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# Glycogen and its metabolism: some new developments and old themes

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## Abstract

Glycogen is a branched polymer of glucose that acts as a store of energy in times of nutritional sufficiency for utilization in times of need. Its metabolism has been the subject of extensive investigation and much is known about its regulation by hormones such as insulin, glucagon and adrenaline (epinephrine). There has been debate over the relative importance of allosteric compared with covalent control of the key biosynthetic enzyme, glycogen synthase, as well as the relative importance of glucose entry into cells compared with glycogen synthase regulation in determining glycogen accumulation. Significant new developments in eukaryotic glycogen metabolism over the last decade or so include: (i) three-dimensional structures of the biosynthetic enzymes glycogenin and glycogen synthase, with associated implications for mechanism and control; (ii) analyses of several genetically engineered mice with altered glycogen metabolism that shed light on the mechanism of control; (iii) greater appreciation of the spatial aspects of glycogen metabolism, including more focus on the lysosomal degradation of glycogen; and (iv) glycogen phosphorylation and advances in the study of Lafora disease, which is emerging as a glycogen storage disease.

## INTRODUCTION

Glycogen is a branched polymer of glucose that serves as an osmotically neutral means to store glucose in cells in times of nutritional plenty for utilization in times of need [1]. It is present in organisms from bacteria and archaea to humans. Plants synthesize related glucose polymers in the form of starch which is composed of amylopectin, a polysaccharide chemically similar to glycogen, and amylose, which is an essentially unbranched linear polymer of glucose [2,3]. Therefore polymerization of glucose may be a universal mechanism for energy storage in Nature.

The discovery of liver glycogen in 1857 is attributed to Claude Bernard (reviewed in [4]). A century and a half later, several of his original tenets are still accepted and study of glycogen metabolism in the second half of the 20th Century introduced a series of novel biochemical concepts, now engrained in current thinking about biological regulation, and resulted directly in the award of four Nobel Prizes (Carl and Gerty Cori in 1947, Louis Leloir in

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1970, Earl Sutherland in 1971, and Edwin Krebs and Edmond Fischer in 1992; see http://nobelprize.org/nobel_prizes/). Among the scientific advances intertwined with 20th Century research on glycogen are the discovery of NDP-sugars as intermediates in polysaccharide synthesis, reversible protein phosphorylation, the first protein kinases and protein phosphatases, the role of allosteric control of enzymes by ligands, hormonal controls of intracellular enzymes by insulin, and hormonal control by cAMP produced via activation of G-protein-coupled hormone receptors.

The present review does not attempt to be comprehensive, rather it highlights certain major themes and areas of recent progress on eukaryotic glycogen metabolism. The focus is on muscle rather than liver and on synthesis rather than degradation via the phosphorylase pathway. The reader is referred to earlier reviews for additional background [1,5–20]. Wilson et al. [21] provide a recent review of glycogen metabolism in bacteria and yeast. Literature coverage is roughly until July 2011.

## GLYCOGEN STRUCTURE

The primary polymerization in glycogen is provided by $\alpha$-1,4-glycosidic linkages between glucose residues; branchpoints are introduced by $\alpha$-1,6-glycosidic linkages (Figure 1). Glycogen isolated from biological sources is polydisperse, existing as a population of molecules of different sizes. In addition, branchpoints are not in precisely defined locations, so that molecules of identical mass need not have identical chemical structures. Therefore precise three-dimensional structures of glycogen cannot be determined by the classical approaches of structural biology. The best that can be done to analyse the chemistry of glycogen molecules is to define molecular mass distributions, average chain lengths and average branching frequencies. Nonetheless, important insights into glycogen structure have emerged. A well-accepted model for glycogen structure [22–25] categorizes the chains as inner B-chains, which would normally contain two branchpoints, and outer A-chains, which are unbranched (Figure 1). Chemical analysis of mammalian glycogen suggests that the average chain length is ~13 residues [23,24]. In this model, glycogen would consist of a series of tiers. An important feature is that the outermost tier of any molecule completely formed in this way would contain 50% of the total glucose residues of the molecule as unbranched A-chains (Table 1). Note, however, that only a fraction of these outer-chain glucose residues are accessible to the degradative enzyme glycogen phosphorylase, which stalls four residues from a branch without the intervention of the debranching enzyme [AGL (amylo-$\alpha$-1,6-glucosidase, 4-$\alpha$-glucanotransferase)]. It is sometimes noted that branchpoints in glycogen occur every ~12 residues, in apparent contradiction of the idea of B-chains containing two branches per 13 residues. However, if half of the glycogen molecule is composed of unbranched outer A-chains, then the 1 in 12 average number is consistent. It has been calculated that addition of a 13th tier to a glycogen molecule would add an impossible density of glucose residues, making 12 tiers a theoretical maximum [25]. Therefore a full-size glycogen molecule in this model would consist of 12 tiers, for a total of ~55000 glucose residues, a molecular mass of ~$10^7$ kDa and a diameter of ~44 nm. In fact, careful analysis of the sizes of glycogen particles present in skeletal muscle by electron microscopy has indicated that few full-size glycogen particles exist and the average diameter is closer to 25 nm or seven tiers [15]. The model described above (Figure 1B) of necessity is

highly stylized, and the actual lengths of the chains, especially the outer chains, would have a major impact on the overall structure and average branching statistics of the population. Indeed, the stochastic nature of glycogen synthesis and the randomness of its metabolism could well mean that individual glycogen molecules deviate significantly from these theoretical formulations, and could be a lot less symmetrical than in the model, with not all tiers being intact for example. Some more detailed structural information has been obtained with simpler oligosaccharides. Polymeric glucose forms helical structures [25]. In amylopectin, parallel helices are thought to form semi-crystalline regions, excluding water and making the polysaccharide quite insoluble. In line with this idea, a crystal structure has been obtained for a 26-residue cyclodextran [26]. In this constrained structure, two antiparallel 13-residue helices are formed, and each is stabilized by a network of intrahelical hydrogen bonds; it is likely that some features of these structures are relevant to those of both natural amylopectin and glycogen.

Individual glycogen molecules are too small to be detected by normal light microscopy. Histochemical staining for glycogen in cells or tissues can therefore only reveal conglomerates of glycogen particles. However, glycogen molecules are large enough to be detected by electron microscopy and have been described as rosette-like $\beta$-particles in muscle and larger $\alpha$-particles in liver [27]. The $\beta$-particles would appear to correspond to the type of structures just described. The $\alpha$-particles seen in liver appear to be formed of aggregates of $\beta$-particles, although the chemical basis for the aggregation is not well understood [28,29]. Analysis of gently purified liver glycogen by size-exclusion chromatography techniques suggests a covalent linkage [29]. This conclusion is also supported by stochastic modelling of the assembly of such large glycogen structures [30]. One possibility would be the presence of infrequent extended polyglucose chains, as is seen in amylopectin [2,3]. However, this is an area that clearly deserves further exploration.

Although predominantly composed of glucose residues, glycogen contains other trace constituents, notably glucosamine [31,32] and phosphate. The most studied of these is covalent phosphate. For many years, phosphate in glycogen was thought to be no more than a minor contaminant and the amount of phosphate in purified glycogen was thought to correlate with purity. Not until the early 1980s was the first convincing report that this ‘minor contaminant’ was an integral part of the polymer [33]. Whelan and colleagues also documented the presence of phosphate in glycogen and postulated that it existed as a $C_6$-monoester, thereby blocking a potential branchpoint, and as a $C_1$–$C_6$ bridging phosphodiester, representing an alternative branchpoint [34,35]. Recent analyses indicate a frequency of glycogen phosphorylation of one phosphate per ~650 glucoses in rabbit skeletal muscle and one per ~1500 residues in mouse muscle glycogen [36,37]. From analysis of phospho-oligosaccharides purified from digested rabbit muscle glycogen, the phosphate has been determined by MS and NMR to exist as $C_2$- and $C_3$-phosphomonoesters, probably introduced as side reactions during the action of the normal synthetic enzyme, glycogen synthase [38] (see below). No evidence was found for the presence of $C_6$-phosphoesters. Excessive phosphorylation of glycogen is associated with a form of epilepsy, called Lafora disease [39–41] (see below). A glycogen molecule undergoes multiple cycles of expansion and contraction. Thus chemical and metabolic insults throughout its lifetime may accumulate and result in an aberrant structure that sometimes escapes normal

metabolism and degenerates into an insoluble deposit, much as other insoluble cellular structures form in various neurological disorders. Whelan and colleagues had proposed exactly this scenario, that the chemical modifications in glycogen might mark the age of the molecule and target the need for its disposal [34]. In fact, in normal aging tissues, even in the absence of overt disease, glycogen-like deposits distinct from normal glycogen have been described in brain, as corpora amylacea [42], and in heart as basophilic degeneration or cardiac colloid [43].

Glycogen forms higher-order assemblages with associated proteins [9,15,18,44,45]. Fischer and colleagues were the first to partially purify from muscle what they termed ‘glycogen particles’ which contained glycogen, several proteins and elements of sarcoplasmic reticulum [46–48]. The particles result from the ability of associating proteins to bind to glycogen, sometimes also to each other and to membranes (Figure 2). Known glycogen-associated proteins are the initiator glycogenin, the metabolic enzymes glycogen synthase, glycogen phosphorylase and the debranching enzyme AGL, and several regulatory proteins including phosphorylase kinase and members of the PP (protein phosphatase) 1G family. In addition, the $\beta$-subunit of AMPK (AMP-activated protein kinase) has a CBM20 (carbohydrate-binding module 20) domain [49,50] and has been shown to bind glycogen [51,52]. More recently identified glycogen-associated proteins are laforin [53,54] and Stbd1 (starch-binding domain protein 1)/genethonin 1 [55]. Laforin and Stbd1 also bind to glycogen via a CBM20 domain. A recent proteomics study of gently purified liver glycogen [45] identified most of the above proteins, except phosphorylase kinase and AMPK, as well as some other surprising ones, including ferritin light and heavy chains. Such novel candidates for glycogen binding, of course, require further validation, but virtually all *bona fide* glycogen-associating proteins, until now, have been shown to have a functional role in glycogen metabolism. Notably absent from the list is the branching enzyme, which can obviously interact with glycogen, but which does not appear to form a stable association [56]. The glycogen particle is unlikely to be a complex with a rigorous stoichiometry as is observed, for example, in ribosomes or the pyruvate dehydrogenase complex. Furthermore, there is a strong likelihood of compositional differences between and even within cell types, for example, skeletal muscle where glycogen particles are found in different subcellular locations [57]. Neither can we exclude alterations in composition controlled by physiological conditions.

High-molecular-mass glycogen is insoluble in ethanol, a property frequently used for its purification. In contrast, treatment of cell extracts with TCA (trichloroacetic acid) precipitates protein, but leaves high-molecular-mass glycogen in solution. A portion of the glycogen, however, is precipitated by TCA and has been termed ‘proglycogen’. Lomako et al. [58–60] suggested that proglycogen was a distinct molecular and metabolic entity, possibly with its own metabolic pathways. The matter has been somewhat controversial [10,61–63], and no distinct proglycogen synthase has been identified at the molecular level. A strong argument against the existence of a genetically separate proglycogen synthase comes from the fact that a mouse with the muscle glycogen synthase gene disrupted has undetectable muscle glycogen levels [64]. Also, evidence from a cell model [61] and from analyses of muscles [57] suggested instead a continuum of glycogen species of different sizes with no discrete lower-molecular-mass form. This is not to say that smaller

(proglycogen) particles might not behave differently than larger molecules and some studies make this argument [65]. Unquestioned is the operational definition of proglycogen, reflecting the fact that smaller glycogen particles contain a sufficient proportion of protein to make them TCA-insoluble.

## OVERVIEW OF GLYCOGEN METABOLIC PATHWAYS

In mammals, the major deposits of glucose are in skeletal muscle and liver, although many other tissues are capable of glycogen synthesis, including kidney, heart, fat and brain. The precursors for glycogen synthesis are either glucose, derived from newly ingested carbohydrate, or gluconeogenic precursors, such as lactate or alanine, a process sometimes termed ‘glyconeogenesis’ or the ‘indirect pathway’ [66]. The latter pathway is carefully reviewed by Agius [17]. The direct pathway requires transport of glucose into cells, by one or more of several GLUTs (glucose transporters) [67]: GLUT1 is widely distributed and provides basal glucose transport; GLUT4 is up-regulated by insulin and is important in insulin-sensitive tissues such as skeletal muscle and adipose tissue; and GLUT2 is prominent in liver and the $\beta$-cells of the pancreas and admits glucose based on a positive glucose gradient between the blood and the tissue.

The immediate glucose donor for glycogen synthesis is the activated NDP-sugar UDP-glucose, which is produced by UDP-glucose pyrophosphorylase at the expense of converting UTP into UDP [12] (Figure 3). There is a specialized initiation step whereby glycogenin [68–71] self-glucosylates to form an oligosaccharide primer chain. Via its extreme C-terminus, glycogenin can interact directly with glycogen synthase which is responsible for the formation of the large majority of the $\alpha$-1,4-glycosidic linkages of glycogen, also utilizing UDP-glucose as the glucosyl donor. The $\alpha$-1,6-glycosidic branchpoints are formed by the action of the branching enzyme. Yagi et al. [72] described a Nudix hydrolyase that they proposed was relatively specific for UDP-glucose. This UGPPase (UDP-glucose pyrophosphatase), encoded by the *NUDT14* gene, is widely distributed in mammalian tissues and has the potential to modulate glycogen synthesis by setting the level of the glucosyl donor UDP-glucose, even if this would be energetically costly [73]. Whether UGPPase has a physiological role in glycogen metabolism remains an open question. Glycogen is degraded by two distinct pathways. In the first, retrieval of glucose, in muscle to fuel contraction or in liver to provide glucose for export to the bloodstream, is mediated by the actions of glycogen phosphorylase and the debranching enzyme AGL. The immediate products of glycogenolysis are glucose 1-phosphate from $\alpha$-1,4-linkages and free glucose from $\alpha$-1,6-linkages. A primary stimulus for hepatic glycogenolysis is nutritional deprivation, with corresponding elevation of counterregulatory hormones such as glucagon [11]. Breakdown of muscle glycogen accompanies exercise, under conditions of increased cAMP and $Ca^{2+}$ [11]. In the second pathway for degradation, glycogen is transferred to the lysosome and hydrolysed to glucose by the lysosomal $\alpha$-glucosidase [also known as GAA (acid $\alpha$-glucosidase), acid maltase] [74].

# GLYCOGEN SYNTHESIS

## Glycogenin

Glycogenin is a self-glucosylating protein that transfers glucose from UDP-glucose first to a tyrosine residue within the protein itself and then forms $\alpha$-1,4-glycosidic linkages until the oligosaccharide chain is extended to a length of 10–20 residues [75–78]. It is a member of the family 8 retaining glycosyltransferases [79,80]. Humans have two genes that encode glycogenin: *GYG1*, which is more widely expressed, and *GYG2*, whose expression is restricted to liver, heart and pancreas [81]. Rodents, on the other hand, have a single glycogenin gene. There is a report [82] of a patient with mutations in *GYG1*, a nonsense mutation in one allele and a missense mutation, T83M, in the other allele, leading to inactive glycogenin-1. The patient had muscle weakness and had presented with cardiac abnormalities following a bout of exercise. The patient's skeletal muscle completely lacked glycogen, but abnormal PAS (periodic acid–Schiff) staining structures, indicative of polysaccharide, were seen in cardiomyocytes, possibly resulting from expression of the *GYG2* gene.

Structurally, glycogenin is a member of the A-type glycosyltransferase family which is characterized by a single Rossmann-fold domain with additional elaborations of secondary structure that control both donor nucleotide sugar specificity and acceptor specificity [83] (Figure 4A). UDP-glucose is bound in a metal-dependent fashion at the C-terminal ends of the central $\beta$-sheet structure (Figure 4B). The metal ion, most likely $Mn^{2+}$, is co-ordinated by two aspartate residues in the canonical DXD motif, a histidine residue from the protein and two phosphate oxygen atoms contributed by the pyrophosphate group of UDP. The primary function of the metal ion is to stabilize the UDP leaving group during glucosyl transfer. Glycogenin is an example of a retaining glycosyltransferase, in that the $\alpha$-configuration of the $C_1$-anomeric carbon is retained in the product. The chemical mechanism of glucosyl transfer in retaining glycosyltransferases generates considerable debate. The simplest explanation for retention of stereochemistry is through the use of a $S_N2$ double-displacement mechanism and an enzyme-bound intermediate with inverted stereochemistry. However, the identification of an enzyme active-site nucleophile with sufficient catalytic impact has proved elusive, although $Asp^{159}$ and $Asp^{162}$ seem to play important roles in glycogenin [84]. More recently, there has been some speculation that this class of enzyme achieves retention of configuration by means of an $S_Ni$-type mechanism where the nucleotide diphosphate serves as the general base to activate the acceptor hydroxy group [85]. The acceptor for glucose transfer by glycogenin is itself, either $Tyr^{195}$ in the initial phase of the reaction or the terminal glucose residue of the growing chain subsequently [84,86,87].

The different chemistries of these reactions dictate that there are two phases to the overall process, for two reasons. First, activation of the initial tyrosine hydroxy group and the subsequent 4′-hydroxy groups of glucose as nucleophiles are not energetically equivalent. Secondly, $Tyr^{195}$ in the unglucosylated protein is too far away from an active site to permit an intramolecular reaction. The simplest explanation of the available data is that the initial transfer of glucose from UDP-glucose to $Tyr^{195}$ occurs through an intermolecular reaction

that switches to an intramolecular reaction as the chain length increases [87]. The initial stages of the glycosyl-transfer reaction appear to follow a processive reaction mechanism where the mono-, di-, tri- and penta-glucosylated species do not accumulate to any significant extent [87]. However, there are slow steps associated with the generation of the tetra- and hexa-glucosylated forms [87]. Subsequent products are more distributive in nature, with all species between seven and sixteen forming a normal distribution. In this manner, glycogenin generates the primer for bulk glycogen synthesis by glycogen synthase.

The association between glycogenin and glycogen synthase is mediated by two mechanisms: (i) association of the glucosyl-primer chain with the active site of glycogen synthase during catalysis; and (ii) protein-mediated association, minimally through a conserved amino acid sequence in the C-terminal domain of glycogenin [88]. The C-terminal 33 amino acids of glycogenin are sufficient for purification of glycogen synthase from tissue extracts and this interaction may be mediated by the relatively conserved sequence motif ($WEX_{2–4}DYL/M$). However, the data suggest that other sites of association may also exist [88].

## Glycogen synthase

In eukaryotes, glycogen synthase is responsible for the bulk synthesis of glycogen by formation of the $\alpha$-1,4-glycosidic linkages with UDP-glucose as the glucosyl donor. Note that glycogen synthesis in bacteria and starch synthesis in plants utilize ADP-glucose [1]. Eukaryotic glycogen synthase is allosterically activated by glucose 6-phosphate and negatively regulated by covalent phosphorylation. The presence of glucose 6-phosphate overcomes inactivation due to phosphorylation and can restore full activity. This property led to the use of assays of the enzyme in the presence or absence of glucose 6-phosphate to give the ratio of activity with and without glucose 6-phosphate as a surrogate measure of phosphorylation state (see [89]) even though not all phosphorylation sites affect activity. Various modifications of the assay have been used to increase sensitivity to changes in phosphorylation of inactivating sites, such as measuring at low and high glucose 6-phosphate concentrations or altering the UDP-glucose concentration (see, for example, [90]). In the literature, reference to the activity or activation state of glycogen synthase is normally to some version of this assay and, when the enzyme source is a cell or tissue extract, it is taken as an indicator of phosphorylation *in vivo*. It must be recalled that, in the cell, the momentary activity of a glycogen synthase molecule is minimally a function of the concentrations of UDP-glucose, glucose 6-phosphate and glycogen, and its phosphorylation state, of which only phosphorylation is reflected in these standard assays. Measured activity and inferred *in vivo* activity need to be distinguished, although we are all sometimes careless in our usage.

Mammals have two genes that encode glycogen synthase: *GYS1*, which is expressed in skeletal muscle and most other cells capable of glycogen synthesis, and *GYS2*, which appears to be restricted to liver [91,92]. The yeast *Saccharomyces cerevisiae* also has two genes for this enzyme, *GSY1* and *GSY2*, with Gsy2p normally the predominant isoform [93]. Mammalian glycogen synthase was one of the first examples of a multiply phosphorylated enzyme [94] and efforts to identify the responsible protein kinases by several groups in the late 1970s and 1980s contributed significantly to protein kinase

discovery in the days before cDNA cloning. A number of protein kinases were linked to the *in vitro* phosphorylation of subsets of the nine sites of muscle glycogen synthase (Figure 5 and Table 2). The sites are located at the N- and C-termini of the protein. From Ser→Ala mutagenesis, four of the sites, 2, 2a, 3a and 3b, were identified as the most important in determining enzyme activity of rabbit muscle enzyme [95,96]. Similar analysis of liver glycogen synthase, however, suggested a dominant role for phosphorylation of site 2 [97]. The yeast glycogen synthases lack the N-terminal phosphorylation and have three C-terminal sites: $Ser^{650}$ and $Ser^{654}$, that resemble mammalian sites 3a and 3b, and $Thr^{667}$ which is unique to yeast [98]. Phosphorylation of $Thr^{667}$ by the cyclin-dependent kinase Pho85p appears to dominate inactivation ([99], and W.A. Wilson and P.J. Roach, unpublished work). Work on glycogen synthase phosphorylation also led to the concept of hierarchal phosphorylation [100,101], whereby the introduction of one phosphate enables the addition of a second. The prototype for this mechanism was the requirement of prior phosphorylation of glycogen synthase by protein kinase CK2 in order that GSK3 (glycogen synthase kinase 3) could add four successive phosphates per subunit [102] (Figure 5). The molecular basis for the phenomenon is that GSK3 recognizes the sequence -SXXXpS- in glycogen synthase [103], a conclusion that was supported by solution of the three-dimensional structure of GSK3 [104,105]. There are two isoforms, GSK3$\alpha$ and GSK3$\beta$ [106]. In relation to glycogen metabolism, GSK3$\alpha$ appears to be more important in liver [107] and GSK3$\beta$ appears to be more important in muscle [108]. Both GSK3 isoforms are inactivated by phosphorylation of an N-terminal regulatory site by Akt/PKB (protein kinase B) [109]. A second example of hierarchal phosphorylation was provided by the observation that protein kinase CK1 (casein kinase 1) preferred sites with the motif -pSXXS- in glycogen synthase [110]. A second covalent modification of glycogen synthase has been proposed, namely O-linked attachment of *N*-acetylglucosamine, with the suggestion that this modified enzyme is less sensitive to activation by phosphatases [111]. This idea is still somewhat controversial. More recently, Zhao et al. [112] reported on a proteomic analysis of the acetylation of lysine residues in human liver proteins. Included among the acetylated proteins identified was glycogen synthase, along with glycogen phosphorylase and UDP-glucose pyrophosphorylase. Two modified lysine residues of glycogen synthase, $Lys^{387}$ and $Lys^{397}$, are conserved in metazoans, but not in yeast, and are located in the long helix that forms the tetramer interface (see below). Other acetylations are at $Lys^{694}$, $Lys^{695}$ and $Lys^{696}$ close to the C-terminus in a region of lower conservation. The ramifications of this novel finding for the physiological control of glycogen metabolism await further investigation.

Dephosphorylation of glycogen synthase, and other glycogen-metabolizing enzymes, is thought to be mediated by members of a family of glycogen-associated PP1Gs composed of a catalytic subunit (PP1c) bound to a glycogen-targeting subunit [113,114]. To date, seven such glycogen-targeting subunits have been identified by a combination of biochemical and bioinformatic analyses. Three of these targeting subunits have been more extensively studied. The $R_{GL}$ or $G_M$ subunit, product of the *PPP1R3A* gene, is restricted to skeletal and heart muscle [115]. The $G_L$ subunit, coded for by the *PPP1R3B* gene, is primarily expressed in the liver, but is also found in human muscle [116,117]. Its expression in liver is induced by insulin [118]. PTG (protein targeting to glycogen) (or R5), encoded by the *PPP1R3C* gene, is more ubiquitously expressed, and is found in several insulin-sensitive tissues,

including skeletal muscle, liver and fat [119]. PTG was proposed to act as a scaffold, interacting directly with glycogen synthase, glycogen phosphorylase and phosphorylase kinase [120], although other work questions this assessment [121]. Less is known of other family members. PPP1R3D (R6) is widely expressed, with high levels in heart and muscle, and lower levels in liver [122]. PPP1R3E is expressed predominantly in human heart and muscle, but in rat heart and liver [123]. Its hepatic expression was reduced in diabetic rats and the phosphatase activity associated with PPP1R3E was restored by administration of insulin [123]. Munro et al. [123] detected *PPP1R3G* mRNA only in brain, but a recent report described a role for PPP1R3G in liver glycogen metabolism, being paradoxically induced by fasting [124].

## Glycogen synthase structure

Like glycogenin, eukaryotic glycogen synthases are retaining glycosyltransferases that utilize UDP-glucose as the nucleotide-sugar donor. However, unlike glycogenin, they are not metal-ion-dependent and possess the other common glycosyltransferase core structure, the GT-B fold [125]. The GT-B fold is characterized by the presence of two Rossmann-fold domains with an interdomain cleft that harbours the active site. It is an interesting evolutionary observation that glycogen phosphorylase, which breaks down glycogen to glucose 1-phosphate, is also a member of the GT-B fold family, suggesting a divergent structural relationship for these enzymes that catalyse opposing reactions [126]. The overall GT-B fold is conserved between bacterial, archaeal and eukaryotic glycogen synthases [125–128]. However, the eukaryotic enzymes differ in oligomeric state, specificity for nucleotide-sugar donor, activation by glucose 6-phosphate and inactivation by phosphorylation.

Each of the distinct properties of the eukaryotic enzymes is associated structurally with sequence insertions or deletions relative to the bacterial and archaeal enzymes (Figure 6). A large insertion of approximately 100 amino acids is present in the C-terminal Rossmann-fold domain and forms the subunit interaction surfaces for the tetrameric eukaryotic glycogen synthases [125]. In addition to this large sequence insertion, a smaller insertion (residues 481–492 in Gsy2p) forms a loop structure that provides the selectivity for UDP-glucose, rather than ADP-glucose, in eukaryotic forms. Regulation by phosphorylation is mediated through N- and/or C-terminal sequence extensions (Figure 5).

Surprisingly, the acquisition of glucose 6-phosphate regulation is not related to addition of structural elements as the binding site is composed of conserved secondary structure. Nor is it related to the glucose 6-phosphate-binding site in glycogen phosphorylase [129], where it is located in the N- rather than C-terminal Rossmann domain, although the conceptual framework for regulation by binding at a subunit interface and the involvement of multiple arginine residues anchoring the phosphate moiety is retained. The glycogen synthase glucose 6-phosphate-binding site is created by a deletion, relative to bacterial and archaeal enzymes, within the loop connecting the N- and C-terminal domains (residues 225–235 in the *Agrobacterium* enzyme, Figure 6) and residue exchanges within the conserved elements of secondary structure. However, the ability to communicate this binding event between subunits requires the tetrameric interface that is generated by the large C-terminal sequence insertion mentioned above. The binding of glucose 6-phosphate within this interface triggers

a large subunit rotation and translocation at the subunit interface, such that the active-site clefts in the individual subunits are now open and accessible for glycogen binding [125] (Figures 7A and 7B). The driving force for this extensive conformational change is provided by relatively few contacts mediated by the glucose moiety of the allosteric activator (Figure 7C). Glucose 6-phosphate is anchored into its binding site through five hydrogen-bonding interactions mediated by the phosphate moiety (Figure 7C), hydrophobic contacts with the faces of the glucose and a hydrogen bond to the 1′-hydroxy group. In contrast, a single hydrogen bond between the 2′-hydroxy group and $His^{280}$ in the opposing subunit, as well as an interaction between adjacent $Asn^{284}$ side chains, are the only strong contacts across the subunit interface that appear to stabilize the activated state (Figure 8).

A three-state model for Gsy2p activation was developed mainly on the basis of kinetic data [130]. Dephosphorylated enzyme in the absence of glucose 6-phosphate was in an intermediate basal-activity state. Phosphorylation decreased the activity, whereas addition of glucose 6-phosphate to either phosphorylated or unphosphorylated enzyme generated a high-activity state. Although structural information is available for the basal and activated states of yeast glycogen synthase, as noted above, information about the inhibited state is mostly through kinetic inference using the two available structural models as a basis for extrapolation (Figure 8). Phosphorylation occurs at serine/threonine sites outside the catalytic core (residues 2–630 of Gsy2p) of glycogen synthase to inactivate the enzyme (Figure 5). It is an interesting structural observation that the key regulatory arginine residues between residues 580 and 592 in Gsy2p are grouped on opposing sides of the same $\alpha$-helix. $Arg^{580}$, $Arg^{583}$ and $Arg^{587}$ are adjacent to the glucose 6-phosphate-binding site and form interactions with the allosteric activator, whereas $Arg^{581}$, $Arg^{589}$ and $Arg^{592}$ lie on the opposing face of the helix and are oriented away from the protein surface [125]. Upon activation, the helices in which these arginine residues reside are pushed apart relative to their positions in the basal state conformation. By inference, we propose that the inhibited conformation positions these same helices closer together, creating a greater conformational tension to overcome in order to achieve an activated state. Evidence for this more closed state comes from two observations. First, mutation of the two arginine residues directed towards the helical interface ($Arg^{589}$ and $Arg^{592}$) to alanine renders the enzyme in a nearly inactive state (activity ratio of 0.11 [125]). Secondly, an alternative conformational state was observed for our basal-activity structure in which a sulfate, which could be mimicking a phosphate, is positioned between these regulatory helices and, when bound in this position, the helices are 3 Å (1 Å = 0.1 nm) closer than in the other basal-state conformation. Both observations suggest that charge neutralization of the arginine residues not involved in glucose 6-phosphate binding leads to a collapse of the regulatory helices toward each other at this crucial subunit interface, pushing the structure toward a more inhibited state. Like the charge neutralization due to sulfate binding or mutation, binding of a phosphorylated residue from the C-terminal (Gsy2p) or either the N-terminal or the C-terminal (higher eukaryotes) regulatory sequences to these same arginine residues would be expected to promote a similar collapse to the inhibited conformation. Because the conformational changes brought about by glucose 6-phosphate activation only affect the distance between the C-termini and regulatory helices in the opposing subunits, we favour a mechanism whereby the

phosphorylated residues are contributed across this interface from the opposite subunit and form a ‘locking strap’ to constrain the enzyme to its inhibited state [125].

An interesting property of glycogen synthase is that it remains associated with glycogen even when inactivated, such that both the dephosphorylated and phosphorylated forms of glycogen synthase can be recovered by simple precipitation of glycogen. This tight association with its substrate has led to speculation that the enzyme must possess some type of carbohydrate-binding module, such as those found in glycogen-binding subunits of PP1, laforin or the branching enzyme. However, simple sequence searches have failed to find such a distinct functional domain. When the first glycogen synthase structures were solved, the realization that it had the same protein fold as glycogen phosphorylase led to a thought that, like phosphorylase [131], perhaps glycogen synthase has its carbohydrate-binding sites integrated into its catalytic domain. Indeed, this has now been shown in the *Escherichia coli*, *Pyrococcus abyssi* and yeast Gsy2p enzymes [128,132,133]. These studies have revealed that glycogen phosphorylase and many forms of glycogen synthase retain a glycogen-association site on the surface of their respective N-terminal Rossmann-fold domains that is completely independent of the acceptor binding site within the catalytic cleft (Figures 9A–9C).

Gsy2p from *S. cerevisiae* has four such glycogen-association sites (Figure 9D): one is located on the surface of the N-terminal domain (site-1), two are located on the surface of the C-terminal domain (site-2 and site-3) and one is located near the interdomain cleft (site-4) that leads to the active site [133]. Conservation of amino acids within these glycogen-association sites in higher eukaryotes suggests that the mammalian enzymes possess these same sites. Mutational data, as well as yeast strain complementation experiments, indicate that all sites contribute to efficient utilization of glycogen as a substrate. However, site-4 appears to be unique in its ability to significantly affect the capacity of Gsy2p to utilize smaller oligosaccharides, such as malto-octaose, as an alternative acceptor substrate [133]. These findings support the idea that glycogen synthase integrates its carbohydrate-binding surfaces into the catalytic domain of the enzyme and these sites contribute to the high catalytic efficiency of glycogen synthase towards glycogen as its substrate.

It is likely that eukaryotic site-1–site-3 keep the enzyme tightly coupled to its substrate whether the enzyme is active or not, whereas site-4 serves a role in the proper positioning of the acceptor end within the active-site cleft, which apparently has a relatively low affinity for the acceptor end. This strategy solves the problem of rapid binding and release of actively extending chains, not requiring complete dissociation from glycogen to reset the position of the acceptor within the active site. It also increases the local concentration of non-reducing ends at or near the active site such that the lower affinity of the acceptor site does not negatively affect the overall efficiency of catalysis.

Díaz et al. [132] recently suggested that binding at accessory sites on the enzyme surface contributes to the processivity of glycogen synthase for chain elongation. Although this may be true, processivity towards a heterogeneous substrate such as glycogen is difficult to assess, since there are many non-reducing ends within a single glycogen particle where

catalysis can be directed without dissociation from the particle itself. The currently available data, including those of Baskaran et al. [133], would support this definition of processivity. However, there are no unequivocal methodologies currently available to assess the more strict definition of processivity where glycogen synthase remains associated with a single acceptor chain for multiple rounds of catalysis. The new elongation assay utilizing malto-octaose as a substrate demonstrates a distributive mechanism for elongation of this substrate [133]. Thus processivity with respect to glycogen must be a function of the additional points of contact between the enzyme and this complex substrate.

## Catalytic mechanism and introduction of phosphate into glycogen by glycogen synthase

As mentioned for glycogenin, the chemical mechanism underlying catalysis in retaining glycosyltransferases remains an enigma. This is especially true for glycogen synthases. Unlike metal-dependent enzymes such as glycogenin, where a metal ion is the primary means through which the UDP leaving group is stabilized, glycogen synthases are metal-ion-independent and rely on hydrogen bonds to amino acid side chains and main-chain atoms to provide charge stabilization of the UDP product (Figure 10). The available nucleotide-bound structures suggest that these roles are filled by conserved arginine and lysine residues (320 and 326 respectively in Gsy2p) and the helical dipole created by the N-terminus of the helix (residues 513–521 in Gsy2p) immediately adjacent to the conserved glutamate residue ($Glu^{509}$) that is strongly implicated in the glycosyl-transfer mechanism [128,134]. A number of catalytic roles have been proposed for this residue, including charge polarization of $Lys^{326}$ for UDP stabilization, charge stabilization of the oxonium ion intermediate in an $S_N1/S_Ni$ mechanism, proper positioning of the glucosyl-donor sugar and as the catalytic nucleophile in an $S_N2$ mechanism [126–128,134]. Whatever the precise role of this residue in the catalytic chemistry, it is clear that its presence in the active site contributes at least $10^4$ towards the catalytic power of the enzyme and thus must be considered a nearly essential catalytic contributor.

Independent of the precise catalytic roles that active-site amino acids play in catalysis, the stereochemistry of the reaction dictates that the acceptor substrate is directed towards the same face of the glucosyl moiety from which the UDP leaving group departed. Thus the ability of the C-1 carbon atom to release the leaving group and undergo nucleophilic attack is an essential component of the reaction mechanism. However, there are other atoms of UDP-glucose that can undergo nucleophilic attack, including the phosphorus atoms in the diphosphate moiety. It has been proposed that the trace levels of phosphate found in glycogen arise from the inherent reactivity of the $\beta$-phosphate of UDP-glucose, leading to the incorporation of glucose-phosphate esters into glycogen [38].

Whelan's group first reported an enzymatic activity from rabbit skeletal muscle that was capable of transferring the $\beta$-phosphate of UDP-glucose to glycogen [35]. However, the enzyme was never characterized at the molecular level. To assay and purify the proposed glycogen:glucose-1-phosphate transferase, Tagliabracci et al. [38] synthesized [$\beta$-$^{32}$P]UDP-glucose and demonstrated transfer of the $\beta$-phosphate from UDP-glucose to glycogen by a mouse muscle extract, but unexpectedly discovered that a muscle extract from a $Gsy1^{-/-}$ mouse {MGSKO (muscle-specific glycogen synthase-knockout) mouse [64]}, that lacks

glycogen synthase, did not catalyse this reaction. Purified glycogen synthases could also phosphorylate glycogen *in vitro*, with one phosphate introduced for every ~10000 glucose residues. This reaction might be viewed as an enzymatic error, not unlike those that occur during DNA and RNA biosynthesis [38]. The chemical mechanism for transfer of phosphate from UDP-glucose to the C-2 and C-3 hydroxy groups in glycogen awaits experimental confirmation, but it was speculated that it involves the formation from UDP-glucose of cyclic glucose phosphates (Figure 11), first described over 50 years ago by Paladini and Leloir [135]. Rarely, within the active site of glycogen synthase, the 2′ or 3′ hydroxy groups of UDP-glucose would spontaneously form glucose 1,2-cyclic phosphate or glucose 1,3-cyclic phosphate through nucleophilic attack of the respective hydroxy groups on the *β*-phosphate of UDP-glucose. At this point, the standard mechanism of glycogen synthase would operate, with attack by the activated C-4′ hydroxy group of the terminal glucose at the C-1 position of the cyclic phosphate, opening the ring to form a phosphoglucose that is added to the growing chain.

## Naturally occurring mutations of glycogen synthase and glycogen storage disease 0

Naturally occurring mutations in both *GYS1* and *GYS2* have been detected in humans. Mutation of *GYS2*, which causes hepatic glycogen deficiency, is better documented, and was named glycogen storage disease 0 (OMIM ID #240600). Patients have relatively mild symptoms, postprandial hyperglycaemia and fasting hypoglycaemia, consistent with the observed deficit in liver glycogen stores [136]. Hepatic glycogen synthase activity is reduced, consistent with the analysis of a number of the naturally occurring *GSY2* mutations by expression in COS cells [137]. A mouse model, LGSKO (liver-specific glycogen synthase-knockout) mice, in which the *Gsy2* gene is disrupted, reproduces much of the phenotype of the human disease [138]. The animals have mild fasting hypoglycaemia, but dispose glucose less well in a glucose-tolerance test. Fed animals have lower capacity for exhaustive exercise than their wild-type littermates, but the difference is lost after fasting. The mice have elevated basal gluconeogenesis and are predisposed to transition to the fasted state. Liver glycogen is therefore significant for normal blood glucose homoeostasis.

Loss-of-function mutations in muscle glycogen synthase have been reported in two families [139,140]. The disease has been designated muscle glycogen storage disease 0. In the first study [139], three siblings were identified with homozygous R462X mutations in *GYS1*. One sibling died of cardiac arrest at age 10.5 years and a second exhibited poor exercise performance and cardiac abnormalities at 11 years of age. Glycogen synthase and glycogen were absent from muscle. In the second case [140], an 8-year-old patient collapsed and died during exercise and was found to have a homozygous two-base deletion in exon 2 of *GYS1*. Many of the symptoms are consistent with cardiac problems observed with MGSKO mice in which *Gys1* is disrupted [64,141]. In crosses of $Gys1^{-/+}$ mice, 90% of the homozygous progeny died perinatally and had abnormal heart development. The surviving MGSKO mice had normal exercise capacity [142], normal lifespans with no overt cardiac symptoms and, paradoxically, enhanced glucose disposal in glucose-tolerance tests. It is likely that substantial developmental and metabolic differences between mice and humans underlie the observed discrepancies in phenotype between species. For example, in humans, it is possible that the perinatal mortality seen in mice can be delayed into early childhood. In any event,

this work underscores the possibility that *GYS1* mutation may be an under-recognized cause for unexplained cardiac problems and deaths in newborns and young children.

In horses, PSSM (polysaccharide storage myopathy) has been recognized for many years as a debilitating glycogen storage disease prevalent in several genetically diverse breeds [143]. Breeding for desired traits in horses has also led to the concomitant accumulation of undesired genetic characteristics. PSSM is associated with excessive glycogen accumulation in muscle; symptoms vary widely, but can be very severe, leading to the inability of the animal to rise from a lying position. Recently, a mutation responsible for PSSM in multiple horse breeds was identified as an R309H-coding mutation in *GYS1* and was associated with increased muscle glycogen synthase activity in the absence of the allosteric activator glucose 6-phosphate, to generate a constitutively activated form of the enzyme. From inspection of the Gsy2p structure, this mutation is quite distant from the glucose 6-phosphate-binding site, but does occur at the interface between the catalytic domain and the long $\alpha$-helices involved in subunit– subunit interactions. The mutation may therefore indirectly favour transition to the activated state, but more specific experiments would be needed to confirm this idea.

## REGULATION OF MUSCLE GLYCOGEN SYNTHESIS

### Rate-determining steps

Glycogen synthesis normally occurs after a meal, when blood glucose and insulin levels are elevated, or after exercise to restore depleted glycogen reserves. In muscle, a major issue is whether the rate of glycogen synthesis is determined by the rate of glucose entry into the cell or the activity of intracellular enzyme(s). In the terminology of MCA (Metabolic Control Analysis) [144], which steps have greatest flux control? Relatively few proteins are involved in the glycogen synthetic pathway (Figure 3) and the main candidates to control muscle glycogen synthesis are GLUT4, hexokinase and glycogen synthase. The other enzymes, i.e. phosphoglucomutase, UDP-glucose pyrophosphorylase, glycogenin and branching enzyme, do not appear to be either strongly regulated or rate-determining under normal circumstances. For many years, it was thought that dephosphorylation and activation of glycogen synthase by insulin was the dominant factor, following from the seminal study of Villar-Palasi and Larner [145]. This fundamental observation has been reproduced many times in animal- and cell-based experiments, and there is a good consensus on this point. When specialized glucose transporters were identified, attention focused also on glucose transport, especially with the finding that insulin promoted GLUT4 translocation to the plasma membrane in muscle [67]. Several mouse models have been constructed in which different components of the glycogen biosynthetic pathway have been mutated. The resting muscle glycogen content of a fed mouse provides a key primary index of the ability to synthesize glycogen under physiological conditions of insulin action. One of the earliest challenges to the primacy of glycogen synthase control came from transgenic overexpression of GLUT1 or GLUT4 in skeletal muscle which caused significant overaccumulation of glycogen [146,147]. However, muscle-specific knockout of GLUT4 paradoxically also led to elevated muscle glycogen [148]. The phenotype is ascribed to a variety of secondary effects in this mouse, including activation of PP1Gs with concomitant activation of glycogen synthase, and increased hexokinase leading to elevated glucose 6-phosphate. The complexity

of the phenotype made it difficult to assess the contribution of glucose transport to glycogen storage. Mice heterozygous for GLUT4-knockout, however, did exhibit decreased muscle glycogen levels [149]. Other mouse models have addressed glycogen synthase. Transgenic overexpression of glycogen synthase with two key inactivating phosphorylation sites (2a and 3a) disabled (GSL mice) led to hyperaccumulation of glycogen in muscle [150]. More subtle genetic manipulation of glycogen synthase activity was achieved by overexpression [151] or knockout [152,153] of the gene encoding the PP1 glycogen-targeting subunit $R_{GL}$ so as to modify the phosphorylation state of glycogen synthase. Overexpression of $R_{GL}$ increased muscle glycogen approximately 3-fold, whereas the absence of $R_{GL}$ reduced muscle glycogen to 10% of wild-type. However, in these mice, the phosphorylation of both glycogen synthase and phosphorylase was modified. In humans, a common nonsense mutation in the *PPP1R3A* gene, which encodes $R_{GL}$, was identified in 1.4% of the white population in the U.K. [154]. The mutation resulted in a truncated $R_{GL}$ protein and a decrease in muscle glycogen accumulation. A corresponding mutation engineered in mice reduced muscle glycogen by 50% and caused mis-targeting of $R_{GL}$. In mice lacking the PTG PP1-targeting subunit, a ~30% reduction in muscle glycogen was observed [155]. An earlier study [156] had reported that homozygous disruption of PTG was lethal, but this result appears in contradiction to the more recent data. Other investigations sought to manipulate glycogen synthase phosphorylation via protein kinases. This approach is *a priori* complex, since multiple protein kinases can phosphorylate glycogen synthase and multiple sites can influence activity (Table 2). Muscle-specific disruption of the gene encoding GSK3$\beta$ resulted in a 2-fold increase in glycogen accumulation, with an increase in glycogen synthase activation by insulin, but no effect on glucose transport; the animals had improved glucose disposal and insulin-sensitivity [108]. Taken together, the preceding results with mouse models suggest that genetic modulation of either glucose transport or glycogen synthase activity is capable of affecting muscle glycogen stores.

Shulman and colleagues have addressed this question using a combination of *in vivo* NMR and MCA [157–159]. Their conclusion was that, within the formalism of MCA, flux control was concentrated at the stage of glucose transport. They argue that glycogen synthase activation via dephosphorylation serves not to increase the metabolic flux to glycogen, but rather to allow a higher glycogen synthetic rate without elevation of glucose 6-phosphate levels [160]. It was pointed out many years ago that there is limited solvent capacity in cells and that it is therefore necessary to minimize metabolite concentrations [161]. Another MCA analysis of insulin-stimulated glycogen synthesis in rat muscle indicated flux control distributed between transport and synthesis [162]. So, where are we in resolving this issue? The different experimental approaches have their advantages and drawbacks. Genetically modified mice have the power of any genetic system, but suffer from the possibility of adaptive responses to the gene modifications; a kind of biological Heisenberg uncertainty principle, if you will. The elegance of non-invasive analysis of glycogen metabolism by NMR has to be balanced with its limitations [163], which include sensitivity and inability to address spatial heterogeneity. The application of MCA in complex animal models is also based on necessarily simplistic assumptions. We would argue that regulation, defined as an evolved mechanism to modify activity, of both glucose transport and of glycogen synthase

can contribute to the rate of glycogen accumulation, possibly to different extents that may vary under different circumstances [164].

## Mechanism of glycogen synthase activation by insulin

More challenging has been the effort to determine which of the *in vitro* protein kinases and/or phosphatases mediate the activation of glycogen synthase by insulin (Figure 12). GSK3, whose action potently inactivates glycogen synthase *in vitro* by phosphorylation of four C-terminal sites [165], has long been considered an important candidate to regulate glycogen synthase. Insulin controls GSK3 activity by promoting phosphorylation of its N-terminal inhibitory phosphorylation site via the Akt/PKB pathway, by a well-established mechanism [166,167]. Insulin thus inactivates GSK3, leading to decreased phosphorylation at its target sites in glycogen synthase due to phosphatase action. Muscle-specific knockout of GSK3$\beta$ in mice increased glycogen levels and potentiated insulin-mediated activation of glycogen synthase [108]. Conversely, overexpression of GSK3$\beta$ in muscle resulted in reduced glycogen accumulation and reduced the glycogen synthase activation state [168]. Earlier studies had suggested that insulin caused dephosphorylation of both N- and C-terminal phosphorylation sites of glycogen synthase [169,170]. The argument was therefore made that insulin must promote dephosphorylation of non-GSK3 sites by other mechanisms (see, for example, [12]). Possibilities would include inactivation of a site 2 kinase, for which there are numerous candidates (Table 2), or activation of a phosphatase, probably of the PP1G family. Knockout of the gene encoding either $R_{GL}$ [152,153] or PTG [155] reduced basal glycogen synthase activity and glycogen accumulation, but did not disable insulin-mediated activation of glycogen synthase. If a phosphatase is involved, it must involve a different regulatory subunit. Another potential complication is redundancy of protein kinases for a given site. For example, in COS cells, sites 3a and/or 3b of glycogen synthase can be phosphorylated by a mechanism independent of GSK3 [95]. There are three candidates from *in vitro* experiments, a DYRK (dual-specificity tyrosine-phosphorylated and -regulated kinase) family kinase [171], p38$\beta$ [172] and PASK [PAS (Per/Arnt/Sim) domain-containing protein kinase] [173]. Some of the uncertainty may have been resolved by the study of McManus et al. [174] who made knockin mice in which either or both GSK3$\alpha$ and GSK3$\beta$ had their inhibitory N-terminal phosphorylation sites ($Ser^{21}$ and $Ser^{9}$ respectively) mutated to alanine. Surprisingly, muscle glycogen levels were unaffected in the double-knockin homozygous mice in which GSK3 is constitutively active. Basal glycogen synthase activity was also unchanged, as was phosphorylation of key GSK3 sites, $Ser^{641}$ and $Ser^{645}$, but insulin-stimulated activation of glycogen synthase was suppressed. The authors argue that the results prove not only that GSK3 (in particular GSK3$\beta$) is the primary means by which insulin activates muscle glycogen synthase, but also that no other protein kinases, phosphatases, or phosphorylation sites are involved unless they too are controlled by GSK3. Perhaps the latter conclusion is a little strong since insulin did activate glycogen synthase in GSK3$\beta$ muscle-specific knockout mice without compensatory changes in GSK3$\alpha$ protein levels or insulin-induced phosphorylation [108]. Therefore there may still be room for GSK3-independent controls of glycogen synthase activity by insulin, but the GSK3-knockin mouse models make a strong case for the importance of GSK3 in insulin signalling to glycogen synthase.

### Mechanism of glycogen synthase control by exercise

Exercise depletes glycogen which must subsequently be replenished, requiring activation of glycogen synthesis (Figure 12). Depending on the experimental conditions, both inactivation and activation of glycogen synthase have been observed in response to exercise [175,176]. Furthermore, activation has been observed both during exercise and after its cessation [175]. Activation of glycogen synthase could be considered paradoxical since this is, overall, a period of glycogen utilization. Several rationalizations can be advanced: (i) activation could 'prime' glycogen synthase for rapid glycogen synthesis once contractile activity lessens; (ii) other factors besides phosphorylation may prevail to avoid glycogen synthesis and 'futile cycling' of glycogen; and (iii) glycogen is actually synthesized and utilized during exercise [177]. Nielsen and Richter [175] have emphasized how various negative and positive signals elicited during exercise must be integrated to determine glycogen synthase activity. Still, the exact mechanism for how exercise controls glycogen synthase phosphorylation is not yet clear, although there is probably a consensus that it differs significantly from insulin-mediated regulation. First, exercise-induced activation of glycogen synthase occurs in muscle-specific insulin-receptor-knockout mice [178]. Secondly, exercise activation of glycogen synthase is normal in the GSK3-double-knockin mice described above [174]. Knockout of the PP1 glycogen-targeting subunit $R_{GL}$ in mice does block activation of glycogen synthase by exercise or *in situ* electrical stimulation, suggesting a role for the phosphatase [151]. How $R_{GL}$ is regulated is not known. An earlier model had proposed that PKA (protein kinase A), activated by adrenaline (epinephrine), phosphorylates $R_{GL}$ and causes its dissociation from the glycogen particle [179]. However, during exercise, the PKA pathway is likely to be activated, which would decrease phosphatase activity in this model.

The relationship between glycogen synthase phosphorylation and activity during muscular activity is complex and exemplifies the notion that inhibitory and activating signals can converge. It has been observed that exercise causes both phosphorylation of sites 2 and 2a, correlating with decreased activity, whereas, upon more prolonged activity, sites 3a and 3b are dephosphorylated, resulting in a net activation [176,180]. Dephosphorylation of sites 3a and 3b could result from the action of $R_{GL}$-PP1c, as described above. Site 2, *in vitro*, is a substrate for multiple protein kinases (Table 2), several of which could conceivably be activated under conditions of muscle contraction. Phosphorylase kinase and AMPK are known to associate with glycogen and have well-established mechanisms for activation during exercise. Phosphorylase kinase is activated by $Ca^{2+}$ and PKA. PKA is activated by cAMP, whose level is likely to be increased under conditions of glycogenolysis. Note also that phosphorylation of a C-terminal PKA site, site 1b, may have a function in the translocation of glycogen synthase (discussed below). Furthermore, CAMKII ($Ca^{2+}$ / calmodulin-dependent protein kinase II) is also activated by $Ca^{2+}$.

Attention has been paid recently to the possible role of AMPK as a site 2 kinase. AMPK, whose activity increases with depletion of the adenylate pool for ATP, is thought to be a monitor of cellular energy status [181] and is indeed activated by muscular activity [182]. AMPK phosphorylates glycogen synthase *in vitro* [183], and disruption of the gene encoding the $\alpha 2$-subunit, but not the $\alpha 1$-subunit, of AMPK resulted in decreased basal phosphorylation of glycogen synthase at site 2 + 2a by ~60% and increased its activation

state [184]. AICAR (5-amino-4-imidazolecarboxamide riboside), an oft used, although relatively non-specific, activator of AMPK, caused a modest increase in site 2 phosphorylation that was not seen in the $\alpha$2-knockout muscle [184]. The $\beta$-subunit of AMPK, as noted, contains a CBM20 domain that allows it to bind glycogen, certainly *in vitro* [51,52,185]. There is some controversy over the stability of the association and its effects on activity. Stapleton and colleagues attempted to isolate glycogen using relatively gentle methods and could not document co-purification of the $\beta$-subunit [45,186]. They had previously failed to show inhibition of AMPK activity by glycogen [52]. However, the study of McBride et al. [185] provides fairly compelling evidence for inhibition of AMPK activity *in vitro* by glycogen, although they note some variability between glycogen preparations. Their bovine liver glycogen inhibited half-maximally at 30 mM glucose equivalents. Smaller oligosaccharides also bound and some could inhibit depending on their structure. For example, linear oligosaccharides such as maltohexaose or maltoheptaose bound, but did not inhibit, AMPK, whereas synthetic oligosaccharides containing an $\alpha$-1,6-linkage were effective inhibitors in the 100–200 $\mu$M range. This observation led McBride et al. [185] to propose that AMPK bound to extended outer chains of glycogen would be active and able to phosphorylate and inactivate glycogen synthase, whereas glycogen with outer chains degraded by phosphorylase, would have more exposed $\alpha$-1,6-linkages that could inhibit AMPK, leading to activation of glycogen synthase. Of course, one weakness in this hypothesis is that, *in vivo* in the presence of debranching enzyme AGL, phosphorylase-limit outer chains would surely have a fleeting existence. Also, site 2 phosphorylation alone cannot fully inactivate glycogen synthase. The broader notion, that AMPK acts as a sensor of glycogen, is, however, appealing. It could help to explain the inverse correlation between glycogen synthase activation state and glycogen level that goes back to the early study of Danforth [187]. Particularly in muscle, there is a correlation between increased AMPK phosphorylation (and presumably activity) and decreases in glycogen level caused by exercise [182] or genetic manipulation [141]. In liver, matters may be less clear since depletion of glycogen in the LGSKO mice, if anything, decreased AMPK phosphorylation [138]. The concept of glycogen level and concentration is itself a particularly difficult one, as acknowledged by McBride and Hardie [188]. Even without invoking differences in affinity for particular oligosaccharide structures, the surface of a large glycogen particle is likely to be very different than that of a smaller one, presenting a different local concentration, if you will, of chains to inhibit or not inhibit AMPK. Future work will require clever experiments to test this intriguing hypothesis.

A rather different connection between AMPK and glycogen comes from study of Hampshire pigs [189] and *PRKAG2* (AMPK $\gamma^2$ non-catalytic subunit) mutations in humans [190] which cause massive overaccumulation of glycogen in muscle and heart respectively. In the pigs, excess glycogen has a deleterious effect on meat quality. In humans, it causes PRKAG2 cardiomyopathy. The mutations are in the $\gamma$3 and $\gamma$2 subunits of AMPK respectively, and, although there has been some debate, it appears that the mutations are effectively gain-of-function [190]. That such mutations cause increased glycogen storage cannot be easily reconciled with glycogen synthase phosphorylation at site 2. In addition, in some studies, AMPK activation by exercise correlates with activation of glycogen synthase [151,191]. It is possible that a different function of AMPK is involved, such as its role in autophagy, as

discussed below, or its stimulation of glucose transport in muscle. It remains to be established whether AMPK is really a predominant glycogen synthase site 2 kinase.

## Allosteric compared with covalent control of glycogen synthase

Since glycogen synthase is regulated by both phosphorylation and allosteric activation by glucose 6-phosphate, the relative importance of these mechanisms has been much discussed, especially for liver where glucose 6-phosphate is believed to play an important role in driving glycogen synthesis [7,14,17]. Pederson et al. [130] used an alanine-scanning mutagenesis approach to identify basic residues of yeast Gsy2p involved in activation by glucose 6-phosphate. Two mutant enzymes were identified that, *in vitro*, were unaffected by glucose 6-phosphate; one retained inactivation by phosphorylation and the other was unaffected by phosphorylation. The mutations were of arginine residues in the highly conserved arginine cluster (Figure 5) that is involved in binding glucose 6-phosphate and setting the activity state of the enzyme (Figures 7 and 8). Expression of the unregulated mutant Gsy2p in yeast resulted in hyperaccumulation of glycogen, whereas the mutant that retained phosphorylation control accumulated less glycogen than wild-type enzyme [192]. The study provided evidence for the importance of glucose 6-phosphate in normal glycogen synthesis *in vivo* and also affirmed the relevance of phosphorylation in the absence of glucose 6-phosphate control [192]. A more recent discussion of mammalian systems began with the work of McManus et al. [174], discussed above, on the double-knockin homozygous mice with constitutively active GSK3 in which glycogen levels were normal. Bouskila et al. [193] suggested that, in these double-knockin mice, glucose 6-phosphate control might be the key reason for the normal glycogen accumulation. Hanashiro and Roach [194] had transferred the glucose 6-phosphate-desensitizing mutations studied in yeast Gsy2p to mammalian glycogen synthase and Bouskila et al. [193] refined the analysis to identify R582A and R586A as point mutations that conferred insensitivity to glucose 6-phosphate activation, sensitivity to phosphorylation and the ability to be normally expressed in mammalian cells. A *Gys1*-knockin mouse encoding the R582A mutation was then constructed. Muscle glycogen levels were ~50% of wild-type, demonstrating that glucose 6-phosphate activation of glycogen synthase plays an important role in glycogen metabolism. This result, in fact, is quite similar to what was observed in yeast [192]. The muscle from the R582A mice had elevated glucose 6-phosphate and UDP-glucose levels, consistent with diminished flux towards glycogen, and *ex vivo* analyses of muscles indicated reduced insulin-stimulated glycogen synthesis. Bouskila et al. [193] concluded that insulin stimulation of glycogen synthesis in muscle is driven predominantly by elevation of glucose 6-phosphate, not glycogen synthase dephosphorylation. Hunter et al. [195] reported that AICAR-induced glycogen synthesis was abolished in the R582A mice, arguing that glucose 6-phosphate was driving glycogen accumulation. These studies raise interesting questions. It would be useful to revisit the role of phosphorylation control using knockin of glycogen synthase mutated at site 2 and/or 3a to see whether the results mimic the earlier less sophisticated GSL mouse models that used transgenic overexpression [150].

# SPATIAL ASPECTS OF GLYCOGEN METABOLISM

## Heterogeneity of glycogen stores in cells and tissues

Glycogen is usually described as primarily cytosolic. In typical biochemical fractionations of extracts of tissue or cells, the bulk of the glycogen remains in a low-speed supernatant after centrifugation, although some is in the pellet, depending on the biological source and metabolic conditions. Glycogen particles are large enough that high-speed centrifugation of the low-speed supernatant can sediment them into a high-speed pellet, together with membranous structures. Although it is possible and even likely that small-molecule metabolites are not homogeneously distributed within cells, it is especially easy to make the case with glycogen, since it can be visualized by electron microscopy as discrete particles, each one a potentially autonomous metabolic machine. In skeletal muscle, cellular localization leads to the definition of distinguishable pools of glycogen granules, sarcolemmal, intermyofibrillar and intramyofibrillar [57,196]. Graham and colleagues have pioneered efforts to quantify these subcellular pools (reviewed in [15,19]), and evidence is emerging that different pools may have different metabolic behaviours [197,198]. A further level of heterogeneity enters when one considers entire tissues. Different muscle fibre types, which can be intermingled in any given muscle, can have different metabolic regimes [199], glycogen metabolism being more important in fast twitch type II fibres. In the liver, periportal and perivenous hepatocytes have significantly different metabolic capacities [200], including differences in the metabolism of glycogen [201]. Obviously, measurements of glycogen by gross biochemical or NMR methods cannot address such heterogeneity.

## Translocation of metabolic enzymes during glycogen synthesis

In liver, it is generally accepted that glucose entry into cells is driven by the concentration gradient between the blood and the hepatocyte, based on the kinetic properties of the high-$K_m$ GLUT2 [67]. Unlike the hexokinase of muscle, the liver glucokinase (hexokinase IV) is not inhibited by its product glucose 6-phosphate, but by a separate regulatory protein [GKRP (glucokinase regulatory protein)] [202]. Glucokinase, under basal glucose conditions, is sequestered in the nucleus where it is bound to GKRP and is held inactive [202]. Acute activation of glucokinase involves its glucose-induced translocation from the nucleus to the cytosol. Guinovart and colleagues have championed the idea that hepatic glycogen synthase also translocates upon exposure of hepatocytes to glucose towards the cell periphery, where new glycogen is synthesized at the site of glucose entry (reviewed in [14]). Glucose 6-phosphate appears to be critical to the translocation process as well as being a direct activator of glycogen synthase.

Hers and colleagues (reviewed in [203]) many years ago proposed a mechanism whereby glycogen phosphorylase and synthase phosphorylation states were controlled in an interdependent fashion in the liver. Central to this hypothesis was allosteric inhibition of glycogen synthase phosphatase by the phosphorylated and active form of phosphorylase. Thus activation of glycogen synthase by a glycogenic stimulus would only be enabled once phosphorylase was first inactivated. Cohen and colleagues revisited this concept armed with more modern understanding of the nature of the phosphatases likely to be involved [116,117]. A predominant glycogen synthase phosphatase in liver consists of the PP1

catalytic subunit in association with the $G_L$ glycogen-targeting subunit [116,117]. Armstrong et al. [121] localized the phosphorylase interacting sequence to the C-terminal 16 amino acids of $G_L$ and Kelsall et al. [204] showed that mutation of $Tyr^{284}$ to phenylalanine disrupted binding to phosphorylase. Kelsall et al. [205] went on to produce Y284F-knockin mice and demonstrated that liver glycogen was increased modestly, but both glycogen synthase and phosphorylase activities were significantly increased. *In vitro* assays showed that thiophosphorylase was unable to inhibit the glycogen synthase phosphatase activity of mutant $G_L$-PP1c. There was an improvement in glucose disposal in the Y284F mice, especially in males, leading to efforts to target interference of the $G_L$–phosphorylase interaction as a therapeutic approach for Type 2 diabetes [206].

Translocation of glycogen synthase has also been observed in other cells. Cid et al. [207] showed that the muscle glycogen synthase isoform was localized in the nucleus in several cell types, including primary human myoblasts, when glucose was low or absent, but was cytosolic in punctate structures in the presence of glucose. Interestingly, phosphorylation does not appear to be relevant to this nuclear localization whereas the arginine-rich cluster is important. In *S. cerevisiae*, glycogen synthase localization correlated strongly with cellular glycogen content [208], changing from a uniform distribution in the cytoplasm when glycogen was abundant to discrete well-defined spots when glycogen was scarce. In a strain lacking glycogenin, and hence unable to synthesize glycogen, Gsy2p was nuclear, echoing the results discussed above. In yeast, however, mutation of the arginine-rich cluster did not affect nuclear localization. Other studies have linked glycogen synthase phosphorylation to subcellular distribution. In 3T3-L1 adipocytes, Ou et al. [209] reported that insulin caused activation of glycogen synthase accompanied by a shift from a diffuse distribution to punctate cytosolic structures. Other correlations between glycogen synthase phosphorylation and subcellular localization have come from studies of rabbit and human muscle. Prats et al. [210] reported that, after glycogen depletion by electrical stimulation, rabbit muscle glycogen synthase dephosphorylated at site 1b and sites 2 + 2a redistributed to specialized spherical structures as a prelude to resynthesis. In a subsequent study of human muscle, further correlation between glycogen synthase phosphorylation and subcellular localization was made [211]. Enzyme phosphorylated at site 1b was mainly associated with intramyofibrillar particles, whereas phosphorylation at site 2 + 2a was associated with intermyofibrillar and subsarcolemmal particles. It was proposed that site 1b phosphorylation was mediated by adrenaline activation of PKA following exercise. This would provide a function for site 1b, which has no direct effect on activity and which is absent from liver glycogen synthase.

## Lysosomal disposal of glycogen

Cytosolic degradation of glycogen by phosphorylase and debranching enzyme AGL has commanded much attention, perhaps because it has been such fertile ground for the discovery of biochemical and hormonal mechanisms. However, glycogen is also disposed via a lysosomal pathway, the importance of which is underscored by Pompe disease (glycogen storage disease type II), in which the lysosomal $\alpha$-glucosidase (GAA) is mutated [74]. Glycogen overaccumulates in lysosomes and vesicular structures [212]. In its most severe form, Pompe disease is fatal within the first year of life, with cardiomyopathy and

muscular hypotonia (OMIM ID #232300). It has been reported that as much as 10% of hepatocyte glycogen is present in the lysosome [28,213]. The exact mechanism by which glycogen is normally transferred to the lysosome is unknown, but is most likely to involve some autophagic or autophagy-like vesicular trafficking (Figure 13). Autophagy comprises a family of processes initially described as a mechanism for random recycling of cellular materials under conditions of nutritional deprivation [214,215]. The type of autophagy that has been most studied, macroautophagy, involves engulfment of cargo within a double membrane to form autophagosomes that ultimately fuse with the lysosome where the cargo is degraded [214–216]. From the intense research activity in this area, it is becoming clear that there are many variants of the basic autophagic process that can have separate controls, specific cargoes and different functions [217,218]. Glycogen has been linked specifically to autophagy. The term ‘glycogen autophagy’ has been applied particularly to the liver of newborns (reviewed in [219]). Glycogen has been reported to accumulate in several organs, notably liver and heart, of the fetus before term, presumably to provide energy reserves for use after the trauma of birth [220]. For the liver, and the newborn in general, the hepatic glycogen reserves are especially important since gluconeogenesis is not well developed at birth [220] and therefore lysosomal degradation of liver glycogen is critically important. Another connection with autophagy is provided by the work of Raben et al. [221] on Pompe disease. From studies of muscle in a mouse model of Pompe disease, $GAA^{-/-}$ mice, they have shown that massive overaccumulation of glycogen in lysosomes, characteristic of the disease, was accompanied by a dramatic increase of glycogen-containing autophagosomes and late endosomes, which they referred to as ‘autophagic build-up’ [221]. Subsequently, mice were generated that combined a homozygous null *GAA* mutation with muscle-specific disruption of the genes encoding Atg5 [222] or Atg7 [223], important autophagy proteins. Loss of Atg5 function indeed diminished autophagic build-up in muscle, but made the clinical phenotype worse. Loss of Atg7 had a similar effect and decreased glycogen by 50–60%. Enzyme replacement therapy (delivery of GAA directly to the animals) normalized glycogen levels in the autophagy-deficient mice [223], making selective suppression of autophagy a promising therapeutic approach for Pompe disease patients.

A totally different link between glycogen and autophagy came from analysis of yeast glycogen metabolism where an unbiased genetic screen showed that two prototypical yeast autophagy genes, *ATG1* and *ATG13*, could restore defective glycogen accumulation to a *snf1* mutant strain [224]. In yeast, defective Snf1p, the orthologue of mammalian AMPK catalytic subunit, causes failure to accumulate glycogen. Snf1p was then found to be a positive regulator of autophagy acting upstream of Atg1p; in yeast, transport of glycogen to the vacuole, the approximate equivalent of the mammalian lysosome, actually serves to store glycogen which is only used very late in starvation, possibly for sporulation, when the yeast vacuolar GAA is induced. A genome-wide survey of yeast for genes whose deletion affected glycogen metabolism revealed that the second largest category of genes identified, ~60, were involved in some way in vesicular trafficking or vacuolar function [225]. Subsequent studies of mammalian autophagy showed that AMPK is also a positive regulator of autophagy [226] and, more recently, implicated AMPK as an upstream regulator of ULK1 [227–230], a mammalian orthologue of Atg1p [231]. One might speculate whether activating mutations of the AMPK γ-subunit might be causing excessive autophagy that is associated with the

pathological overaccumulation of glycogen, via mechanisms such as described below. If a downstream trafficking event, for example, the fusion of glycogen-laden autophagosomes with the lysosome, became limiting, there could be a build up of glycogen vesicles such as is seen in PRKAG2 cardiomyopathy or Pompe disease.

Mechanistically, how is glycogen transported to lysosomes? If vesicular transport is involved, one would expect a means to associate glycogen with membranes. Using electron microscopy, glycogen particles are often reported to be close to membranes, endoplasmic reticulum in liver [232] or sarcoplasmic reticulum in muscle [15]. Recently, Stbd1 has emerged as a candidate to anchor glycogen to membrane locations [55]. In independent work, Stbd1 was identified as being associated with liver glycogen in a proteomics study and proposed to be involved in locating glycogen to membrane compartments in cells [45]. Stbd1 has a highly conserved N-terminal 24-residue hydrophobic sequence and a C-terminal CBM20 domain [233], with an intervening sequence predicted to be disordered [55]. *In vitro*, Stbd1 binds to polysaccharides, preferentially interacting with less branched structures such as the amylopectin of plant starch or the glycogen isolated from laforin-knockout mice (see the next section). In MGSKO and LGSKO mice, lacking muscle and liver glycogen respectively, Stbd1 levels are decreased, suggesting a connection between Stbd1 and glycogen metabolism. Expression of Stbd1 in COS cells resulted in the formation of large perinuclear structures, as judged by immunofluorescence, co-localized with glycogen identified by PAS staining [55]. Point mutations in the CBM20 domain designed to disable glycogen binding did not alter Stbd1 perinuclear localization, but eliminated glycogen co-localization. Deletion of the hydrophobic N-terminus of Stbd1 led to a diffuse cytosolic distribution, supporting the idea that it serves to direct Stbd1 to intracellular membranes.

This first effort at assessing the function of Stbd1 provides plausible evidence for a role in glycogen metabolism, with the hypothesis that Stbd1 anchors glycogen to intracellular membranes. Yeast two-hybrid screens using Stbd1 as bait identified Stbd1 itself as an interacting protein, suggesting an oligomeric structure, GABARAP (γ-aminobutyric acid type A receptor-associated protein) and GABARAPL1 (GABARAP-like 1). The last two are members of the mammalian ATG8 family of autophagy proteins [234,235]. Interaction of both GABARAP and GABARAPL1 with Stbd1 was validated by co-expression in COS cells and co-immunoprecipitation. By immunofluorescence, Stbd1 was strongly co-localized with GABARAPL1, less so with GABARAP, in the perinuclear structures described above. Similar co-localization was observed between Stbd1 and endogenous GABARAPL1. The association between Stbd1 and GABARAPL1 provides a tantalizing connection with autophagy. Yeast have a single *ATG8* gene that is thought to be critically involved in the formation of autophagosomes [234]. The emerging sense is that selectivity can be conferred by interaction of Atg8 with specific receptor proteins [217,218]. In mammals, the situation is more complex as there are six orthologues of Atg8, the best studied being LC3 (light chain 3), whose lipidation is often used as a marker for autophagy induced by nutritional stresses [236]. The interaction of ATG8 family members with different cargo-specifying receptors may thus allow for a variety of selective autophagy pathways whose operation need not mimic exactly the traditional macroautophagy process mediated by LC3. Little is known about the function of GABARAPL1 [237]. A current hypothesis, then, is that Stbd1 tethers glycogen to membranes and, by an as yet undefined mechanism involving interaction with

GABARAPL1, participates in the trafficking of glycogen to the lysosome, the process of ‘glycogen autophagy’ or ‘glycophagy’. Since Stbd1 *in vitro* shows a preference for binding to poorly branched and/or highly phosphorylated glycogen, it could favour the disposal of aberrant glycogen particles and be part of a quality-control mechanism. Alternatively, it might be controlled by conditions or stimuli that tend to produce aberrant glycogen. Much remains to be learned about the function of Stbd1 and the phenotype of an Stbd1-knockout mouse, currently under construction, will be of considerable interest.

## LAFORA DISEASE AND GLYCOGEN PHOSPHORYLATION

A century ago, Gonzalo Lafora reported autopsy results from patients with teenage-onset myoclonus epilepsy with associated dementia [238]. He observed in ganglion cells ‘amyloid bodies’, a term used at the time to describe material that stained like starch. We now know that these ‘Lafora bodies’ are actually deposits that contain poorly branched insoluble glycogen-like carbohydrate, sometimes called polyglucosan, and are a hallmark of Lafora disease, an autosomal recessive fatal neurodegenerative disorder categorized as a progressive myoclonus epilepsy. Symptoms start in early adolescence and progress as stimulus-sensitive grand mal tonic–clonic, absences, and visual and myoclonic seizures. Rapid progressive dementia develops leading to death typically within 10 years of the first symptom [39–41,239]. Lafora disease is very rare, found most often in parts of the world where consanguinity is common [240]. Lafora bodies are found in many tissues, including the brain, skeletal muscle, heart and liver; their presence in neurons is widely considered causative of the disease. Mutations in two genes, *EPM2A* (epilepsy progressive myoclonus type 2A) and *EPM2B* (epilepsy progressive myoclonus type 2B), account for approximately 90% of Lafora cases [241]. *EPM2A* or *EPM2B* encode the proteins laforin and malin respectively.

### Laforin

Minassian et al. [53] identified the laforin gene by positional cloning. Laforin is ubiquitous, with the highest expression levels in skeletal muscle, liver, kidney, heart and brain, tissues with abundant Lafora bodies in Lafora patients [54,242]. Laforin contains the signature DSP (dual-specificity phosphatase) catalytic motif, HCXXGXXRS/T [243], and can dephosphorylate phosphoserine/phosphothreonine, phosphotyrosine [244] and the generic phosphatase substrate PNPP (*p*-nitrophenyl phosphate) *in vitro* [245]. Additionally, laforin contains a CBM20 domain that binds complex carbohydrates including glycogen, amylopectin and polyglucosan [245–247]. Laforin is conserved in all vertebrates and in a small defined group of invertebrates and protists, including *Toxoplasma gondii*, that are of red algal descent, possess a true mitochondrion and produce floridean starch, a complex carbohydrate resembling amylopectin [248,249].

In mammals, laforin has been reported to bind several proteins involved in glycogen metabolism, including glycogen synthase, GSK3, PTG and malin [250–253]. HIRIP5 [HIRA (histone cell cycle regulation defective homologue A)-interacting protein 5], a cytosolic protein involved in iron metabolism [254], *Epm2a*-interacting protein 1, a protein with unknown function [255], the Alzheimer’s disease protein tau [256], and the $\alpha$2 and $\beta$2

subunits of AMPK [257] also interact with laforin. Some 59 disease-causing mutations and several polymorphisms in the *EPM2A* gene have been described. Of those tested, almost all affect polysaccharide binding or phosphatase activity (The Lafora Progressive Myoclonus Epilepsy Mutation and Polymorphism Database; http://projects.tcag.ca/lafora/). One mutation was described that had no effect on phosphatase activity and polysaccharide-binding activity, but was reported to affect interaction with PTG [251]. Laforin is extremely sensitive to inhibition by polysaccharides, when assayed using PNPP, but 20% of activity remains at saturation [247]. One disease mutation, W32G [258], is located in the conserved carbohydrate-binding domain and the recombinant phosphatase harbouring this mutation eliminates glycogen binding while retaining significant PNPPase activity [245,247]. Therefore impaired glycogen binding by laforin may be sufficient to cause disease. Another link between laforin and glycogen comes from the observation that laforin protein levels correlate with the amount of glycogen in a series of mouse models in which the muscle glycogen content was genetically manipulated [259].

A prominent hypothesis in Lafora research has been that polyglucosan formation results from an imbalance between the activities of glycogen synthase and branching enzyme. There is precedent since polyglucosans accumulate in other glycogen storage diseases. For example, Andersen disease and adult polyglucosan body disease both result from mutations in the *GBE1* gene, encoding the branching enzyme [260]. Impaired branching activity leads predictably to a less branched form of glycogen. In Tarui disease [261], the *PFKM* gene, encoding the muscle form of phosphofructokinase, is mutated. A build-up of glycolytic intermediates in Tarui patients is thought to drive excessive glycogen synthesis through elevation of glucose 6-phosphate, also resulting in polyglucosan formation, the imbalance in this case caused by increased glycogen elongation. In the GSL mice, described earlier, which overexpress hyperactive glycogen synthase in muscle, overaccumulation of glycogen is accompanied by the development of structures reminiscent of Lafora bodies [262,263]. These observations prompted several groups to seek mechanisms whereby laforin could affect glycogen-synthesizing enzymes, more specifically, how laforin might increase glycogen synthase activity. One candidate is the protein kinase GSK3 which contains an inhibitory phosphorylation site ([109], and see above). Two groups proposed that the inhibitory phosphate of GSK3 can be removed by laforin [252,264], thus potentially leading to activation of glycogen synthase to cause the biosynthetic imbalance. Lohi et al. [252] suggested that laforin's role is to detect polyglucosan appearance during glycogen synthesis and to initiate mechanisms to down-regulate glycogen synthase. Several studies, however, argue against GSK3$\beta$ being a substrate for laforin [36,37,191,250,265]. Perhaps most convincing is the observation that the GSK3 phosphorylation state is unchanged in three different genetic mouse models of Lafora disease, in which the *Epm2a* gene is disrupted [36,37], dominant-negative laforin is overexpressed [265] or the *Epm2b* gene is disrupted [191]. Measurements of glycogen synthase and branching enzyme AGL activities in these mice also argued against the 'branching imbalance' hypothesis [265].

Recently, from analyses of *Epm2a*$^{-/-}$ mice, Sanz and colleagues suggested that laforin is a more general regulator of insulin-sensitivity and proposed that laforin is a new component of the insulin signalling cascade [266]. They reported that *Epm2a*$^{-/-}$ mice had enhanced glucose disposal, most prominently in the heart, and many insulin-dependent processes were

hyperactivated when compared with commercial C57BL/6 wild-type controls. GSK3$\beta$ phosphorylation was increased, but the authors suggest that this is due to increased insulin signalling rather than a direct result of the inability of laforin to dephosphorylate GSK3$\beta$. In contrast, other experiments comparing *Epm2a*$^{-/-}$ mice with wild-type controls with a matched genetic background revealed no differences in glucose- or insulin-tolerance tests or in the insulin signalling pathway (A.A. DePaoli-Roach, D. Segvich, C. Meyer, Y. Rahimi, C.A. Worby, M.S. Gentry and P.J. Roach, unpublished work). A role for laforin in insulin signalling is thus controversial and needs further investigation.

## Laforin and autophagy

As discussed above, autophagy or an autophagy-like process is an important mechanism for glycogen disposal. Independent of this function, autophagy appears to be essential for central nervous system function as its inhibition leads to neurodegeneration, behavioural changes and early death in mice [267]. Autophagy is also thought to remove disease-associated cytoplasmic aggregate-prone proteins, which stain positive with anti-ubiquitin antibodies and accumulate in Lafora disease, most prominently in the vicinity of Lafora bodies [268,269]. Consistent with this theme, Aguado et al. [270] reported that laforin activates autophagy by acting upstream of TSC (tuberous sclerosis complex) 2, a tumour suppressor mutated in patients with TSC [271]. Loss of TSC2 leads to activation of mTOR (mammalian target of rapamycin), which, in addition to activating protein synthesis and cell growth, also potently inhibits autophagy [272]. The authors concluded that laforin inhibits mTOR and thus activates autophagy. Therefore, when laforin is disabled, activation of mTOR would inhibit autophagy to cause the disease phenotype. However, the fact that patients with TSC do not develop Lafora bodies argues against laforin acting upstream of TSC2 as a mechanism for the development of Lafora bodies. Likewise, there are no reports of TSC-like tumours in Lafora patients or the Lafora disease mouse models. Laforin may, however, have multiple functions.

## Laforin as a glycogen phosphatase

A promising hypothesis in Lafora research was introduced by Dixon and colleagues [250] by demonstrating that laforin dephosphorylates amylopectin, the major component of plant starch. Amylopectin has chemistry comparable with that of glycogen ($\alpha$-1,4-glycosidic linkages with less frequent $\alpha$-1,6-branch points) and contains a significant amount of covalent phosphate in the form of $C_6$- and $C_3$-phosphomonoesters [273]. Worby et al. [250] also attempted to measure dephosphorylation of commercially available rabbit liver glycogen by laforin, but were unsuccessful, possibly because of the lack of assay sensitivity and/or the low phosphate content of liver glycogen. Tagliabracci et al. [37] were able to demonstrate that laforin could dephosphorylate rabbit muscle glycogen *in vitro*. Muscle glycogen contains approximately 10-fold more phosphate than rabbit liver glycogen [34,35]. Laforin released ~25% of the phosphate present in undigested rabbit muscle glycogen. Digestion of the glycogen with glucosidases in the presence of laforin led to release of 90% of the phosphate, suggesting that the majority of phosphates in the native particle are sterically protected from the phosphatase. Laforin activity towards glycogen is dependent on a functional carbohydrate-binding domain, as W32G mutant laforin was unable to bind and dephosphorylate glycogen, yet still retained significant activity toward PNPP [37]. The

hypothesis that laforin is a physiological glycogen phosphatase was strengthened by analysis of highly purified glycogen from laforin-knockout mice. Glycogen phosphate levels were 4–6-fold higher in the muscle of *Epm2a*$^{-/-}$ mice compared with their wild-type counterparts [36,37]. Furthermore, glycogen from *Epm2a*$^{-/-}$ mice displayed a progressive deterioration in structure and solubility that paralleled the formation of Lafora bodies. Laforin-dependent hydrolysis of the phosphate in glycogen from *Epm2a*$^{-/-}$ mice largely reversed the abnormal appearance of the polysaccharide by electron microscopy [36]. How phosphate disturbs glycogen structure and chemical properties is not fully understood, but a possibility is that phosphate disrupts the elaborate hydrogen-bonding network associated with helical polyglucose [25,26].

## Malin

*EPM2B* (also called *NHLRC1*) encodes malin, a 395-residue protein that contains an N-terminal RING finger domain followed by six NHL domains [274]. Some 56 disease-causing mutations and several polymorphisms in the *EPM2B* gene have been described (The Lafora Progressive Myoclonus Epilepsy Mutation and Polymorphism Database; http://projects.tcag.ca/lafora/). The RING finger domain is characteristic of E3 ubiquitin ligases [275] and Gentry et al. [253] reported that malin interacts with laforin and catalyses its polyubiquitylation *in vitro* and in cultured cells resulting in its proteasome-dependent degradation. As noted, laforin protein levels correlate with glycogen in a series of mouse models in which the muscle glycogen content was genetically manipulated. Thus, the increased laforin levels in patients carrying malin mutations [276] as well as *Epm2b* knockout mice [191] may be a consequence of over-accumulation of glycogen. In any event, if the physiological function of malin is to mediate the destruction of laforin, it is hard to reconcile the fact that Lafora disease is caused by recessive mutations in either the *EPM2A* or *EPM2B* genes. On the basis of the model of Gentry et al. [253], defective malin would up-regulate laforin protein levels.

Recent studies, mainly using cell culture systems, have reported that several proteins involved in glycogen metabolism are substrates for malin, including glycogen synthase, the PP1 glycogen-targeting subunit PTG, the debranching enzyme AGL and AMPK [277–280]. Two independent laboratories have reported that co-expression of malin and laforin resulted in the ubiquitylation and proteasome-dependent degradation of PTG [277,278]. They proposed that laforin, via its glycogen-binding domain, could recruit malin to the glycogen particle to promote the degradation of PTG and glycogen synthase, thereby inhibiting glycogen synthesis. They also suggest that neurons contain the enzymatic machinery for synthesizing glycogen, but do not do so because glycogen synthase is in an inactivated hyperphosphorylated form. Loss of laforin or malin would inhibit malin-mediated degradation of PTG and glycogen synthase, driving excessive glycogen accumulation by dephosphorylation of glycogen synthase. Interestingly, co-expression of the catalytically inactive form of laforin had the same effect on the degradation of PTG. However, patients with mutations in laforin that abolish phosphatase activity still develop Lafora bodies and the neurological sequalae. Perhaps the phosphatase domain is required for an independent, but interrelated, function. Cheng et al. [279] identified the debranching enzyme AGL as a substrate for malin-mediated ubiquitylation and proteasome-dependent degradation. This

ubiquitylation event, unlike the ones discussed above, was independent of laforin. The authors proposed that mutations in malin would prevent the ubiquitylation and proteasome-dependent degradation of AGL, resulting in increased AGL protein, removal of $\alpha$-1,6-glycosidic linkages and hence polyglucosan formation. However, on the basis of the two-stage degradation of glycogen by phosphorylase and debranching enzyme AGL, excessive AGL activity should only reduce branching frequency if phosphorylase, normally an abundant enzyme, becomes limiting. Other reports [257,281] have proposed that AMPK phosphorylates laforin, enhances its association with malin and thereby regulates laforin and malin targets. AMPK was also reported to phosphorylate the PTG phosphatase subunit and target it for degradation by the malin–laforin complex [282]. However, DePaoli-Roach et al. [191] showed that physiological activation of AMPK in muscle by exercising mice did not alter the levels of PTG, AGL, laforin or glycogen synthase, arguing against AMPK-mediated degradation of these proteins, at least on this time scale. In addition, there is a report that malin catalyses $Lys^{63}$ ubiquitylation of the AMPK $\beta$-subunit when it is part of a trimeric complex in cells, a modification that would not target degradation [280]. Other studies have suggested that laforin and malin are recruited to aggresomes upon proteasomal inhibition [283]. The authors proposed that the centrosomal accumulation of malin and laforin enhances the ubiquitylation of malin substrates, facilitating their efficient degradation by the proteasome. In addition, laforin and malin were shown to form a functional complex with HSP70 (heat-shock protein 70) to suppress the cellular toxicity of misfolded proteins by promoting their degradation through the unfolded protein response [284].

Analysis of *Epm2b*$^{-/-}$ mice challenges a number of the proposed mechanisms of action of malin, since one would expect that loss of the E3 ubiquitin ligase would result in increased protein levels of the physiological substrates [191]. At 3 months of age, AGL and PTG levels were unchanged, as was the glycogen synthase activity ratio, arguing against AGL and PTG being substrates for malin-mediated degradation. Laforin protein, however, was significantly increased, especially in brain, and was redistributed from a soluble to an insoluble fraction [191]. This finding could be consistent with laforin being a malin target. The other interpretation is that increased insoluble glycogen sequesters the laforin, protecting it from degradation. Extensive analysis of glycogen-metabolizing enzymes in these mice failed to reveal any significant alterations, despite the fact that Lafora bodies were present in neurons, skeletal muscle and heart. Turnbull et al. [285] reported a 1.5-fold increase in skeletal muscle and liver glycogen phosphate levels in 6-month-old mice from an independently generated *Epm2b*$^{-/-}$ mouse model. These results suggest that malin regulates laforin activity and/or distribution, leading to the hyperphosphorylation of glycogen and subsequent formation of polyglucosan seen in Lafora patients.

## Lafora bodies and Lafora disease

There has been some debate as to whether Lafora bodies are the cause or the consequence of Lafora disease [240]. One argument stems from the observation that laforin-knockout mice, at least in one study, develop neuronal degeneration before Lafora body formation, both of which precede behavioural dysfunctions [286]. However, the recent generation of a double-knockout mouse model lacking both laforin and PTG makes a strong case that glycogen, and Lafora bodies in particular, is linked to the neuropathology [287]. *Ppp1r3c*$^{-/-}$ mice have a

30% reduction in muscle glycogen, correlated with decreased glycogen synthase activation state and no change in phosphorylase [155]. Brain glycogen is reduced by 75%. As noted above, in older *Epm2a*$^{-/-}$ mice, glycogen overaccumulates. In the study of Turnbull et al. [287], total glycogen was elevated 4–5-fold in brain and muscle of 12-month-old *Epm2a*$^{-/-}$ mice. This correlated with massive increases in the number of Lafora bodies observed histochemically and with severe neurological symptoms. In the *Epm2a*$^{-/-}$ *Ppp1r3c*$^{-/-}$ double-knockout mice, glycogen and Lafora body abundance were dramatically decreased and the neurological defects of *Epm2a*$^{-/-}$ mice were resolved. Very recently, the presence of a genetic variant of PTG that decreased glycogen levels was associated with a slower progression of Lafora disease [288]. By epistasis, laforin is therefore upstream of PTG with regard to Lafora body formation. The simplest explanation is that reduced capacity to synthesize glycogen due to defects in PTG is sufficient to suppress polyglucosan formation. However, other roles for PTG cannot be excluded.

## Current status of Lafora research

The molecular era of Lafora research began in 1998. In only a dozen years or so, a vibrant research enterprise has emerged around what is a very rare disease. The primary focus of most work has been to understand the functions of the products of the two major causative genes, laforin and malin, and how their impairment could explain the disease process. The results in this young research area, as one might expect, provide numerous hypotheses (Figure 14), some inconsistent results and much healthy debate. The study of the *Epm2a*$^{-/-}$ *Ppp1r3c*$^{-/-}$ mice would appear to make a strong case that Lafora disease is indeed a glycogen storage disease [287]. The evidence in our view supports the idea that laforin evolved as an *in vivo* glycogen phosphatase, removing $C_2$- and $C_3$-phosphomonoesters from glycogen, and associating with glycogen via its CBM20 domain. The properties of hyperphosphorylated glycogen in *Epm2a*$^{-/-}$ mice match the reduced solubility of polyglucosans. Considering also the observation of increased glycogen phosphorylation in *Epm2b*$^{-/-}$ mice, we propose that glycogen phosphorylation is a central causative factor in Lafora disease and that laforin functions in a repair or damage-control mechanism, to remove the rare phosphate introduced into glycogen by glycogen synthase. Such a function for laforin can also explain why the onset of symptoms in Lafora patients is not immediately at birth. Even without functional laforin, glycogen particles can undergo normal cycles of degradation and resynthesis, and it is not until the phosphate exceeds a threshold that the glycogen structure in some particles is disturbed and destined to become a Lafora body. Unresolved is whether laforin has other substrates or functions *in vivo*. Besides the obvious genetic link between laforin and malin, direct physical interaction between the two proteins has been suggested by several studies so that defects in laforin might also have an impact on malin function. This may help to explain some of the other roles proposed for laforin.

What is the status of the imbalance theory? In young *Epm2a*$^{-/-}$ mice, there are no great changes in glycogen synthase and branching enzyme [36,265]. In old *Epm2a*$^{-/-}$ mice, there is a hyperaccumulation of glycogen synthase, sequestered with polyglucosan, but its activity is not correspondingly increased. Therefore there is little supporting evidence for the theory at this time. What is definitely unclear is the relationship between glycogen phosphorylation and its acquisition of a sparsely branched structure. In the absence of laforin, does

phosphorylation precede the diminished branching or do they both progress simultaneously? Is there any mechanistic relationship, with phosphate blocking branching enzyme action or being a normal part of the branching process, for example? Is there an unappreciated control of branching enzyme?

Perhaps the biggest unknown is the function of malin. As noted, there is no shortage of proposed targets (Figure 14), based mainly on experiments with cells. The levels of several of these protein targets are unchanged in tissue from *Epm2b*$^{-/-}$ mice making it hard to reconcile with the idea that the E3 ubiquitin ligase activity of malin targets these proteins for degradation. Perhaps the ubiquitylation has a different role. Perhaps the physiologically critical target for malin has yet to be discovered. Or, like laforin, perhaps malin has functions not obvious from its primary structure. An area that will probably receive more attention in the future is the possible connection between Lafora disease and autophagy, whether generalized macroautophagy or a more specialized process specific to glycogen, i.e. glycophagy. If lysosomal disposal is a means to eliminate damaged glycogen, for example excessively phosphorylated glycogen, malin or a malin–laforin complex might play a role in that process.

## CONCLUSIONS

After 150 years, one might have imagined that there would not be much more to learn about glycogen and its metabolism. Indeed, we now know much about the biochemistry, molecular biology and genetics, we have considerable molecular insight into the structure and mechanisms of the metabolic enzymes, and we have gained significant understanding of the control of glycogen metabolism in relation to nutritional status. Knowledge of the signalling pathways is still incomplete, however, especially with regard to muscle contraction and the role of AMPK. And there have been some unexpected new findings. The introduction of phosphate into glycogen as an error in glycogen synthase action underlies a possible link to Lafora disease, and leads to discussion of a totally new aspect of glycogen metabolism, namely that of its covalent phosphate and the role of the laforin phosphatase. The function of the other Lafora disease gene, which encodes malin, is a major unresolved question. Lysosomal disposal of glycogen, long recognized in relation to Pompe disease, is now being revisited to elucidate the molecular mechanism of the transport of glycogen to lysosomes. Other spatial aspects of glycogen metabolism, again first described many years ago, are beginning to be examined with modern methods for imaging subcellular localizations. The glycogen particle may be a prime example of a metabolic machine, accepting substrates and releasing products in response to signals from the cell, but still functioning autonomously wherever it is built.

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## Abbreviations

| | |
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| **AGL** | amylo-$\alpha$-1,6-glucosidase, 4-$\alpha$-glucanotransferase |
| **AICAR** | 5-amino-4-imidazolecarboxamide riboside |
| **AMPK** | AMP-activated protein kinase |
| **CBM20** | carbohydrate-binding module 20 |
| **GAA** | acid $\alpha$-glucosidase |
| **GABARAP** | $\gamma$-aminobutyric acid type A receptor-associated protein |
| **GABARAPL1** | GABARAP-like 1 |
| **GKRP** | glucokinase regulatory protein |
| **GLUT** | glucose transporter |
| **GSK3** | glycogen synthase kinase 3 |
| **LC3** | light chain 3 |
| **LGSKO** | liver-specific glycogen synthase-knockout |
| **MCA** | Metabolic Control Analysis |
| **MGSKO** | muscle-specific glycogen synthase-knockout |
| **mTOR** | mammalian target of rapamycin |
| **PAS** | periodic acid–Schiff |
| **PKA** | protein kinase A |
| **PKB** | protein kinase B |
| **PNPP** | *p*-nitrophenyl phosphate |
| **PP** | protein phosphatase |
| **PRKAG2** | AMPK $\gamma$2 non-catalytic subunit |
| **PSSM** | polysaccharide storage myopathy |
| **PTG** | protein targeting to glycogen |
| **Stbd1** | starch-binding domain protein 1 |
| **TCA** | trichloroacetic acid |
| **TSC** | tuberous sclerosis complex |
| **UGPPase** | UDP-glucose pyrophosphatase |

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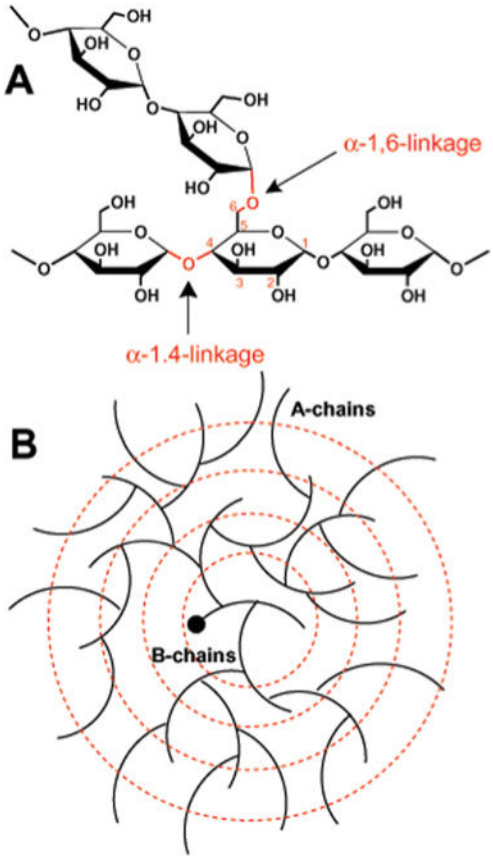


**Figure 1. Glycogen structure**

(**A**) Polymerizing $\alpha$-1,4-glycosidic linkages and a branching $\alpha$-1,6-glycosidic linkage are shown. (**B**) The tiered model for glycogen organization in which inner B-chains on average carry two branches and the outer A-chains are unbranched. The black circle denotes glycogenin.


**Figure 2. Glycogen particles**

Shown are the well-established glycogen-associated proteins: the metabolic enzymes (mauve) glycogenin (GN), glycogen synthase (GS), phosphorylase (PH) and debranching enzyme (DBE); the protein kinases (red) phosphorylase kinase (PH kinase) and AMPK; the phosphatases (green) type 1 catalytic subunit (PP1c) and laforin (LF); the PP1 glycogen-targeting subunits (blue) $R_{GL}$, $G_L$ and PTG; and the putative membrane-anchoring protein Stbd1. Malin has been suggested to bind glycogen via interaction with laforin. Phosphorylase kinase, Stbd1 and $R_{GL}$ bind membranes. Numerous protein–protein interactions are either known or proposed to exist among these glycogen-binding proteins.

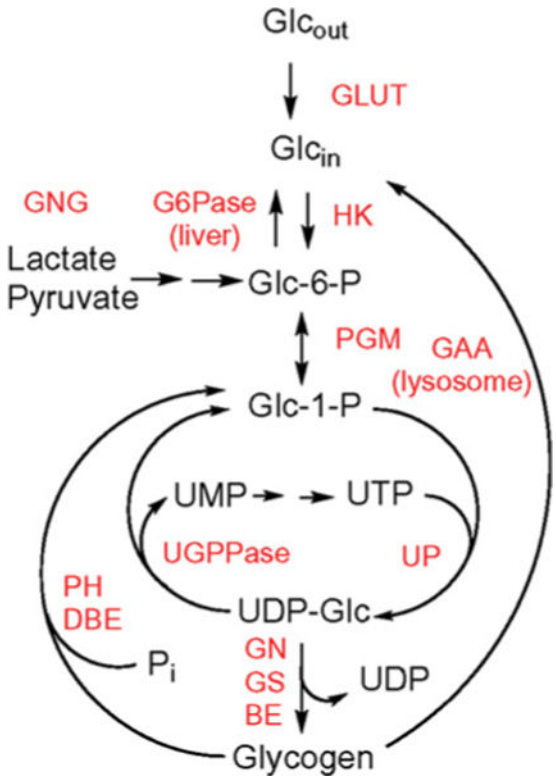


**Figure 3. Overview of glycogen metabolism**

$Glc_{out}$, extracellular glucose; $Glc_{in}$, intracellular glucose; HK, hexokinase; G6Pase, glucose-6-phosphatase; PGM, phosphoglucomutase; UP, UDP-glucose pyrophosphorylase; UGPPase, UDP-glucose pyrophosphatase; GN, glycogenin; GS, glycogen synthase; BE, branching enzyme; PH, glycogen phosphorylase; DBE, debranching enzyme; GAA, lysosomal $\alpha$-glucosidase; GNG, gluconeogenesis.

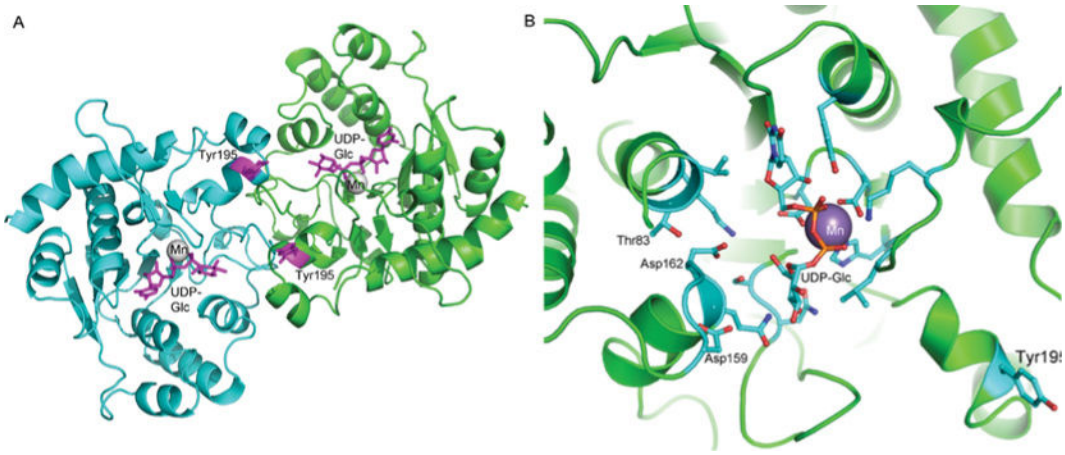


**Figure 4. Structure of mammalian glycogenin**

(**A**) Ribbons representation of the glycogenin dimer. The active sites are denoted by the bound substrate UDP-glucose (magenta). The location of $Tyr^{195}$ near the dimer interface is indicated using magenta colouring of the residue. (**B**) The active site of glycogenin. Residues discussed in the text are labelled, and the position of the catalytically essential $Mn^{2+}$ ion is shown using a purple sphere.

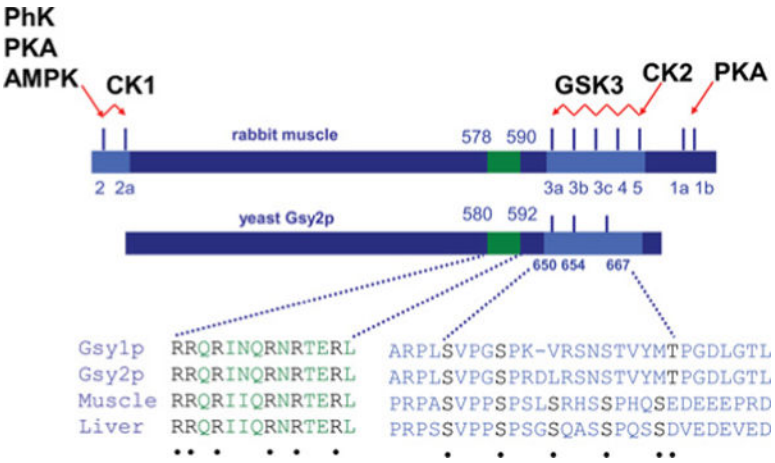


**Figure 5. Regulatory features of glycogen synthase**

Shown is a comparison of the general architecture of yeast and mammalian glycogen synthases in terms of phosphorylation sites (light blue, not to scale) and the arginine-rich cluster implicated in conferring sensitivity to activation by glucose 6-phosphate (green). The conserved arginine residues and the phosphorylated residues are in black and marked by dots. Some of the protein kinases involved in phosphorylating the mammalian enzyme are linked to sites they modify. See the legend to Table 2.

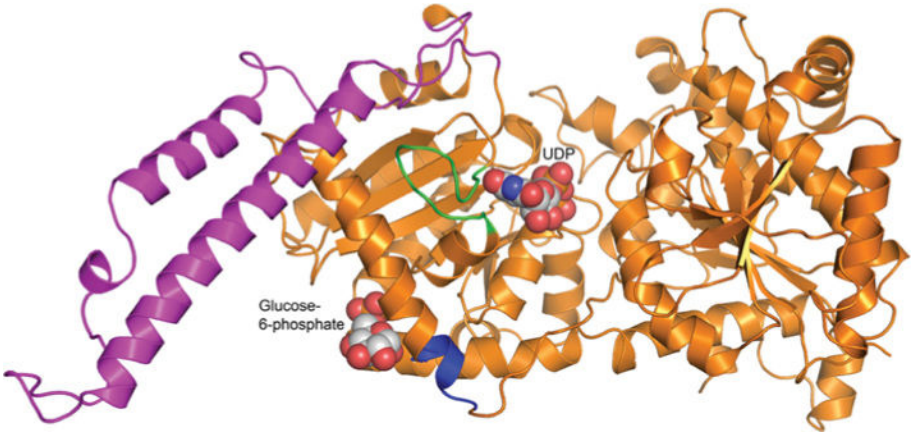


**Figure 6. Ribbons diagram of eukaryotic glycogen synthase**

The diagram highlights the sequence insertions and deletions of glycogen synthase that confer its allosteric regulation and preference for UDP-glucose. The secondary-structural elements conferring its tetrameric arrangement are coloured magenta. The location of the ten-residue deletion relative to the bacterial enzymes that conveys glucose 6-phosphate regulation is coloured blue. The inserted loop of residues that confer preference for UDP-glucose is coloured green.

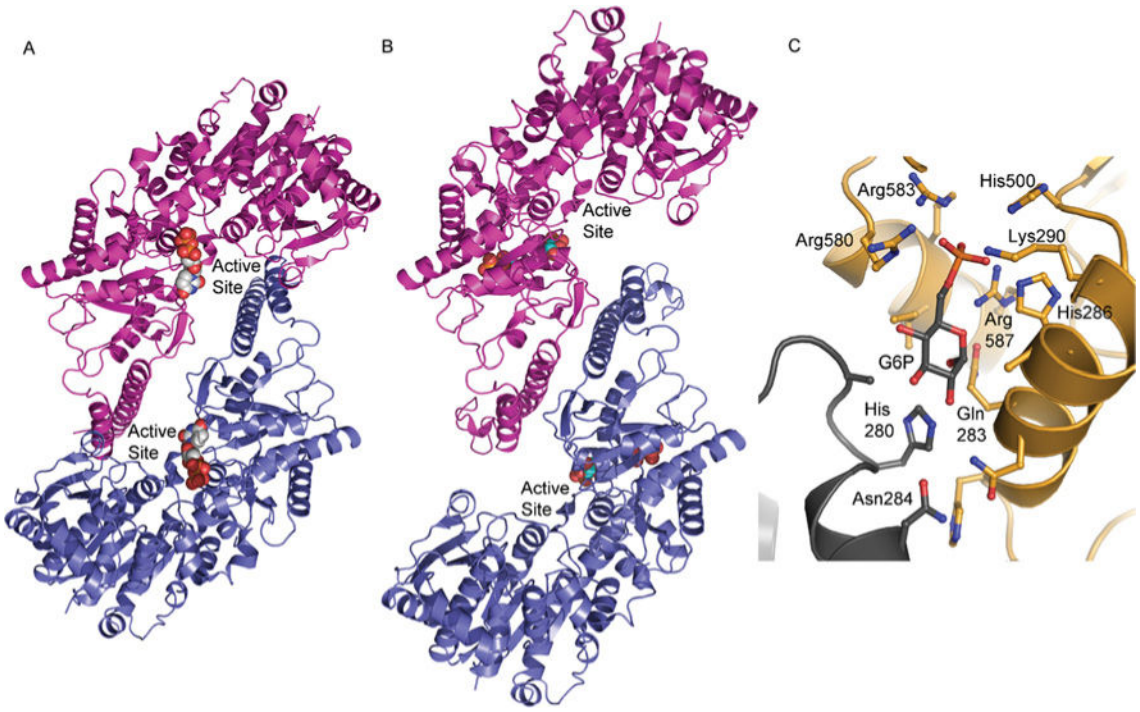


**Figure 7. Glucose 6-phosphate activation**

The active sites of glycogen synthase are occluded in the absence of glucose 6-phosphate (**A**), but are opened and freed for glycogen access in the activated state (**B**). Glucose 6-phosphate is bound at the interface between subunits with multiple charged residues interacting with the phosphate moiety and relatively few contacts with the glucose moiety (**C**).

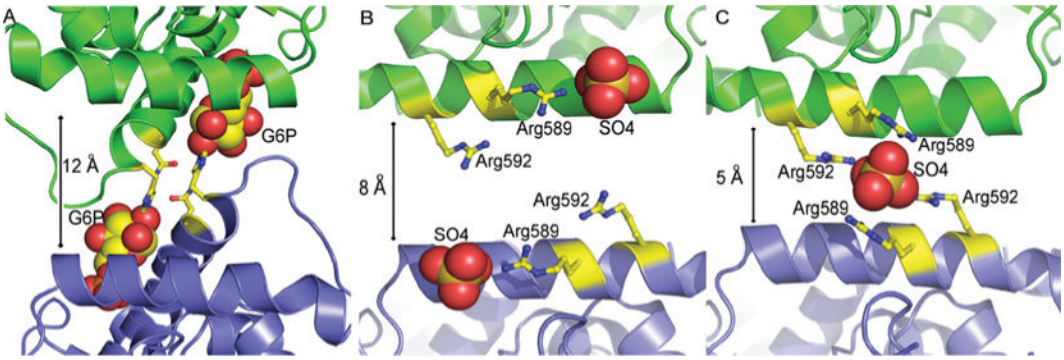


**Figure 8. Interactions at the regulatory interface in different conformational states of yeast glycogen synthase**

(**A**) The binding of glucose 6-phosphate reorganizes the interface and positions the regulatory helices approximately 12Å apart. (**B**) One of the basal state conformations of yeast glycogen synthase where two sulfate molecules are bound next to $Arg^{589}$ on the opposite face of the regulatory helix from where glucose 6-phosphate is bound. The regulatory helices are positioned approximately 8 Å apart in this conformation. (**C**) Another conformational state observed for glycogen synthase when a single sulfate molecule is bound between the regulatory helices and pulls the helices to within approximately 5 Å of one another. This state may resemble the inhibited phosphorylated state.

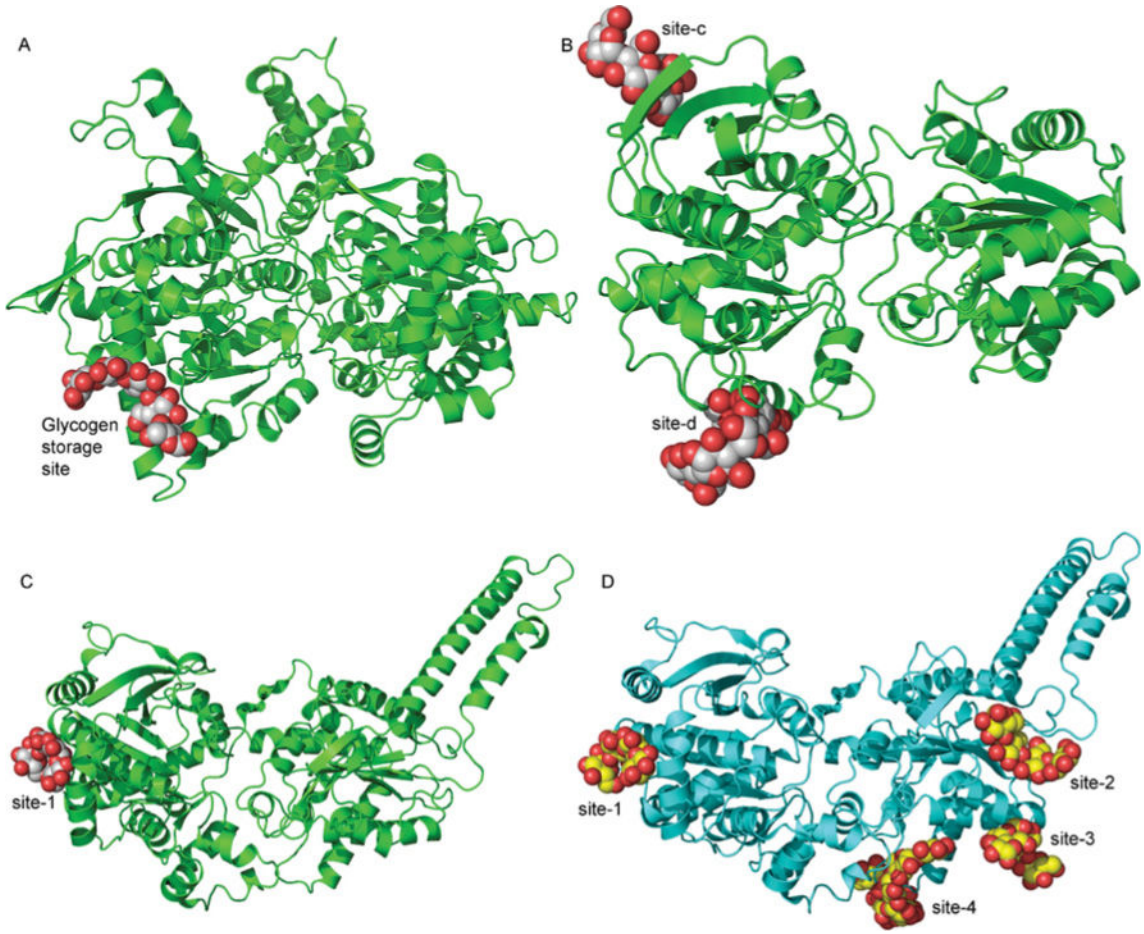


**Figure 9. Maltodextran binding in glycogen phosphophorylase and glycogen synthases**

(**A**) A ribbons representation of phosphorylase with a maltodextran bound in the ‘glycogen storage site’. (**B**) A ribbons representation of the glycogen synthase monomer from *E. coli* displaying maltodextran-binding sites ‘c’ and ‘d’ that are located on its N-terminal domain. (**C**) A ribbons representation of a single subunit in yeast glycogen synthase displaying the maltodextran bound to site-1 located in the N-terminal domain. (**D**) A ribbons representation of a single subunit of yeast glycogen synthase displaying the locations of all four maltodextran-binding sites.

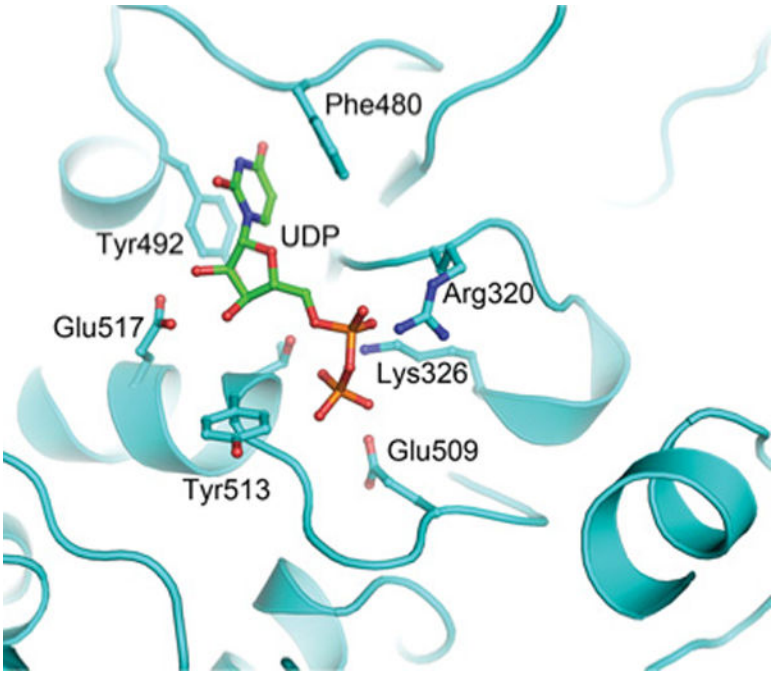


**Figure 10. Active site of yeast glycogen synthase**

Those residues in Gys2p responsible for recognizing and binding the donor nucleotide sugar substrate are labelled. The glutamate residues present in the $EX_7E$ motif probably participate in glucosyl transfer from the donor to acceptor substrate ($Glu^{509}$) and in positioning the uridine ribose moiety ($Glu^{517}$).

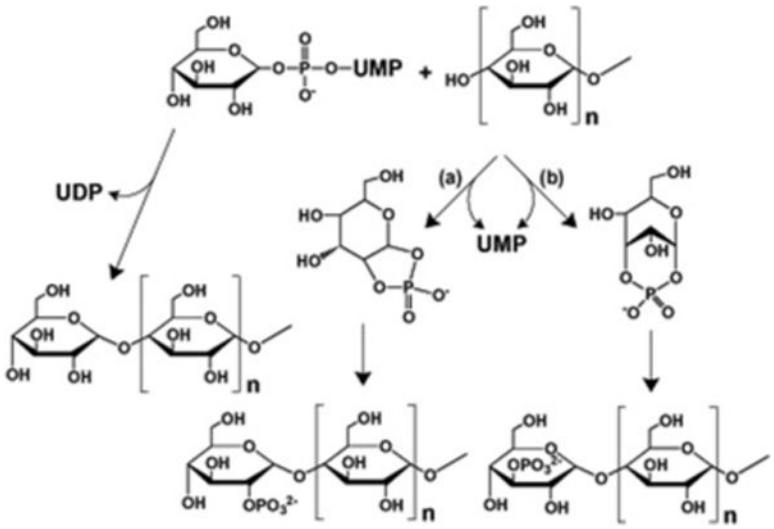


**Figure 11. Possible mechanism for glycogen phosphorylation**

The usual glycogen synthase reaction is shown on the left where glucose from UDP-glucose is added to the non-reducing end to form a new $\alpha$-1,4-glycosidic linkage. The proposed mechanism for the introduction of phosphate would involve the formation of either glucose-1,2-cyclic phosphate (a) or glucose-1,3-cyclic phosphate (b) in the enzyme active site. Reaction of C-1 of the cyclic phosphate would lead to addition of either a glucose 2-phosphate or a glucose 3-phosphate to the non-reducing end.

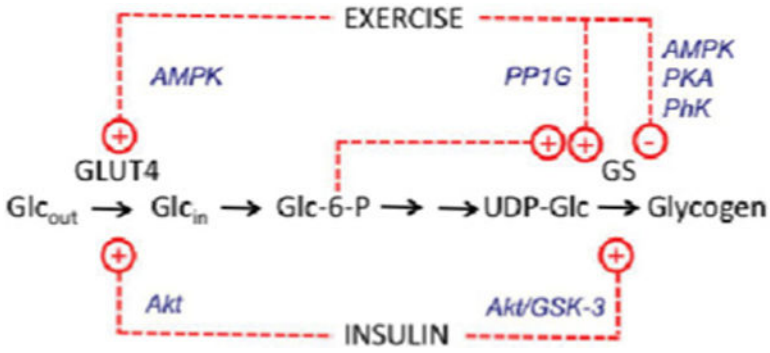


**Figure 12. Control of glycogen synthesis in muscle**

Both insulin and exercise increase glucose uptake via GLUT4. Increased glucose 6-phosphate (Glc-6-P) levels provide feedforward activation of GS (glycogen synthase). Insulin also causes dephosphorylation and activation of glycogen synthase by promoting the inactivation of GSK3 by Akt. The effect of exercise on glycogen synthase phosphorylation is more complex, potentially dephosphorylating via a PP1G containing $R_{GL}$ and well as increasing phosphorylation via activation of protein kinases such as AMPK. PhK, phosphorylase kinase.

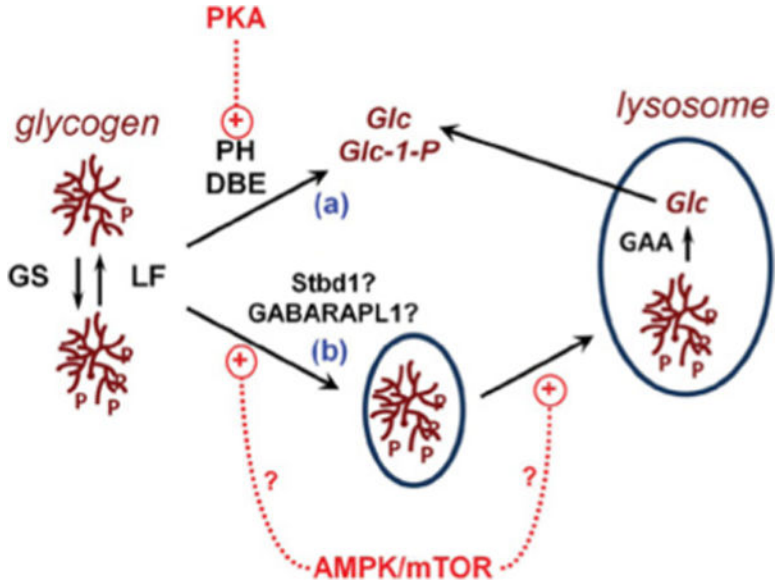


**Figure 13. Degradation of glycogen**

Glycogen is converted into glucose (Glc) by two pathways: (a) the classic cytosolic pathway controlled by cAMP and PKA, and mediated by glycogen phosphorylase (PH) and debranching enzyme (DBE); and (b) the lysosomal pathway in which degradation is ultimately catalysed by the lysosomal GAA. The latter pathway is poorly understood mechanistically, but probably resembles autophagy. It may be an example of selective autophagy with cargo specificity conferred by Stbd1 which would anchor glycogen to membranes and interact with the ATG8 family member GABARAPL1. The model also depicts the possibility that abnormally phosphorylated and/or branched glycogen is preferentially disposed of by this pathway. GS, glycogen synthase; LF, laforin.

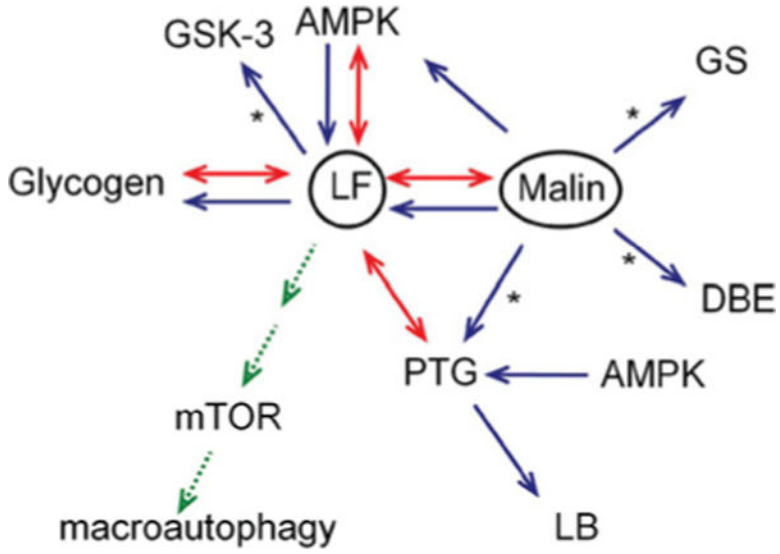


**Figure 14. Reported interactions among laforin, malin and glycogen-metabolizing proteins**

The Figure summarizes some of the interactions reported for laforin and malin, as discussed in the text, based on studies *in vitro*, in cell systems and genetically modified mice. Blue single-headed arrows depict an enzyme–substrate relationship. Red double-headed arrows indicate a protein–protein interaction. Dashed green arrows indicate signalling pathways. Asterisks indicate some instances where analyses of *Epm2a* $^{-/-}$ and/or *Epm2b* $^{-/-}$ mice do not seem consistent with the proposed interaction. LF, laforin; LB; Lafora bodies; GS, glycogen synthase; DBE, debranching enzyme.

**Table 1**

Glucose and phosphate distribution within a glycogen particle

| Tier* | Glucose/tier† | Chains/tier‡ | Total glucose§ | Phosphate/tier‖ |
|---|---|---|---|---|
| 1 | 13 | 1 | 13 | 0.03 |
| 2 | 26 | 3 | 39 | 0.08 |
| 3 | 52 | 7 | 91 | 0.2 |
| 4 | 104 | 15 | 195 | 0.4 |
| 5 | 208 | 31 | 403 | 0.8 |
| 6 | 416 | 63 | 819 | 1.6 |
| 7 | 832 | 127 | 1651 | 3.3 |
| 8 | 1664 | 255 | 3315 | 7 |
| 9 | 3328 | 511 | 6643 | 13 |
| 10 | 6656 | 1023 | 13299 | 27 |
| 11 | 13312 | 2047 | 26611 | 53 |
| 12 | 26624 | 4095 | 53235 | 106 |

*Based on the model of Figure 1.

†Based on a chain length of 13.

‡Number of chains per tier; chains(tier $n$) = $2^n - 1$

§Sum of the number of glucose residues up to and including this tier.

‖Covalent phosphates, assuming one phosphate per 500 glucose residues and an even distribution of phosphates between the inner and outer tiers. Total phosphates in a 12-tier molecule on this model would be ~212.

## Table 2
### Muscle glycogen synthase phosphorylation sites

CAMKII, $Ca^{2+}$/calmodulin-dependent protein kinase II; CK1, protein kinase casein kinase 1; CK2, protein kinase CK2; DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase; PASK, PAS (Per/Arnt/Sim) domain-containing protein kinase; PhK, phosphorylase kinase; PKC, protein kinase C.

| Phosphorylation site | | | |
|---|---|---|---|
| **Common designation*** | **Residue (rabbit)†** | **Residue (mouse)†** | ***In vitro* kinase(s)** |
| Site 2 | 7 | 8 | AMPK, CAMKII, PhK, PKA, PKC |
| Site 2a | 10 | 11 | CK1 |
| Site 3a | 640 | 641 | GSK3, PASK, DYRK |
| Site 3b | 644 | 645 | GSK3, p38$\beta$ |
| Site 3c | 648 | 649 | GSK3 |
| Site 4 | 652 | 653 | GSK3 |
| Site 5 | 656 | 657 | CK2 |
| Site 1a | 697 | 698 | PKA, PKC |
| Site 1b | 710 | 711 | PKA, CAMKII |

*Site designations made before the protein sequence was known, but still used in many publications.

†Residue numbers were first derived from biochemical studies of rabbit muscle enzyme. More recent studies of mouse (and human) glycogen synthase generally follow HUGO recommendations that begin with the translational start site and thus differ by one. Both usages are present in the literature.