space where the N- and C- terminal regions of two adjacent GS protomers meet and traverse away from the core (**Fig. 2b**). Local resolution analysis shows that this area is resolved at lower resolution than the core of the GS tetramer (**Supplementary Fig. 4b**). Analysis of this ~25 kDa region by focussed 3D classification (without applying symmetry) reveals that the region is highly flexible, as seen by the various different conformations, explaining the lower resolution and uninterpretable density (**Fig. 2c and Supplementary Fig. 4f**). Interestingly, these “spike” regions were present in all the refined classes, and suggests that GS exists as a continuum of structures with a core inactive

tetramer and “dynamic spikes” buttressed on either side, thus constraining the opening of GS to an active tetramer.

To explore the flexibility and mobility of GS, we performed 3D variability analysis³⁵ using cryoSPARC³⁶. The dynamic movements of the “spike” region and concurrent movements of the GS tetramer are highlighted in Movie S1. While some flexibility was observed within each GS protomer, an opening of the GS tetramer towards an active, relaxed state was not observed, highlighting the crucial role of the spike regions in maintaining an inactive, tense state GS tetramer.

Fig. 2 Cryo-EM structure of human GS-GN complex

a Human GS cartoon model shown in ribbons and a 4 Å reconstructed cryo-EM map shown in transparent surface and coloured by corresponding chain. A model of the last 34 residues of the C-terminus of human GN (GN³⁴) is displayed in green. **b** Cryo-EM density map shown at a lower threshold (left). The N- and C-terminal tails of two protomers converge and form the “spike” region (right). **c** Focussed 3D classification of the phosphoregulatory “spike” region reveals flexibility of this inter subunit domain. 3D classes and percentage population are indicated with different colours and the 3D refined map of the core region is coloured blue.

Phosphorylated human GS is in the inactive state

GS is regulated by both allosteric activation by G6P and inhibition via phosphorylation of its N- and C-terminal tails². Mechanistic and structural studies of the yGS have elegantly dissected its allosteric activation by G6P, whereby G6P binding induces large rotational and translational movements, leading to a more accessible active site for the growing glycogen chain to be fed into GS for elongation^{27,30}. However, the mechanisms governing GS activation by G6P and inactivation by phosphorylation, and how these opposing regulatory pressures are coupled at the molecular level remains unknown.

An advantage of expressing HsGS in insect cells is that these preparations are heavily phosphorylated. We mapped phosphorylation sites by tandem mass spectrometry (MS/MS) following proteolysis with either trypsin, chymotrypsin or elastase resulting in a sequence coverage of 97%, which is higher than the 73%³¹ and 65%³² sequence coverage achieved in previous studies (**Table 1 and Supplementary Fig. 7b**). This analysis identified canonical sites 2, 3a, 3b, 4 and 5 (S8, S641, S645, S653, S657), and also non-conventional sites (S412, S652, S727, S731). In addition, we could detect HsGS site 2 (S8) phosphorylation for the first time in a recombinant enzyme preparation. Together, these results show that expression in insect cells is sufficient to achieve a high level of phosphorylation providing suitable enzyme preparations for understanding inactive GS.

As expected, our highly phosphorylated enzyme has a low basal (-G6P) level of activity (**Fig. 3a**). Protein phosphatase 1 (PP1) and lambda phosphatase (lambda PP) treatment resulted in faster migration of GS in an SDS-PAGE and also a reduction in signal for specific phosphorylation site antibodies (**Fig. 3b and Supplementary Fig. 7a**). We observed over 10-fold increase in basal activity when GS is dephosphorylated by both PP1 and lambda PP (**Fig. 3a and 3b**). Both the phosphorylated and dephosphorylated GS forms were similarly active after addition of G6P (**Fig. 3a**), which is consistent with studies using GS from endogenous sources^{37,38}.

Table 1: Summary of GS phosphorylation site analysis. LTP and HTP refer to low throughput site determination (methods other than mass spectrometry) and high throughput analysis (mass spectrometry only), respectively³⁹

Site	Sequence	Peptide modifications	ptmRS Score	MASCOT score	Enzyme	PhosphoSite
S8	PLNRTLsMS	S7(Phospho)	S7: 100	40	Elastase	15 LTP & 1 HTP
S8	PLNRTLsMS	S7(Phospho); M8(Oxidation)	S7: 100	31	Elastase	
S8	sMSSLPLGLEDW	S1(Phospho)	S1: 99.71	13	Chymotrypsin	
S412	ESLLVGsLpDMNKMLEDKF	S7(Phospho)	S7: 100	31	Chymotrypsin	17 HTP
S412	ESLLVGsLpDMNKMML	M14(Oxidation)	S7: 100	23	Chymotrypsin	
S641	QGYRYPRPAsVPPSPS	S10(Phospho)	S10: 99.99	20	Elastase	32 LTP & 30 HTP
S641 & S645	QGYRYPRPAsVPPSPS	S10(Phospho); S14(Phospho)	S10: 100; S14: 100	24	Elastase	(645) 21 LTP & 39 HTP
S652	RHsSPHQSEDEEDPRNGPL	S3(Phospho)	S3: 99.78	34	Elastase	15 HTP
S657	RHsSPHQSEDEEDPRNGPL	S3(Phospho); S4(Phospho); S8(Phospho)	S8: 100	19	Elastase	(653) 13 LTP & 27 HTP
S652 & S657	RHsSPHQSEDEEDPRNGPL	S3(Phospho); S8(Phospho)	S3: 99.67; S8: 100	53	Elastase	(657) 14 LTP & 50 HTP
S727	RNSVDATSSSLSTPSEPLsPTSSLGEER	S20(Phospho)	S20: 100	66	Trypsin	1 LTP & 22 HTP
S727	STPSEPLsPTSSL	S8(Phospho)	S8: 99.63	20	Chymotrypsin	
S731	STPSEPLsPTSSLGEERN	S12(Phospho)	S12: 99.79	73	Chymotrypsin	6 HTP
S731	TPSEPLsPTSSL	S11(Phospho)	S11: 100	22	Elastase	
S727 & S731	NSVDATSSSLSTPSEPLsPTSSLGEER	S19(Phospho); S23(Phospho)	99.52	60	Trypsin	see above
S727 & S731	STPSEPLsPTSSLGEERN	S8(Phospho); S12(Phospho)	S8: 100; S12: 100	42	Chymotrypsin	see above

GS contains a dynamic and tuneable phosphoregulatory apparatus

Comparisons between human, *C. elegans* and yeast GS structures are consistent with the human structure in the inactive state. Each HsGS protomer shows a closed conformation of its active site, and a regulatory loop, that only becomes ordered upon G6P binding, is disordered in the human structure (**Supplementary Fig. 8**). Previous studies have suggested that phosphorylated tails may be able to engage the G6P binding site and directly compete with G6P. However, our EM density maps show no extra density within the G6P binding site (**Supplementary Fig. 8a**). Thus, we see no evidence to support the hypothesis that the phosphorylated tails directly interact with residues lining the G6P pocket to directly compete with G6P binding. Instead, we posit that the phosphoregulatory regions indirectly affect G6P binding by constraining the opening and closing of the GS tetramer.

When comparing HsGS to previous yeast crystal structures, the distance between regulatory helices ($\alpha 23$ in humans, $\alpha 22$ in yeast) in adjacent monomers changes according to the activity state of GS (**Fig. 3c**). Using the C α atoms on residue 591 on chain A and residue 580 on chain B (and the corresponding in the yeast proteins) the helices are furthest apart, at 16 Å, when G6P is bound and GS is in its high activity state, as there is better access for accepting the substrate^{27,30}. When no G6P is bound and there is no phosphorylation, GS is in the basal state and the helices lie 11 Å apart. In a yGS structure of a mimic of the inhibited state, where residues R589 and R592 were mutated to Ala, the helices are closest together, at 8 Å, leading to the closure of the N-terminal domain and thus acceptor access is hindered³⁰. HsGS produced in insect cells is phosphorylated^{31,32} (**Fig. 3b, Supplementary Fig. 7**) and the helices lie 8 Å apart, thus confirming that we have reconstructed the first phosphorylated, auto-inhibited structure (**Fig. 3c**).

The N- and C-terminal tails of each GS protomer lie parallel to each other, and travel side by side along the GS tetrameric core to reach centre. Here, they meet the N- and C-terminal tails

from an adjacent GS protomer, which have travelled from the opposite direction (**Fig. 3d**). From the electron density map generated without the application of symmetry averaging, it appears that one C-terminal tail disengages with the GS core earlier than the other C-terminal tail from the adjacent chain. The C-terminal tail from chain B continues to travel further across the regulatory helices than chain A, prior to traversing away from the core to form the “spike” region (**Fig. 3d**). This allows chain B to engage with the regulatory helices $\alpha 23$, specifically interacting with residues R588 and R591, which come from two GS protomers to form a positively charged pocket we have termed the “arginine cradle” (**Fig. 3e**). Judging by the distance from the last discernible residue of the C-terminal tail and the available cryo-EM density we have modelled a phosphorylated S641 (pS641) in chain B in interacting distance with the arginine cradle (**Fig. 3d**). The position of S641 is consistent with the AlphaFold⁴⁰ predicted model of HsGS (**Supplementary Fig. 8d**) suggesting that this interaction may also be possible in the non-phosphorylated state, although the negative charge on the phosphate group would naturally provide stronger interactions with the positively charged arginine cradle. S641 is a major phosphorylation site involved in activity regulation, and interaction of pS641 with the arginine cradle from helix $\alpha 23$ provides a crucial activity switch mechanism from a tense (phosphorylated) state to a relaxed (G6P-bound) state. The involvement of helices $\alpha 23$ which also interacts with G6P via the nearby arginine residues R582 and R586 (**Supplementary Fig. 8a**) is significant, since this could provide a possible explanation between G6P-binding and its ability to override inactivation by phosphorylation.

Fig. 3 Dynamic and tuneable phosphoregulatory region of human GS

a Activity of GS-GN(Y195F) with and without the addition of lambda phosphatase (lambda PP) and protein phosphatase 1 (PP1). Upon G6P saturation, GS reaches similar activity levels regardless of phosphorylation state. Data are mean $\pm$ S.E.M. from $n=2$ and representative of two independent experiments. **b** Western blot for the indicated human GS phosphorylation sites and total GS. **c** Comparison of distances between regulatory helices of adjacent monomers of human GS (reported here), low activity inhibited mimic (PDB ID 5SUL), basal state (PDB ID 3NAZ) and G6P activated (PDB ID 5SUK) yeast crystal structure. Distances were measured from the C α of residues 591 chain A and 580 chain B (and corresponding yeast residues). **d**. The N- and C- terminal tails of one protomer lie parallel and move towards the bottom of the “spike” region, meeting the N- and C-terminal tails of the adjacent GS protomer **e** Electron density for phosphorylated S641 (pS641) interacting with R588 and R591 on the regulatory helices α 23. **f** Residues that are interacting with the N- and C-terminal tails that are mutated in this study are shown.

Aligning the structure of the C-terminal domain residues of human, yeast and *C. elegans* GS (residues 294-597 in HsGS, residues 283-598 in yGS and residues 312-679 in CeGS) revealed that helix α 3 (human residues 85-96) is shifted towards the C-terminal domain, compared to the yeast basal state. This N-terminal closure observed when comparing the yeast basal and mimic of the phosphorylated state crystal structures was suggested to contribute to the inhibited state of GS³⁰. Consistent with these analyses, we observe an even bigger shift of helix α 3 and a tight N-terminal closure in the phosphorylated human GS (**Supplementary Fig. 8c**).

The position of the extreme N-terminus is noticeably different in human and *C. elegans* GS structures compared to yeast (**Supplementary Fig. 6c**). The majority of the first β sheet in all structures is in the same place, however human residues before 26 (residue 7 in yeast) move in the opposing direction to yeast (**Supplementary Fig. 6c**). This positioning of the human GS N-terminus is directed towards the regulatory helices (α 23). Previous structural investigation of CeGS-GN³⁴ suggested a hypothesis where phosphorylation could enable the N-terminus to engage with regulatory helices¹⁹. Our human phosphorylated structure supports this hypothesis, although the current density does not allow model building before residue 14. However, weak density for the N-terminus is present showing that this region also interacts with the globular GS domain and regulatory helices, thus explaining N-terminal phospho tail contribution to GS inactivation (**Fig. 3d and Supplementary Fig. 6d**). Collectively, our structural analyses support a model by which phosphorylated N- and C-terminal tails inhibit the GS tetramer by constraining a tense conformation through inter-subunit interactions.

Dislodging the GS phosphoregulatory regions

Due to the flexibility evident in the N- and C-terminal tails, we were unable to build phosphorylated residues in the cryo-EM map other than pS641. However, we can see the

beginning of the flexible phosphoregulatory “spike” region and residues from the GS “core tetramer” which interact with this regulatory region (**Fig. 3f**). To investigate the relationship between allosteric regulation and inhibitory phosphorylation and elucidate the mechanism of inactivation, we mutated residues in GS that contact the beginning of the phosphoregulatory region. We selected residues which are not involved in G6P binding and mutated these in order to “dislodge” the regulatory tails (**Fig. 3f and Supplementary Fig. 8e**). If the phosphorylated tails are indeed holding GS in an inactivated state, weakening the interaction between the core tetramer and the “spike” regions should create an enzyme with higher basal activity in comparison to the WT. Consistent with our hypothesis, we observed a marginal increase in basal (-G6P) GS activity in R588A+R591A, Y600A, R603A, H610E and W18A mutants, that was reflective of the phosphorylated state at residues S8, S641 and S645 (**Fig. 4a and 4b**). These mutants were unaffected in terms of GN co-purification and with the exception of R588A+R591A mutant, they had similar melting (T_m) profiles and oligomeric state to the WT GS complexes (**Fig. 4e, Supplementary Fig. 3b, 9a and 9b**). All mutants except Y600A could still be activated to similar levels to the WT upon addition of G6P (**Fig. 4a**).

Upon addition of PP1 and lambda PP, the GS mutants R588A+R591A, R603A and W18A were more easily dephosphorylated at S641 and S8 than WT (**Fig. 4d**). For the W18A mutant, this dephosphorylation by both lambda-PP and PP1 resulted in over a 20-fold increase in basal activity, and also an approximately 3-fold increase in comparison to WT (**Fig. 4c**). The GS R603A-GN(Y195F) mutant has a basal activity similar to WT upon dephosphorylation. However, the robust dephosphorylation at S641 and S8 in GS R588A+R591A was not associated with an increase in activity (**Fig. 4c**). As described above, R588 and R591 lie on the regulatory helices and are also involved in inter-subunit interactions and form the arginine cradle that interacts with pS641 (**Fig. 3c**). In the GS R588A+R591A double mutant we see dissociation of the complex in mass photometry (**Supplementary Fig. 9c**). Therefore, the role of these residues in stabilising the GS tetramer may be the cause for the lack of rescue of activity upon dephosphorylation (**Fig. 4c**). Moreover, dephosphorylated GS had a markedly lower T_m (48 °C) than WT or mutant GS supporting the idea that phosphorylation of the “spike” regions strengthens the inter-subunit interactions within the tetramer and holding the enzyme in the “tense” conformation.

Fig. 4 Dislodging the GS phosphoregulatory region increases basal activity and increases accessibility for phosphatases

a Activity of GS WT and indicated mutants in the GS-GN(Y195F) complex in the presence and absence of G6P (left) and +/- G6P activity ratio (right). Data are mean +/- S.E.M from n=3 and representative of two independent experiments. **b** Western blot for specific human GS phosphorylation sites S641/645, S641, S8, and total GS and GN. **c** Activity of phosphorylated and dephosphorylated GS WT and indicated mutants (left) and +/- G6P activity ratio (right). Data are mean +/- S.E.M from n=2 and representative of two independent experiments. **d** Western blots of GS WT and mutants in the GS-GN(Y195F) complex after dephosphorylation with PP1 and/or lambda phosphatase (lambda PP). **e** Melting temperature (T_m) of GS WT and mutants in the GS-GN(Y195F) complex. Changes in melting temperature upon addition of 12.5 mM G6P ($\Delta T_m = T_m^{+G6P} - T_m^{-G6P}$). Data in **e** are mean +/- S.E.M from n = 3 experiments carried out in technical duplicates (dephosphorylated GS) and triplicates (WT and mutant GS).

Discussion

Here, we provide structural and biochemical analysis of phosphorylated human GS in the full-length GS-GN complex. Cryo-EM analysis reveals an inter-subunit flexible region which holds GS in an inactive conformation, shedding light on how phosphorylation of the N- and C-terminal tails inhibit the enzyme (**Fig. 5**). In addition, negative stain EM maps reveal two GN dimers binding to a GS tetramer, explaining the conformational plasticity of this octameric enzyme complex and the inner workings of how GS-GN cooperate to synthesize glycogen (**Fig. 1 and Fig. 5**). The two GN dimers neighbouring a GS tetramer do not interact in an identical manner, with one GS dimer tilted closer towards GS in comparison to the other (**Fig. 1f**). This observed flexibility of GN shows the inherited dynamic nature within the complex which can be aided by the variable length linker connecting the catalytic domain and the C-terminal GN³⁴ region that anchors GS. The precise functional relevance of this movement is yet to be explored, however the linker length was shown to govern glycogen particle size and molecular weight distribution *in vitro*¹⁹. Thus, the ability for GN to orient and bind flexibly to GS may be a desirable feature facilitating the wide range of size and distribution of glycogen particles seen in multiple species and tissues⁴. Unfortunately, this inherent mobility also prevented a high-resolution reconstruction of GN structure and identification of any additional interfaces between GS and the globular catalytic domain on GN.

GS phosphorylation sites lie outside of the catalytic core on the N- and C- terminal tails (**Fig. 1a and Supplementary Fig. 2**). A mechanism by which phosphorylation could detach this N-terminal region from its position, and move to interact with the arginine residues in the regulatory helices $\alpha 23$ was previously proposed¹⁹. Our structure of the phosphorylated state of GS supports this hypothesis, as the human N-terminal tail lies in the same direction as *C. elegans* N-terminal region situated against the regulatory helices, although the exact position differs (**Supplementary Fig. 6c**). More importantly, in human GS the C-terminus is

responsible for the majority of the observable interactions with helix α 23. R588 and R591 are not conserved in *C. elegans* (**Supplementary Fig. 2**), perhaps explaining the positional differences between human and *C. elegans* GS tails. This exchange of interactions between N- and C-terminus suggests a functional redundancy between the multiple phosphorylation sites and a likely explanation for the dynamic nature of the “spike” regions.

The C-terminus of human GS runs parallel to the N-terminal tail, travelling alongside the GS core, prior to moving away from the core to form the “spike” region (**Fig. 3d**). Unexpectedly, the C-terminal tail of adjacent protomers are not identical. One tail appears to disengage from the GS core sooner than the other. This leaves room for one tail to interact with the regulatory helix, specifically residue pS641 interacting with R588 and R591 on both protomers (**Fig. 3d and 3e**). S641 was identified as a site of phosphorylation by both phosphorylation mapping and western blotting (**Table 1, Supplementary Fig. 7b and Fig. 3b**). This negative moiety was coordinated by multiple arginine residues from the arginine cradle, and suggests a crucial role for phosphorylation in interacting with the regulatory helices. A negative charge from pS641 could be strengthening the interaction between two GS protomers, in comparison to basal and activated GS, thus constraining the GS tetramer and leading to an inactive enzyme (**Fig. 3c**).

The non-identical/different positions of the N- and C-terminal tails as well as the flexibility of the “spike” region indicates a high level of coordination and communication both between the N- and C- termini of a single GS protomer, as well as between protomers (**Fig. 2c and 3d**). Having one tail buttressed against the regulatory helix and the other steering away from the core may allow interchanging of the tails based on the level of phosphorylation, revealing a significance for the existence of multiple phosphorylation sites. Having one tail more accessible to phosphatases may also aid/allow rapid dephosphorylation of GS, leading to an increase in GS activity and thus glycogen synthesis (**Fig. 5**).

Elucidating the role of the inter-subunit domain that house the phosphorylation sites through mutations that weaken the interactions between the core tetramer and the “spike” regions, resulted in basal activity equal to or higher than the WT, yet retaining activation by G6P (**Fig. 4a**). The GS Y600A mutant was not activated by G6P to the same extent as WT (**Fig. 4b**), and although Y600 does not directly bind to G6P, UDP or sugars^{27,41,42}, it is possible that this hydrophobic residue is important for interdomain movements which are required for full GS activation.

Fig. 5 GS and GN cooperate to synthesize glycogen

Glucose is converted into glycogen through the action of glycogenin (GN), glycogen synthase (GS) and glycogen branching enzyme (GBE). GN interacts with GS to feed the initial glucose chain into the GS active site for elongation. GS is regulated by allosteric activation and inhibitory phosphorylation. pS641 from one C-terminal tail interacts with the regulatory helices $\alpha 23$.

413

414 Dephosphorylation of the GS-GN(Y195F) complex resulted in an increase in basal activity,
415 yet there is little difference in the high activity (G6P-bound) state between phosphorylated and
416 dephosphorylated complexes (**Fig. 3a and Fig. 4d**). This is in accordance with previous
417 studies that demonstrate that G6P can overcome inhibition by phosphorylation and restore full
418 activity². C-terminal mutants R588A+R591A and R603A and the N-terminal W18A mutant
419 were more easily dephosphorylated than WT (**Fig. 4c**) suggesting that dislodging of the
420 phosphoregulatory region leads to phosphorylation sites being more accessible to
421 phosphatases.

422

423 The robust dephosphorylation of GS R588A+R591A did not result in an increase in basal
424 activity (**Fig. 4c and 4d**), which mirrors previous results in yeast³⁰. The analogous mutations
425 were used in yGS resulting in low basal activity, yet it could still be fully activated by G6P³⁰. It
426 was proposed that these residues in the regulatory helix are essential for keeping GS in a
427 “spring loaded” intermediate state, and thus charge neutralization by mutation of arginine to
428 alanine leads to the “tense” inactive state³⁰. Our activity data agree with this as we don’t see
429 an increase in basal activity despite dephosphorylation at S641 and S8, although we do see
430 a marginal difference between this mutant and WT in the phosphorylated state (**Fig. 4a and**

4c). However, it is important to note that we also see some complex dissociation with the R588A+R591A mutant as evidenced by a larger dissociated complex peak in mass photometry (**Supplementary Fig. 9c**). It is possible that the dephosphorylated R588A+R591A mutant is also unstable and even more dissociated than the phosphorylated mutant, resulting in a less active preparation.

Mutations of human GS1 and GS2 are prevalent in glycogen storage diseases. Several mutations cause glycogen storage disease 0, which is due to a deficiency in GS and thus reduced levels of glycogen⁴³. These mutations are enriched in key binding pockets of GS, affecting UDP-G, G6P and sugar binding. Some mutations affect the interaction between GS and GN³⁴, consistent with the requirement of this interaction for glycogen synthesis¹⁹. Thus, the structure provides a valuable resource to understand disease mutations and to assess the impact residue changes have on GS-GN activity and/or interaction (**Supplementary Table 2 and Supplementary Fig. 8f**).

Our phosphorylated GS-GN structure reveals that the N- and C-terminal phosphorylation tails constrain inter-subunit movement by coordinating the N-terminal lobes of GS and interaction of the regulatory helices $\alpha 23$ primarily via an arginine-rich pocket (arginine cradle). Thus, GS has evolved a mechanism by which the phosphorylated N- and C-terminal “spike” regions hold GS in an inactive conformation that is relieved by dephosphorylation and/or G6P binding. We propose that the dynamic nature of these “spike” regions serves the purpose of exposing phosphorylated residues to phosphatases, thus allowing a “tunable rheostat” instead of an on/off switch for regulating GS activity. Collectively, our analyses of the human GS-GN enzyme complexes reveal important mechanistic and structural details that could improve our understanding of glycogen storage diseases.

Materials and Methods

Materials

Total GN antibody (S197C, third bleed) was obtained from MRC-PPU Reagents and Services. Total GS (#3898) and p-GS S641 (#47043) antibodies were from Cell Signaling Technologies. p-GS S641/S645 (07-817) is from MerckMillipore. p-GS S8 antibody (YZ5716, first bleed) was generated by (YenZym Antibodies Brisbane, CA, USA) by immunisation with a phosphorylated peptide of the mouse GYS1 sequence (PLSRSL-*S-VSSLPG-amide, the prefix * denotes the phosphorylated residue). Secondary antibodies (711-035-152 and 713-035-147) were

obtained from BioRad. Glucose-6-phosphate (G6P) (10127647001) is from Roche. All other chemicals if not noted otherwise are from Sigma Aldrich.

Cloning, protein expression and purification of GS-GN complex

Genes encoding human GS1 (HsGS:NM 002103) and human GN1 (HsGN:NM 001184720) mutant were cloned into pFL a vector⁴⁴. A single 6 x His purification tag followed by a cleavable site was engineered at the N-terminus of GN WT or GN Y195F mutant. For co-expression of WT GS and mutants the genes encoding human GS1 and human GN1 (Y195F) were cloned in pFastBac vectors, both with a 6 x His purification tag followed by a TEV site at the N-terminus. Recombinant bacmids were generated in DH10Bac™ cells. Virus amplification and cell expression in *Spodoptera frugiperda* (sf9) cells and *Trichoplusia ni* (Tni) cells respectively were carried out using standard procedures⁴⁵. For co-infection of pFastBac clones, a 10:1 ratio of the GS:GN P2 virus ratio was used. A PCR based site directed mutagenesis was used to create the following mutants from the pFastBac GS1 construct: W18A, R588A+R591A, Y600A, R603A, H610E, S614R. All of the alterations were confirmed by DNA sequencing.

Cell pellets containing HsGS-GN, HsGS-GN(Y195F) and mutants were resuspended in lysis buffer (50 mM Tris-HCl pH 7.6, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.075% β -mercaptoethanol, 1mM benzamidine, 0.8 mM phenylmethyl sulfonyl fluoride (PMSF), 0.3 mg/mL lysozyme). Cells were lysed by sonication (1 second on, 3 seconds off for a total of 5 minutes) on ice and the lysate was cleared by centrifugation at 35,000g for 30 minutes at 4 °C. The clarified lysate was sonicated again (1 second on, 3 seconds off for a total of 1 minute), followed by filtering with a 0.45 μ m filter (MerckMillipore). Filtered lysate was loaded onto a pre-equilibrated 1 mL or 5 mL HisTrap HP column (GE Healthcare) charged with Ni²⁺. The loaded column was washed with four column volumes (CV) of low salt buffer (50 mM Tris-HCl pH 7.6, 300 mM NaCl, 20 mM imidazole, 10% glycerol, 0.075% β -mercaptoethanol, 1mM benzamidine), followed by four CV washes of high salt buffer (50 mM Tris-HCl pH 7.6, 500 mM NaCl, 20 mM imidazole, 10% glycerol, 0.075% β -mercaptoethanol, 1mM benzamidine) and finally 4 CV washes in low salt buffer. The column was then attached to the AKTA system (GE Healthcare) and washed with low salt buffer. The protein was then eluted by applying an imidazole gradient with elution buffer (50 mM Tris-HCl pH 7.6, 300 mM NaCl, 300 mM imidazole, 10% glycerol, 0.075% β -mercaptoethanol, 1 mM benzamidine). The fractions containing protein were analysed by SDS-PAGE and then pooled and dialysed overnight (10,000 MWCO SnakeSkin dialysis tubing (Thermo Scientific)) at 4 °C in dialysis buffer with TEV protease added (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 20 mM imidazole, 10% glycerol, 0.075% β -mercaptoethanol, 1 mM benzamidine). The dialysed protein was re-loaded onto the

HisTrap column equilibrated with low salt buffer, for a Ni subtraction step. TEV cleaved protein was eluted in the flow through and low salt washes. The flow through and first low salt wash were pooled and concentrated using a VIVASPIN20 30,000 MWCO (Sartorius, Gerson), followed by centrifugation at 17,000g for 15 minutes at 4 °C. Protein was then injected onto a 16/600 or 10/300 Superdex200 column (GE Healthcare) equilibrated with gel filtration buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP, 10% glycerol). Fractions containing protein were analysed by SDS-PAGE, and fractions containing GN were pooled, concentrated and stored at -80 °C. Some fractions containing GS-GN complex were stored separately at -80 °C and the remaining protein was pooled and concentrated before being stored at -80 °C. Proteins were visualized by Coomassie blue staining, and glucosylated species were detected using the periodic acid-Schiff (PAS) method (Glycoprotein staining kit, Thermo Scientific).

***In vitro* dephosphorylation of GS-GN**

Protein phosphatase 1 (PP1) and lambda phosphatase (lambda PP) were bought from MRC PPU Reagents and Services. Both have an N-terminal GST tag and lambda PP also has a C-terminal 6xHis tag. GS-GN complex was dephosphorylated in reactions containing equal amounts of PP1 and lambda PP in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP, 1 mM MnCl₂ and 10% glycerol for 30 minutes at 30 °C. For subsequent differential scanning fluorimetry experiments, the phosphatases were removed by incubating the reactions with GST beads for 1 hour at 4 °C. Reactions were then passed through an equilibrated 0.45 µm Spin-X column (Costar, 0.45 µm cellulose acetate) and eluted by centrifugation at 16,000g for 2 minutes.

Negative stain electron microscopy – grid preparation and data collection

HsGS-GN WT and Y195F were diluted in buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP, 10% glycerol) to concentrations between 0.01 and 0.02 mg/mL immediately before grid preparation. Carbon-coated copper grids (Formvar/Carbon 300 mesh Cu, Agar Scientific) were glow-discharged for 30 seconds, 10 mA and 0.39 mBar pressure (PELCO easiGlow, Ted Pella). Grids were incubated for 1 minute with 6 µL sample, washed with H₂O three times and stained twice with 2% w/v uranyl acetate for 20 seconds. Excess liquid was removed by blotting with filter paper. Data was collected on a FEI Technai F20 electron microscope operated at 120 keV, equipped with a FEI Ceta (CMOS CCD) camera.

Negative stain electron microscopy – data processing

RELION 3.0 was used for processing of negative stain-EM data⁴⁶. Real-time contrast transfer function (CTF) parameters were determined using gCTF⁴⁷. Approximately 2,000 particles

were manually picked, extracted with a box size of $104 \text{ \AA}^2$, then subjected to reference-free 2D classification to produce initial references to be used for auto-picking. The parameters for auto-picking were optimized and 92,580 particles were extracted. The extracted particles were used for iterative rounds of reference-free 2D classification. Based on visual inspection, best quality 2D average classes were selected to generate a *de novo* 3D initial model, which was used as a reference in unsupervised 3D classification. These classes were then subjected to 3D refinement to generate a final EM density map.

Cryo-electron microscopy – grid preparation and data collection

Quantifoil R2/1 Cu300 or Quantifoil R1.2/1.3 Cu300 (Quantifoil Micro Tools) grids were glow-discharged using the GloQube plasma cleaner (Quorum) at 40 mA for 30 seconds, for GS-GN(Y195F) and GS-GN respectively. A FEI Vitrobot IV was equilibrated at 4 °C at 100% relative humidity. GS-GN(Y195F) complex was diluted in buffer (25 mM HEPES pH7.5, 150 mM NaCl, 1 mM TCEP) to 0.23 mg/mL (1.85 μM) containing 3% glycerol immediately before 3 μL was added to the grid. GS-GN was diluted to 0.36 mg/mL (2.97 μM) containing 8% glycerol. This was followed by immediate blotting and plunge-freezing into liquid ethane cooled by liquid nitrogen.

All data was collected on a FEI Titan KRIOS transmission electron microscope at 300 keV. The total electron dose was $85 \text{ e}/\text{\AA}^2$, a magnification of 75,000x was used and a final calibrated object sampling of $1.065 \text{ \AA}/\text{pixel}$. For GS-GN(Y195F), 8,699 movies were recorded using the EPU automated acquisition software on a FEI Falcon III direct electron detector in integrating mode⁴⁸. Each movie had a total exposure time of 1.8 seconds, collected over 47 fractions with an electron dose of $1.8 \text{ e}/\text{\AA}^2$ per fraction. Three exposures per hole was taken and the defocus values ranged from $-1.7 \text{ }\mu\text{m}$ to $-3.1 \text{ }\mu\text{m}$.

For GS-GN, 3,009 movies were recorded using the EPU automated acquisition software on a FEI Falcon III direct electron detector in integrating mode⁴⁸. Each movie had a total exposure time of 1.6 seconds, collected over 47 fractions with an electron dose of $1.8 \text{ e}/\text{\AA}^2$ per fraction. One exposure per hole was taken and the defocus values ranged from $-1.7 \text{ }\mu\text{m}$ to $-3.1 \text{ }\mu\text{m}$.

Cryo-electron microscopy – data processing

For both GS-GN(Y195F) and GS-GN, drift-corrected averages of each movie were created using MotionCor2⁴⁹ and real-time contrast transfer function (CTF) parameters were determined using gCTF⁴⁷. Both motion correction and CTF estimation were carried out on-the-fly⁴⁸. Particles were picked using the PhosaurusNet general model in crYOLO v1.3.5⁵⁰. For

GS-GN(Y195F), 780,290 particles were picked and for GS-GN, 250,250 particles were picked. Particles were then imported into RELION 3.0⁴⁶, extracted with a box size of 220 pixels and subjected to reference-free 2D classification.

For GS-GN(Y195F), 546,661 particles selected after 2D classification were subjected to 3D classification, applying D2 symmetry. 3D classification gave a multitude of conformations, indicating flexibility and heterogeneity in the dataset. To treat this heterogeneity, iterative rounds of 3D classification were performed, generating 12 classes each round. Carrying all “good”/unambiguous classes forward, 160,687 particles were subjected to 3D refinement and postprocessing, generating a map at 4.11 Å. Followed by iterative rounds of per particle contrast transfer function refinement and Bayesian particle polishing to generate a map at 4 Å. Final resolutions were determined using the gold-standard Fourier shell correlation criterion (FSC=0.143). Local resolution was estimated using the local resolution feature in RELION.

To prevent interpretation of any artefacts created by applying D2 symmetry, the data was also processed in C1 symmetry. The same particles after 2D classification were subjected to 3D classification without applying symmetry. All “good”/unambiguous classes were carried forward for a second round of 3D classification. High quality classes were then subjected to 3D refinement and postprocessing, to generate a 4.8 Å map.

To elucidate the movement of phosphoregulatory regions, an alignment free 3D classification with a mask containing the “spike” density was performed, using a regularisation parameter T of 40⁵¹ (**Fig. 2c and Supplementary Fig. 4f**).

For GS-GN, 84,557 particles selected after 2D classification were subjected to 3D classification. 36,972 particles (from 2 classes) were subjected to 3D refinement and postprocessing, generating a map at 6.0 Å.

To explore the heterogeneity in the dataset, the 3D variability analysis³⁵ tool in cryoSPARC v3.2.0³⁶ was used. The 160,687 particles after 3D classification were imported into cryoSPARC and homogenous refinement with a mask was performed. The subsequent particles were used in the 3D variability analysis to solve 3 modes, using C1 symmetry and with a filter resolution of 7 Å. The subsequent frames were visualized in Chimera⁵². 1 mode is shown in **Movie S1**.

Model building and refinement

A preliminary model of human GS was generated by AlphaFold⁴⁰. This preliminary model was rigid body fitted into the cryoEM density in UCSF Chimera⁵². The model was then built using iterative rounds of manual building in COOT⁵³ and real space refinement in PHENIX 1.14⁵⁴. A model of the last 34 residues of human GN was created by Phyre2⁵⁵ and placed in the approximate location using the *C. elegans* GS-GN³⁴ crystal structure (PDB ID 4QLB) in Chimera⁵² and then fitted into the map by rigid body fitting.

Visualisation, structure analysis and sequence alignments

Visualisation and structure analysis were performed using ChimeraX⁵⁶ or Chimera⁵². Multiple sequence alignments were performed using MUSCLE⁵⁷ and displayed and edited using ALINE v1.0.025⁵⁸.

Mass photometry

Mass photometry experiments were performed using a Refyn One^{MP} mass photometer. Immediately prior to mass photometry measurements, proteins were diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP for a final concentration of 50 nM. For each measurement, (16 µL) buffer was added to a well and the focus point was found and adjusted when necessary. Protein (4 µL) was then added to the buffer droplet, the sample was mixed and movies of 60 seconds were recorded using AcquireMP. Data were analysed using DiscoverMP.

Differential Scanning Fluorimetry

Thermal shift assays were performed using an Applied Biosystems QuantStudio 3 Real-Time PCR system. SYPROTM Orange (Invitrogen) was used as a fluorescence probe. Proteins were diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 1 µM. Varied concentrations of G6P were added and the reaction was incubated at room temperature for 30 minutes. SYPRO Orange was diluted in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP to a final concentration of 2.5 X, in a total reaction volume of 20 µL. The temperature was raised in 0.018 °C intervals from 20 °C to 95 °C. Data were analysed using Protein Thermal Shift v1.4.

Tandem mass spectrometry

Concentrated purified protein complexes (6.75 µg) were diluted 30-fold in 25 mM ammonium bicarbonate pH 8.0 before being subject to reduction with dithiothreitol and alkylation with iodoacetamide, as previously described⁵⁹. The eluent was equally divided into three for

digestion with either: 33:1 (w/w) trypsin gold (Promega), 25:1 (w/w) chymotrypsin (Promega), or 10:1 (w/w) elastase (Promega), using the manufacturer's recommended temperatures for 18 hours with 600 rpm shaking. Digests were then subject to in-house packed, strong cation exchange stage tip clean-up, as previously described by⁶⁰. Dried peptides were solubilized in 20 μ l of 3% (v/v) acetonitrile and 0.1% (v/v) TFA in water, sonicated for 10 minutes, and centrifuged at 13,000g for 15 minutes at 4 °C being separated using an Ultimate 3000 nano system (Dionex) by reversed-phase HPLC, over a 60-minute gradient, as described in⁵⁹. All data acquisition was performed using a Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific), with higher-energy C-trap dissociation (HCD) fragmentation set at 32% normalized collision energy for 2+ to 5+ charge states. MS1 spectra were acquired in the Orbitrap (60K resolution at 200 m/z) over a range of 350 to 1400 m/z , AGC target = standard, maximum injection time = auto, with an intensity threshold for fragmentation of $2e^4$. MS2 spectra were acquired in the Orbitrap (30K resolution at 200 m/z), AGC target = standard, maximum injection time = dynamic. A dynamic exclusion window of 20 seconds was applied at a 10 ppm mass tolerance. Data was analysed by Proteome Discoverer 1.4 using the UniProt Human reviewed database (updated April 2020) with fixed modification = carbamidomethylation (C), variable modifications = oxidation (M) and phospho (S/T/Y), instrument type = electrospray ionization–Fourier-transform ion cyclotron resonance (ESI-FTICR), MS1 mass tolerance = 10 ppm, MS2 mass tolerance = 0.01 Da, and the *ptmRS* node on; set to a score > 99.0.

Glycogen synthase activity assay

1 μ g of purified protein was diluted in ice cold lysis buffer (270 mM sucrose, 50 mM Tris HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 20 mM glycerol-2-phosphate, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM DTT, 0.1 mM PMSF, 1 mM benzamidine, 1 mg/mL microcystin-LR, 2 mg/mL leupeptin, and 2 mg/mL pepstatin A) to a total volume of 100 μ L. 20 μ L of the protein was added to 80 μ L of the assay buffer (25 mM Tris-HCl (pH 7.8), 50 mM NaF, 5 mM EDTA, 10 mg/ml glycogen, 1.5 mM UDP-glucose, 0.125% (v/v) 2-mercaptoethanol and 0.15 mCi/mmol D-[¹⁴C]-UDP-glucose (American Radiolabelled Chemicals, Inc., ARC 0154) with 0 and 12.5 mM G6P. Reactions were incubated for 20 minutes at 30 °C with mild agitation at 300 rpm. The reactions were stopped by spotting 90 μ L of the reaction mix onto 2.5 cm x 2.5 cm squares of filter paper (Whatman 3MM) which were immediately immersed in ice cold 66% ethanol and left to incubate with mild agitation for 20 minutes. The filter papers were washed thrice more in 66% ethanol for 20 minutes each and rinsed in acetone. The dried filters were subjected to scintillation counting.

Immunoblotting

Purified proteins were denatured in Laemmli buffer at 100 °C for 5 minutes. 100 ng of the protein was separated by SDS-PAGE on 4-10% gel and transferred onto nitrocellulose membranes (#926-31090, LiCOR). Membranes were blocked for 45 minutes at room temperature in 5% skim milk (Sigma, 1153630500) followed by incubation in TBST (10 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% (v/v) Tween-20) containing 5% (w/v) BSA and the primary antibody overnight at 4 °C. The membranes were incubated for 45 minutes in HRP conjugated secondary antibodies diluted 1:10,000 in 3% skim milk, after extensive washing in TBST. The membranes were imaged using enhanced chemiluminescence (ECL) reagent (GE Healthcare).

Author contributions:

L.M. performed the majority of experiments (molecular biology, protein production, electron microscopy, differential scanning fluorimetry and mass photometry analysis), D.B. performed glycogen synthase activity and western blot assays, L.D. performed phosphorylation mapping and C.B. provided support with structural biology and protein production. L.M. and E.Z. drafted the manuscript with input from D.B., K.S., L.D. and C.E.E. and all authors revised it. J.P., C.E.E., C.H., J.B., N.A.R., H.K., K.S. and E.Z. provided supervision and project management. E.Z., K.S., C.E.E., C.H. and J.B. designed and interpreted data in consultation with all authors.

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Conflict of Interest

The authors report no conflicts of interest.

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