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Glycogen-autophagy: Molecular machinery and cellular mechanisms of glycophagy

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Autophagy is an essential cellular process involving degradation of superfluous or defective macromolecules and organelles as a form of homeostatic recycling. Initially proposed to be a "bulk" degradation pathway, a more nuanced appreciation of selective autophagy pathways has developed in the literature in recent years. As a glycogen-selective autophagy process, "glycophagy" is emerging as a key metabolic route of transport and delivery of glycolytic fuel substrate. Study of glycophagy is at an early stage. Enhanced understanding of this major noncanonical pathway of glycogen flux will provide important opportunities for new insights into cellular energy metabolism. In addition, glycogen metabolic mishandling is centrally involved in the pathophysiology of several metabolic diseases in a wide range of tissues, including the liver, skeletal muscle, cardiac muscle, and brain. Thus, advances in this exciting new field are of broad multidisciplinary interest relevant to many cell types and metabolic states. Here, we review the current evidence of glycophagy involvement in homeostatic cellular metabolic processes and of molecular mediators participating in glycophagy flux. We integrate information from a variety of settings including cell lines, primary cell culture systems, *ex vivo* tissue preparations, genetic disease models, and clinical glycogen disease states.

Glycogen is a hexose sugar polymer central to systemic and cellular metabolic homeostasis. Cytosolic regulated metabolism of glycogen has been extensively studied. Recently a noncanonical pathway of glycogenolysis involving a selective autophagy pathway trafficking glycogen to the lysosome has received attention. Macroautophagy (from the Greek "self-eating") is an essential cellular process that describes the packaging of cytoplasmic materials into autophagosomes for trafficking to lysosomes for degradation (1). Autophagy was initially conceptualized as a nonselective "bulk" degradation process. More recently the notion of selective autophagy has emerged, with specific protein mediators targeting organelles and macromolecules for destruction (2, 3). The molecular mechanisms of autophagy involve coordination of several protein complexes and vesicle fusion events (reviewed in (1, 2, 4)). Briefly, the cytoplasmic material (autophagy "cargo") is tagged by an autophagy receptor and encaptured into a forming double-membrane autophagosome structure by binding to an autophagy-related protein 8 (Atg8) family protein, which is anchored into the membrane by lipidation. Following fusion of the autophagosome with the lysosome, acid-activated enzymes degrade the autophagosome contents. The most well-characterized autophagy pathway involves degradation of protein macromolecules and aggregates. This process utilizes ubiquitin-tagging of target proteins, recognized by receptor molecules such as p62, which complex with the Atg8 family protein, LC3 (3).

Several selective-autophagy pathways have been identified that target mitochondria (mitophagy), endoplasmic reticulum (ER-phagy), lipids (lipophagy), and glycogen (glycophagy) (5, 6). For several decades, findings regarding the lysosomal degradation of glycogen have been reported, particularly in the context of glycogen storage diseases (7). Although a role for autophagic supply of glycogen to the lysosome for processing has been previously considered, only relatively recently has the concept and terminology of "glycophagy" been identified. An understanding of the specific protein intermediaries of glycophagy is developing. Some insight into the processes of glycogen tagging and recruitment to the glycoautophagosome is available from *in vitro* colocalization studies and proteomic analysis of glycogen-associated proteins. We have reported the first evidence indicating that glycophagy is operational in cardiac muscle and is distinct from LC3-mediated macrophagy (8, 9). In Figure 1; the current state of knowledge relating to glycogen-specific autophagy protein machinery (primarily derived from *in vitro* cell line studies) is depicted. Starch-binding domain-containing protein 1 (Stbd1) functions as the "glycophagy receptor," tagging glycogen for autophagic degradation at the carbohydrate-binding domain (CBM20) (10). Sequence data and colocalization studies support a role

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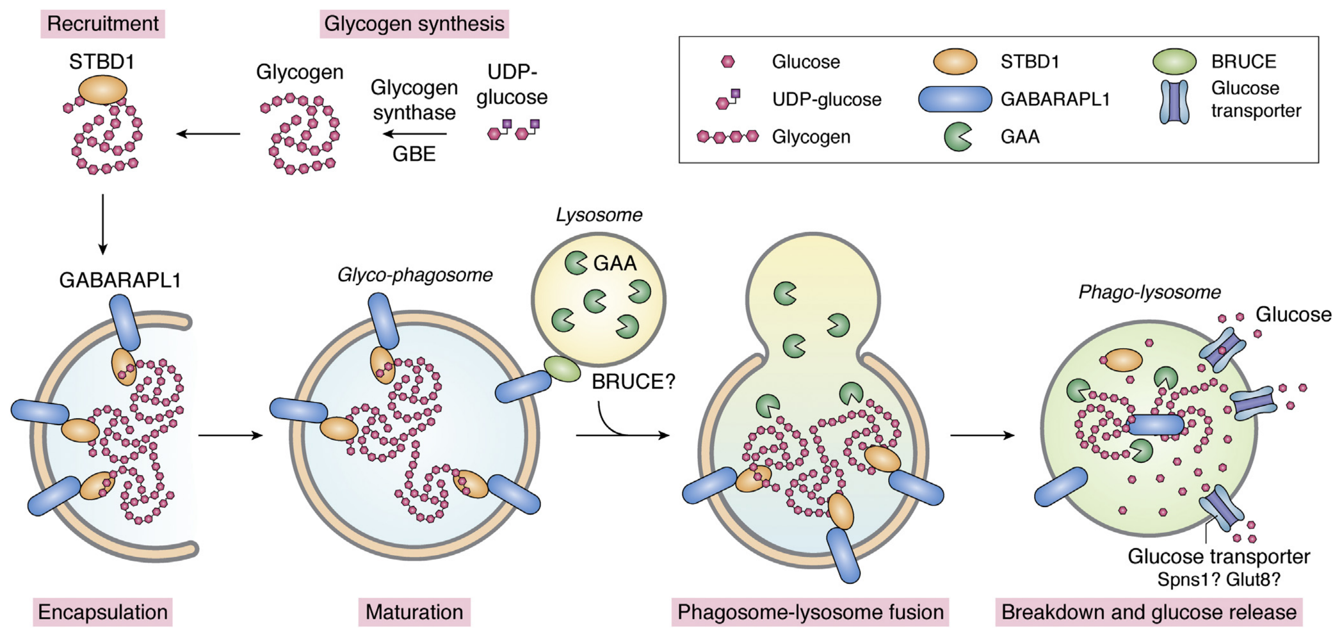

Figure 1. Schematic of the proposed glycophagy process. Using UDP-glucose as a substrate, the glucose chains in glycogen granules are elongated by glycogen synthase (GS) and branched by glycogen-branching enzyme (GBE). Glycophagy involves the tagging of glycogen with the adapter protein STBD1, which recruits glycogen into the autophagosome by binding to GABARAPL1. The mature glycophagosome fuses with a lysosome where GAA degrades glycogen to free glucose for metabolic recycling. BRUCE, baculovirus IAP repeat-containing ubiquitin-conjugating enzyme; GAA, acid α-glucosidase.

for Stbd1 recruiting glycogen to the forming phagosome by binding to the Atg8 partner protein, GABA type A receptor-associated protein like 1 (Gabarapl1) — an unhelpful historical nomenclature (11, 12). Following autophagosome–lysosome fusion, acid α-glucosidase (Gaa) mediates lysosomal glycogen breakdown (13, 14). The available literature has provided characterization of some of these key players in the glycophagy machinery, but an understanding of the pathway is at present limited.

As a glycogen-selective autophagy process, glycophagy is emerging as a key metabolic route of transport and delivery of glycolytic fuel substrate. The study of glycophagy is at an early stage, and enhanced understanding of this major noncanonical pathway of glycogen flux will provide important opportunities for new insight into cellular energy metabolism. Glycogen mishandling is centrally involved in the pathophysiology of several metabolic diseases in a wide range of tissues. Thus, advances in this exciting new field are of broad multidisciplinary interest relevant to many cell types and metabolic states. Here, we review the current evidence of glycophagy involvement in homeostatic cellular metabolic processes and of molecular mediators participating in glycophagy flux. The review integrates information from a range of *in vitro* and *in vivo* settings—cell lines, primary cell culture systems, *ex vivo* tissue preparations, genetic disease models, and clinical glycogen disease states.

Cytosolic *versus* lysosomal glycogen morphology and metabolism

Glycogen is a large elaborate polysaccharide of glucose monomers linked in chains *via* α-1,4-glycosidic bonds, with branching occurring *via* α-1,6-glycosidic bonds. Cellular glycogen particulates occur as small β-granules (<50 nm) or as larger α-granules (up to ∼300 nm diameter). These granule types have also been categorized as low molecular weight and high molecular weight, for β-granules and α-granules, respectively (15). The α-granules are considered to be aggregates of β-granules connected *via* disulfide bonds between glycogen-associated protein backbones (16, 17). The precise mechanism of α-granule formation is not yet fully elucidated. The molecular forms of glycogen differ according to cell type. Hepatic glycogen stores consist primarily of large α-granules with low surface area to volume ratio, consistent with limited polymer enzyme access and slow glucose release (17). In skeletal muscle, the smaller β-granules are predominant with high surface area to volume ratio consistent with greater polymer enzyme access to produce a rapid glucose release to meet a surge in energy demand (17). In cardiac tissues, both granule types are observed, and we have previously reported that a response to metabolic stress involves a decrease in the relative proportion of glycogen α-particle *versus* β-particle sizes (9). Thus, glycogen granule structure is tissue specific and is likely linked to functional outcomes. Further understanding of the structure–function relationship may reveal important granule type-specific (and therefore tissue-specific) processes for glycogen handling.

Glycogen granules are protein-rich structures, forming a dynamic carbohydrate–protein complex termed the "glycosome." Proteomic analysis of enriched or purified mammalian glycogen has identified hundreds of glycogen-associated proteins involved in the maintenance and regulation of the glycogen macromolecule (18, 19). Glycogenin is a central protein in the glycogen granule, providing the priming steps

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for formation of the initial glycogen polymer. Using UDP-glucose as a substrate, the glucose chains are elongated by glycogen synthase and branched by glycogen-branching enzyme (Fig. 1). Cytosolic glycogen breakdown releases glucose-1-phosphate for glycolytic metabolism. The α-1,4-glycosidic bonds are cleaved by glycogen phosphorylase and the α-1,6-glycosidic branch points are cleaved by glycogen-debranching enzyme (GDE) (15, 20). There is some evidence that incorporation of phosphate into the glycogen granule has a role in determining glycogen breakdown processing. Phosphate can be covalently bound to glucose residues within glycogen, by a mechanism yet to be fully characterized (21). Removal of phosphate from glycogen is mediated *via* a glycogen phosphatase, laforin (22, 23). It is observed that increased glycogen phosphate content is associated with limited extent of glycogen branching (reviewed in (15, 24)). There has been speculation that less-branched glycogen may be favored by glycophagy (see Glycogen structure – a key determinant of glycophagic fate?), but whether elevated phosphate levels promote glycogen processing by glycophagy is yet to be resolved.

Glycophagy-mediated glycogenolysis in the lysosome involves bulk degradation of the glycogen granule by Gaa to release free glucose. Gaa is synthesized as a 110 kDa glycoprotein and trafficked to the late endosome/lysosome from the *trans*-Golgi network *via* the mannose-6-phosphate receptor (25). Maturation of Gaa occurs in the endolysosome *via* N-glycan processing and proteolytic cleavage to generate two major active Gaa species (7, 26). Gaa has been shown to cleave both α-1,4 glycosidic strands and α-1,6-glycosidic branch points of the glycogen molecule (13, 14, 27), with a higher efficiency for α-1,4-glycosidic bonds (28). Inherited Gaa deficiency causes a glycogen storage disorder, Pompe disease (29). Preclinical investigations into Pompe disease using Gaa KO mice have clearly demonstrated that Gaa is essential for maintaining glycogen homeostasis and have advanced understanding of the importance of lysosomal glycogenolysis for preserving skeletal and cardiac muscle function (30, 31). Clinically, Pompe disease is characterized by lysosomal glycogen accumulation in nerve and muscle cells, leading to progressive cardiac, musculoskeletal, and respiratory complications and premature death (32, 33). Enzyme replacement therapy delivering recombinant Gaa has demonstrated marked improvement in cardiac disease outcomes for Pompe patients (30). The severity of the phenotype resulting from inherited Gaa mutations establishes that glycophagy and lysosomal degradation of glycogen are essential processes to maintain cellular function and not merely a redundant parallel pathway to phosphorylase-mediated glycogenolysis.

Glycogen structure—a key determinant of glycophagic fate

With cytosolic (phosphorylase-mediated) and lysosomal (glycophagy-mediated) glycogenolysis pathways operating in parallel, a key question arises—what determines whether glycogen is degraded in the cytosol or in the lysosome? The precise regulatory mechanisms involved in targeting glycogen for glycophagy have not yet been defined, but some insight can be gained from reports identifying the structural characteristics of glycogen most closely aligned with glycophagy occurrence and the biochemical properties of glycophagy protein mediators.

From a morphological perspective, evidence suggests that the structural characteristics of glycogen in the lysosome may be different to glycogen in the cytosol. In the adult rat liver, at least 10% of cellular glycogen is confined to lysosome compartments, and lysosome-localized glycogen particles have a higher molecular weight (consistent with α-granules) than those in the cytosol (34). Inhibition of Gaa shifts the relative cellular glycogen content toward a low molecular weight profile (*i.e.*, β-granules) (35). Based on these observations, it can be speculated that high molecular weight glycogen detected in the lysosome is more likely to represent large α-granules than smaller β-granules. Given that α-granules contain protein backbones linked by disulfide bonds, it could be hypothesized that, compared with the cytosol, the lysosome provides an optimized environment for breakdown of α-granules. Specifically, the lysosome contains lysosomal thiol reductase for breaking the disulfide linkages (36), lysosomal proteases for degrading the glycogen-associated proteins, and acid α-glucosidase for cleaving the glucose strands.

From a biochemical perspective, there is some evidence to suggest that glycophagy may have some selectivity for glycogen with low-branching content. *In vitro* studies using recombinant human Gaa have shown that Gaa exhibits higher efficiency for cleavage of α-1,4-glycosidic bonds than the α-1,6-glycosidic branch points (28). Similarly, Stbd1 has higher affinity for less branched polysaccharides, demonstrated by comparing binding of purified recombinant Stbd1 with glycogen and with amylopectin (a plant polysaccharide less branched than glycogen) (10). Further, glycophagy activity is highest in rodents in the neonatal setting where less branched fetal-type glycogen is dominant (37, 38). These observations suggest that in tagging glycogen for glycophagy destination, Stbd1 discriminates between glycogen structural forms.

Taking all these findings together, it could be concluded that glycogen destined for glycophagy degradation is more likely to consist of α-granules (based on morphological evidence) and have low-branching content (based on the binding affinity of Stbd1) and is optimally managed by lysosomal metabolic processes. By extrapolation, it could be hypothesized that cytosolic glycogen metabolism may therefore primarily involve the more branched molecular forms, which are handled by the already characterized branching and debranching enzymes. Further work is required to explore this proposition.

Tagging glycogen for glycophagy—the glycophagy "receptor"

The initial stage in an autophagy process involves recruitment of the cellular material to be degraded (the cargo) to the autophagosome (Fig. 1). Recruitment is mediated by autophagy tagging "receptor" proteins exhibiting binding sites for

both the cargo and Atg8 proteins, which are anchored into the forming autophagosome membrane. Potential glycophagy receptors have been identified using *in silico* and *in vitro* screening and *in vivo* colocalization studies.

Evidence for Stbd1 as a glycophagy receptor

Emerging evidence supports a role for Stbd1 as a glycophagy receptor, tagging glycogen for glycophagy-mediated degradation. Mammalian Stbd1 was discovered in the 1990's (initially named Genethonin1 or GENX-3414) and was classified as an internal membrane protein (39). Stbd1 was identified as a glycogen-binding protein by a proteomic screen of isolated glycogen from mouse and rat liver tissue (*i.e.*, the glycogen proteome) (18). Stbd1 contains both a carbohydrate (glycogen)–binding domain, CBM20 (aa 258–357) and seven binding sites for Atg8 proteins (Fig. 2) (11, 40). The standard sequence of an Atg8-binding site is [Trp or Phe or Tyr]-x-x-[Ile or Leu or Val], which is known as an Atg8-interacting motif (AIM) (41). Functional AIMs are usually present in intrinsically disordered protein regions and assume tertiary conformation following binding to Atg8 proteins (42). Two of the seven AIMs identified on Stbd1 meet these criteria, spanning aa 203 to 206 and aa 212 to 215 (11). Extensive computational analysis of AIMs has shown that the presence of potential phosphorylation sites located N′-terminally to the AIM sequence can stabilize its interaction with Atg8 proteins. On Stbd1, both aa 203 to 206 and aa 212 to 215 AIMs contain potential "upstream" phosphorylation sites (Ser175 and Ser211 respectively, Fig. 2). Ser175, but not Ser211, has been shown to be phosphorylated by AMPK in a chemical genetic screen (43), providing preliminary evidence to support a role for AMPK in regulating the interaction of Stbd1 with Atg8 proteins *via* phosphorylating Ser175 near to the aa 203 to 206 AIM. AMPK is well known to play a key role in regulating both energy metabolism and autophagy processes, and it could be expected that Stbd1-mediated glycophagy induction is also under AMPK signaling control. Further investigation into the link between AMPK signaling and glycophagy is warranted.

A role for Stbd1 in glycophagy is further supported by studies using coimmunofluorescence and lysosomal inhibitor approaches. In COS cells, recombinant Stbd1 colocalizes with the lysosomal-associated membrane protein 1 (Lamp1) (10). Lysosomal inhibitors such as bafilomycin and chloroquine have been used frequently in the autophagy field as experimental tools to monitor autophagy flux and identify proteins involved in autophagy lysosomal processes (44). Bafilomycin inhibits vacuolar H^+-ATPases preventing lysosomal acidification and blocking autophagosome–lysosome fusion. In HeLa cells, treatment with bafilomycin induced accumulation of FLAG-labeled Stbd1 protein in total cell lysates (45). The subcellular location of the accumulated Stbd1 protein was not determined. Interestingly, mutation of the Stbd1 aa 203 to 206 AIM did not affect bafilomycin-induced Stbd1 accumulation (45), suggesting that alternative AIMs on Stbd1 may be employed for Atg8 interaction in this setting. Similar findings relating to Stbd1 AIM redundancy were observed *in vivo* using Stbd1 gene replacement therapy in Stbd1-Gaa double KO mice. Stbd1-KO prevents glycogen accumulation in the liver of Pompe disease mice (Gaa-KO). This effect was reversed by Stbd1 gene replacement, even when Stbd1 with an aa 203 to 206 AIM mutation was used (46). These findings suggest that, at least in the context of disrupted lysosomal glycogen degradation (Gaa deficiency), Stbd1-mediated glycogen recruitment into glycophagosomes in hepatocytes may not be dependent on binding to Atg8 *via* the aa 203 to 206 AIM. The challenge now is to identify alternative mechanisms that may be involved.

Subcellular localization of Stbd1

The subcellular localization of Stbd1 appears to primarily align with glycogen clusters at the ER. In COS cells, visualization of Stbd1 by genetic addition of a hemagglutinin (HA)-tag and demonstrated that Stbd1 is localized to the ER and the *trans*-Golgi network (45). Comparison with COS cells

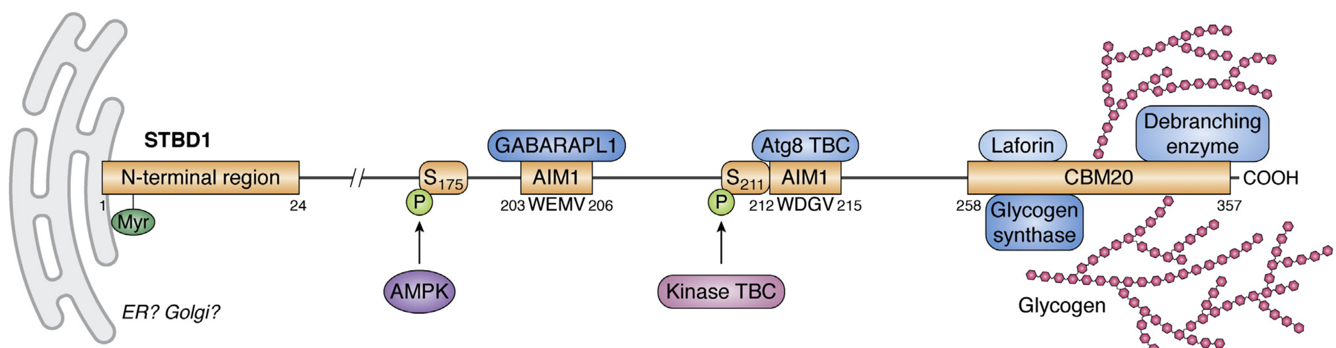

Figure 2. STBD1 protein domains, interacting proteins, posttranslational modifications, and putative subcellular locations. The glycogen-binding protein, STBD1 (*orange*), contains two putative functional Atg8-interacting motifs (AIMs) potentially regulated by phosphorylation of serine (S) residues located N-terminally to the AIM site. The carbohydrate-binding domain (CBM20) binds to glycogen. Evidence suggests that Gabarapl1 is the Atg8 family protein that binds to AIM1. AMP-activated protein kinase (AMPK) has been shown to phosphorylate Ser175 and may be involved in regulating the AIM1–Gabarapl1 interaction. Several glycogen-related proteins bind to the CBM20 site including laforin, glycogen-debranching enzyme, and glycogen synthase. Myristoylation (Myr) of the N terminus may play a role in determining subcellular location, and the endoplasmic reticulum (ER) and/or the golgi apparatus may be the site of STBD1 localization and phagophore nucleation.

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expressing truncation mutant forms of Stbd1 revealed that the N-terminal hydrophobic region of Stbd1 (aa 1–24) is required for ER localization (45). Additionally, it has been demonstrated in HeLa cells that N-myristoylation of Stbd1 plays a role in regulating subcellular localization at the ER and mitochondria (47) (Fig. 2). In mouse myoblasts, Stbd1 is required for ER stress–induced glycogen accumulation at the ER, and Stbd1 overexpression stimulates glycogen clustering at ER sites (48). In this ER stress setting, culture in glucose-deprivation media reduced glycogen to control levels, suggesting that glucose substrate supply is a limiting factor for ER stress–induced glycogen accumulation. Interestingly, the glycogen that accumulated with ER stress did not colocalize with the lysosomal marker Lamp1, and inhibition of the lysosome using bafilomycin did not prevent glucose deprivation-induced glycogen degradation (48). These findings suggest that ER stress–induced glycogen clustering may be driven by cytosolic glycogen processes and the involvement of glycophagy in this ER stress setting is yet to be determined. As depicted in Figure 2, the glycogen-binding CBM20 domain of Stbd1 has also been shown to interact with several glycogen handling proteins such as glycogen synthase, laforin (a glycogen phosphatase mentioned earlier), and GDE (45). It therefore seems likely that the recruitment of Stbd1 to ER glycogen clusters in an ER-stress setting involves recruitment of other glycogen handling proteins, but the functional outcomes of these interactions with Stbd1 have not been determined. These knowledge gaps have important implications for advancing understanding of the relationship between cytosolic glycogen processing and glycophagic glycogen degradation, and further investigation is warranted.

Tissue specificity of Stbd1 involvement in glycophagy

Stbd1 protein expression is highest in the liver and skeletal muscle, then spleen, heart, adipose, and lung tissue (10, 39). Expression of Stbd1 in the brain, kidney, and pancreatic tissues appears very low (10). An extensive comparative tissue analysis of Stbd1 function has not been performed, but there is some evidence of tissue specificity. In preclinical models of Pompe disease (Gaa-KO mice), glycogen accumulation is associated with Stbd1 accumulation in skeletal muscle but not heart or liver tissues (49). Interestingly, despite the report that liver Stbd1 is unchanged in Gaa-KO mice, Stbd1 KO prevents Gaa-KO–induced glycogen accumulation in the liver but not in skeletal muscle or heart tissues (46). In a separate study using otherwise healthy mice, Stbd1-KO did not affect liver glycogen content (50). In brown adipose tissue, Stbd1 and glycophagy may play a role in the link between lipid droplet biogenesis and glycogen dynamics. Findings from *in vitro* experiments involving Stbd1 knockdown and autophagy inhibitors show that Stbd1 and glycophagy are essential for lipid droplet formation in differentiating brown adipocytes (51). It has been proposed that glycophagy-mediated degradation of glycogen provides metabolic substrate for *de novo* lipogenesis in brown adipose tissue, at least in the embryonic state of differentiation (51, 52). Together, this evidence from Pompe disease mice and embryonic adipocytes suggests that Stbd1 may have tissue-specific (and perhaps developmental-specific) roles. More comprehensive studies systematically evaluating developmental stages with tissue comparators are required to specifically link these findings to glycophagy.

Alternative glycophagy receptors

The criteria for a protein to be a potential glycophagy receptor include the presence of binding sites for both the cargo (glycogen) and Atg8 proteins (*i.e.*, *via* an AIM-binding site). As a component of this review, we pursued an exploratory *in silico* sequence analysis to search for the presence of the AIM sequence of amino acids ([Trp or Phe or Tyr]-x-x-[Ile or Leu or Val]) in common glycogen-related proteins, as a first step toward identifying novel glycophagy receptors (Table 1). As expected, Stbd1 emerged as a prominent “hit” in the search, with 10 AIMs. Interestingly, several other glycogen-related proteins also contained AIMs: glycogen synthase, glycogen-branching enzyme, glycogen phosphorylase, glycogenin, GDE, laforin, and malin (an E3 ubiquitin ligase that regulates laforin levels). GDE contained the highest number of AIMs (39 hits). However, it is important to note that the presence of an AIM in a glycogen-binding protein does not necessarily translate to functional binding with Atg8 proteins. For example, conflicting reports on the interaction between glycogen synthase and Atg8 proteins are available. In a proteomic analysis of the autophagy interaction network, glycogen synthase was identified as an interacting partner of several Atg8 family proteins (12). But this was not supported by *in vitro* validation experiments, which showed that glycogen synthase does not directly bind to Atg8 proteins (12). Interestingly, in *Drosophila* skeletal muscle, colocalization of glycogen synthase with Atg8 was demonstrated. Mutation of the glycogen synthase aa 608 to 612 AIM revealed that synthase–Atg8 colocalization and sequestration of glycogen to the phagosome was dependent on this AIM (53). These studies show that conservation of and access to AIM sites may constitute a glycogen autophagy regulatory process, which requires further investigation in a mammalian setting. At present, Stbd1 is recognized as the primary glycophagy receptor. But based on the AIM sequence screening presented in Table 1, involvement of additional glycophagy receptors seems likely, and mapping the molecular mediators of glycophagy is an important step for developing new intervention tools and drug targets.

Glycophagosome formation

In parallel with cargo tagging, initiation of autophagy occurs with the formation of the autophagosome membrane. Whether the process of the early stage of glycophagosome formation is different to other autophagy subtypes has not been directly investigated, but it is likely that the overall concepts are similar. Autophagosome biogenesis involves membrane acquisitions from intracellular compartments, and there has been considerable interest in identifying the subcellular origin of the autophagosome. The growing evidence

Table 1
Atg8-interacting motifs identified in human glycogen-related proteins *in silico*

Protein	Gene	Uniprot identifier	No. of Atg8-interacting motifs (AIMs)
Starch-binding domain-containing protein 1	STBD1	O95210	10
Glycogen synthase 1	GYS1	P13807	12
Glycogen synthase 2	GYS2	P54840	12
Glycogen-branching enzyme	AGL	Q04446	14
Glycogen phosphorylase muscle	PYGM	P11217	10
Glycogen phosphorylase liver	PYGL	P06737	11
Glycogen phosphorylase brain	PYGB	P11216	11
Glycogenin 1	GYG1	P46976	9
Glycogenin 2	GYG2	O15488	7
Glycogen-debranching enzyme	GDE	P35573	39
Laforin	EPM2A	O95278	6
Malin	NHLRC1	Q6VVB1	2

The glycogen-related proteins were screened for AIMs ([Trp or Phe or Tyr]-x-x-[Ile or Leu or Val]) using Expasy ScanProsite tool (https://prosite.expasy.org/scanprosite/).

base suggests that the ER and its associated compartments play an important role in phagophore membrane formation. Several ER-related origin sites appear to be involved in autophagosome biogenesis, including ER exit sites (27, 54), ER subdomain omegasomes (55–58), the ER–Golgi intermediate compartment (59), and mitochondria-associated ER membranes (60). An understanding of the molecular mechanisms of autophagosome biogenesis is developing, but whether this process is distinct for selective autophagy pathways, such as glycophagy, is yet to be determined.

Some evidence suggests that pools of glycogen are localized at the proposed sites of autophagosome biogenesis. Proteomic analysis of hepatic glycogen extracts identified an abundance of ER, mitochondrial, and lysosomal proteins (18). In skeletal muscle, glycogen interacts with several subcellular compartments, including myofilaments, mitochondria, and the muscle specialized form of the ER, the sarcoplasmic reticulum (SR) (61–63). An SR-glycogenolytic complex has been described in fast-twitch skeletal muscle. It is proposed that at this complex, phosphorylase-mediated glycogen breakdown provides glucose-1-phosphate availability for glycolytic ATP production (62). Glycolytic enzymes are colocalized with the SR ATPase Ca^{2+} (SERCA) pumps (64). Thus glycogen-derived glycolytic ATP supply may facilitate Ca^{2+} reuptake into the SR to ensure muscle relaxation during the contractile cycle. Additionally, Stbd1 also contains ER-binding domains (45). Therefore, it seems likely that the ER–SR glycogen complex includes glycophagy protein mediators, and the concept of an ER-glycogen-phagophore "hub" is credible (65). Further work is required to fully characterize the process of glycophagosome initiation and confirm the role of glycophagy in the ER-glycogenolytic complex.

Glycophagosome cargo capture—the role of the glycophagy "Atg8 partner"

After initiation of autophagosome formation, the autophagy receptor protein–cargo complex is captured into the forming autophagosome by binding to an Atg8 protein "partner" (Fig. 1). Yeast have a single Atg8 gene and mammalian cells have an Atg8 protein family consisting of the Lc3 (Lc3a, Lc3b, Lc3c) and Gabarap (Gabarap, Gabarapl1, Gabarapl2) subfamilies, with high sequence similarity (66). Atg8s are ubiquitin-like proteins that participate in membrane trafficking and autophagy. The lipidation of Atg8 anchors them into the autophagosome membrane to mediate capture of the autophagy cargo.

Evidence for Gabarapl1 as a glycophagy Atg8 partner protein

In an autophagy network analysis, the glycophagy receptor, Stbd1, interacted with all six Atg8 family members in HEK293T cells using GST pull-down assays (12). A biotinylated peptide screen revealed that the binding affinity of Stbd1 is ∼10-fold higher for Gabarap compared to Lc3b (other Atg8 subfamily members not assessed) (67). Similarly, an Stbd1 yeast two-hybrid screen identified Gabarap and Gabarapl1 as potential binding partners for Stbd1 (10). Coexpression *in vitro* validation experiments in COSM9 cells indicated that Stbd1 only partially colocalizes with Gabarap while its cellular distribution pattern fully aligns with that of Gabarapl1, producing large perinuclear structures (10). Overexpressed Gabarapl1 localized to the ER and *trans*-Golgi network in CHO cells (68), which is similar to the pattern observed with overexpressed Stbd1 (10, 45). Targeted mutagenesis revealed that the aa 203 to 206 AIM on Stbd1 is essential for Stbd1–Gabarapl1 interaction (11, 69) but not for perinuclear localization (11, 45) while the opposite was evident for the aa 212 to 215 AIM (11). These findings suggest that the aa 203 to 206 AIM might be the primary target for Gabarapl1 interaction, and the aa 212 to 215 AIM is primarily involved in localization of Stbd1 (Fig. 2). Furthermore, mutation of the Stbd1 aa 203 to 206 AIM site induces glycogen accumulation in cancer cells *in vitro* (69), consistent with the contention that Gabarapl1 binding to this Stbd1 AIM is an essential step in glycophagy-mediated degradation of glycogen. Further work is required to understand the complex molecular interplay of these AIMs in mediating glycophagy receptor–Atg8 partner interaction. Collectively, these *in vitro* studies provide a convincing case for Gabarapl1 to be the most likely candidate for a glycophagy Atg8 "partner" protein, recruiting Stbd1-bound glycogen into the forming glycophagosome. Investigations in an *in vivo* setting are now a priority.

Distinct Gabarapl1 actions

A strong evidence base for Gabarapl1 involvement in glycophagy is developing, and there is some indication that Gabarapl1 molecular mechanisms are distinct from other Atg8

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family proteins (70). Despite the high sequence similarity between the six Atg8 proteins, computational analysis has revealed that differences in the 3D conformation of Atg8s correspond to a high level of functional specificity (66). For example, differential interaction with Unc-51 like autophagy activating kinase 1 (Ulk1), an essential mediator of the early stages of autophagosome biogenesis, is evident between different members of the Atg8 family of proteins. This has been shown by the use of nutrient starvation, a potent autophagy stimulus, which increased Gabarapl1 (but not Gabarap or Gabarapl2) binding to Ulk1 in HEK293T cells (71). Similarly, in neural stem cells, insulin withdrawal enhanced Ulk1–Gabarapl1 but not Gabarapl2 interaction (72). *In vitro* systematic deletion of the Atg8 genes demonstrated that Gabarap and Gabarapl1 (but not Gabarapl2) promote Ulk1 activity. In contrast, Lc3b and Lc3c (but not Lc3a) negatively regulate Ulk1 activity (71). Although further work is required to fully link these findings to glycophagy activity, it can be hypothesized that Gabarapl1 promotes glycophagy initiation *via* binding to Ulk1, in a manner distinct from other Atg8s.

Additionally, the mechanisms of autophagosome–lysosome fusion appear to be different between the Gabarap and Lc3 subfamilies, which may have important implications for glycophagy. The "Bruce" protein (baculovirus IAP repeat-containing ubiquitin-conjugating enzyme) has been implicated in autophagosome–lysosome fusion (Fig. 1) (73, 74). In mouse embryonic fibroblasts, Bruce KO led to accumulation of autophagosomes and prevented Atg8 localization in lysosomes (74). Interestingly, Bruce selectively interacts with Gabarapl1 (and Gabarap) and not with other members of the Atg8 protein family (74). Similarly, ectopic P granules protein 5 (EPG5) is involved in autophagosome–lysosome fusion and binds preferentially to the members of the Gabarap subfamily over the Lc3 subfamily, demonstrated by GST pull-down assays (75). These findings suggest that autophagosome–lysosome fusion may have distinct mechanisms for Gabarapl1-tagged autophagosomes, and it could be speculated that these pathways may be selectively involved in glycophagosome processing. It is so far unclear whether autophagosomes exclusively contain Gabarap or Lc3 Atg8s, but these distinct fusion mechanisms may have important implications for delineating glycophagy from other autophagy processes and require further investigation.

Regulation of glycophagy & lysosomal glycogenolysis

Signaling regulation of glycophagy

Very few studies have directly investigated the signaling pathways governing glycophagy induction, but some insights into the regulation of glycophagy can be extrapolated from studies investigating the regulation of lysosomal Gaa activity, that is, at the end-stage glycophagy process. A role for β-adrenergic signaling has been advanced. In neonatal rats, *in vivo* systemic administration of the β-adrenergic agonist, adrenaline, upregulated cardiac and hepatic Gaa activity (37), and a β-adrenergic receptor antagonist, propranolol, decreased hepatic Gaa activity (76). Similarly, administration of the downstream second messenger involved in β-adrenergic signaling, cAMP, activated Gaa *in vivo* (76) and *in vitro* (77). Collectively, these studies suggest that glycophagy responsiveness to β-adrenergic signaling is apparent. Given that β-adrenergic receptors mediate sympathetic nervous system actions in several tissues, the concept that glycophagy may be promoted by β-adrenergic signaling is consistent with the contention that glycophagy-mediated glycogen breakdown may play a role in increasing glucose availability in settings of high energy demand.

In addition, evidence suggests that insulin-mTOR signaling may negatively regulate Gabarapl1 and Gaa. In neonatal rats, *in vivo* treatment with the mTOR inhibitor, rapamycin, increased cardiac Gaa activity (37), and in mouse embryonic fibroblasts, *in vitro* mTOR activation *via* Tsc2 KO was associated with decreased glycophagy activity (78). Insulin-stimulated Akt signaling is a known inhibitor of the forkhead box O (FoxO) transcription factor family, and Gabarapl1 has been identified as a transcription target of FoxO1 and FoxO3. Transfection of constitutively active FoxO1 and FoxO3 in neonatal rat cardiomyocytes upregulated Gabarapl1 mRNA expression (79). Nutrient starvation in this setting downregulated insulin signaling and increased binding of FoxO1 and FoxO3 to the promotor region of Gabarapl1 (79). However, not all studies support a role for insulin-induced inhibition of glycophagy. Activation of mTOR *via* insulin treatment in C2C12 myotubes did not alter Gaa activity (77). In primary cardiomyocytes cultured in high glucose, insulin increased Stbd1 protein, independent of insulin-augmented Akt activation (8). More work is required to understand the nuances of insulin signaling and glycophagy. Given that insulin signaling (involving mTOR and FoxO transcription factors) is a well-known inhibitory pathway of autophagy (80), it could be expected that glycophagy is regulated in similar manner.

Gaa transcription may be regulated by Gabarap Atg8 availability. Evidence for this has been found in some cell lines. Gabarap subfamily (but not Lc3 subfamily) Atg8s promote TFEB translocation to the nucleus in response to nutrient starvation in HeLa cells (81). Gene network analysis in HeLa cells has identified TFEB as a transcription factor for most lysosomal genes, including Gaa (82). In a different cell line, C2C12 myoblasts, contraction-induced TFEB upregulation was not associated with changes in Gaa mRNA expression (77), which may reflect tissue specificity of TFEB regulation. Tissue-specific regulation of glycophagy by Notch1 signaling has also been demonstrated. Inhibition of Notch1 signaling decreased Gaa activity in C2C12 myoblasts (77), but activation of Notch1 decreased Gaa expression in hepatocytes (83). Thus, Notch1 appears to positively regulate Gaa in skeletal muscle cells but negatively regulate Gaa in liver cells. Collectively, these studies provide evidence to suggest that glycophagy and subsequent lysosomal glycogen degradation is regulated by energy signaling pathways involving β-adrenergic and insulin-mTOR signaling, with potential tissue-specific involvement of TFEB and Notch1 pathways. These initial findings provide the basis for future robust interrogation of the regulatory pathways involved, particularly in the context of understanding

glycophagy regulation that may be distinct from other autophagy subtypes.

Glycophagy is linked with energy metabolism

The signaling pathways involved in regulating glycophagy are primarily related to meeting cellular energy demand, and there is accumulating evidence linking glycophagy-mediated glucose availability to energy metabolism. We have shown that glycophagy is involved in cardiac glycogen homeostatic regulation in relation to fasting-induced metabolic stress *in vivo* (9). *In vitro* data evaluating shifts in Stbd1 levels with glycolysis activity are discrepant, possibly reflecting differences in cell origin—oncogenic *versus* developmental metabolic settings (69, 84). Knockdown of Gaa in skeletal muscle cells (C2C12 myotubes) was associated with decreased glycolytic metabolic rate (77), further supporting the contention that glycophagy provides glucose substrate for glycolysis. A shift from glucose oxidation to increased fatty acid oxidation was also evident (77). Downregulation of glucose metabolism in the Gaa knockdown cells was associated with signs of cellular energy stress, demonstrated by increased activity of the key energy sensor, AMPK, and increased signaling *via* PPARα, a major regulator of energy homeostasis (77). AMPK has been identified in an *in vitro* chemical genetic screen to phosphorylate Stbd1 (43) and may be involved in regulation of both induction and end-stage glycophagy steps.

Glycophagy also appears to play a role in mediating skeletal muscle glycogen utilization and recovery with exercise. Elevated Gaa activity is associated with glycogen depletion in contracting C2C12 myotubes, and low Gaa activity is associated with glycogen supercompensation postexercise in human subjects (77). These findings linking glycophagy to energy metabolism suggest that glucose released from the lysosome following glycogen breakdown may provide glycolytic fuel for ATP production. It is clear that glycophagy is closely linked with cellular glucose availability as glycophagy activity is high during the postnatal period of glucose starvation in newborn rats. Systemic administration of glucose during this period abolishes the postnatal glycophagy response (76). Collectively, these studies suggest that glycophagy and subsequent lysosomal glycogenolysis may provide an important route of glucose substrate supply. Advancing understanding of the mechanisms underlying lysosomal glucose release and subsequent metabolism is an important priority.

Lysosomal glucose transport

The mechanism of glucose liberation from the lysosome has received very little attention in the literature. There has been some indication that the Glut8 transporter may be involved in lysosomal glucose release but conflicting reports exist. Glut8 exhibits an endosomal/lysosomal-targeting sequence (85, 86), and in Glut8-stably expressing CHO and MB231 cells, Glut8 colocalized with the lysosomal protein Lamp1 and was detected in lysosomal membranes (85, 87). Furthermore, inhibition of the lysosome with chloroquine or bafilomycin resulted in an accumulation of Glut8 (87). However, not all reports support a role for Glut8 in the lysosome. In PC12 neuronal cells, Glut8 colocalized with markers for the ER but not the lysosome (88). Other studies show that Glut8 is an insulin-sensitive plasma membrane transporter, which participates in cellular glucose uptake in the heart and other tissues (89, 90). Yet, an *in vitro* study screening gain and loss of function of Glut8 showed that knockdown of Glut8 does not influence 2-deoxyglucose uptake (87). Thus, whether Glut8 is operational as a lysosomal glucose transporter and has a role in glycophagy has not been fully elucidated.

An alternative/additional lysosomal glucose transporter candidate is the transmembrane protein, spinster 1 (Spns1, also known as "benchwarmer" in *Drosophila*). *In silico* analysis has identified that the sequence and structure of Spns1 has high similarity with proteins from the sugar transporter subfamily, and Spns1 has been classified as a predicted sugar transporter (91). Spns1 subcellular localization is closely aligned with lysosomes, as evidenced by colocalization of GFP-labeled Spns1 with the lysosome marker, Lamp1, and the acidophilic marker, lysotracker (92, 93). Knockdown of Spsn1 in rat kidney epithelial cultured cells is associated with enlarged lysosomes and increased periodic acid schiff-stained glycogen concentrated in round vesicle-type structures (potentially lysosomes) (92). Collectively, these studies provide some evidence to suggest a role for Glut8 and/or Spns1 in mediating lysosomal glucose release, and more work is required to fully elucidate the protein intermediaries involved in this process.

Open questions & challenges for the field

The glycophagy field is at a developing stage and there are important knowledge gaps providing opportunities for future investigation. While evidence of Stbd1 and Gabarapl1 involvement in glycophagy is becoming more definitive, investigation into alternative/additional glycophagy-specific protein intermediaries involved is an important next step. Precise experimental approaches are required to delineate candidate protein involvement in glycophagy *versus* other autophagy subtypes, and this represents a significant challenge in moving the field forward. There is some evidence (mostly in cell lines) that the lysosomal stage of glycophagy is regulated by β-adrenergic, insulin, and AMPK signaling, and further validation *in vivo* is a priority. The signaling regulation of glycophagy induction (glycogen recruitment and capture into the phagosome) has received very little attention in the literature. At present, the lack of understanding of specific glycophagy regulatory signals has obviated the development (or repurposing) of pharmacological tools to manipulate glycophagy with high specificity.

Another open question in the field relates to what factors determine whether glycogen is degraded in the cytosol or in the lysosome. As detailed in previous sections, some evidence suggests that high molecular weight, less-branched, and hyperphosphorylated glycogen may be preferentially targeted to glycophagy (*versus* cytosolic enzymatic) processing, but the evidence is anecdotal, and the factors which determine

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glycogen fate are not yet fully elucidated. Also awaiting resolution is the matter of whether autophagosomes exclusively contain either Gabarap or Lc3 Atg8's—to what extent does the cargo drive the trafficking journey? Other key questions yet to be addressed relate to the process of lysosomal glucose handling and export following glycogen degradation. Similarly, questions about the cellular localization of glycophagosome formation and delivery of cargo glycogen breakdown products remain to be tackled.

Beyond inherited glycogen storage disorders, there is some indication that disturbances in glycophagy may play a role in disease settings involving energy stress (5, 94, 95). To date, autophagy is an elusive target for disease intervention (96, 97), and modulating selective-autophagy processes may be a more viable therapeutic strategy. Given that glycophagy is a fundamental cellular process positioned at the interface of autophagy and regulated metabolism, it has potential to be an important target for metabolic disease intervention (98). Development of precision tools to target glycophagy with high specificity to test efficacy in disease rescue will be an important challenge in the next phase of this research enterprise.

Conclusion

Recent advances in understanding selective-autophagy pathways have opened up new areas of investigation in relation to the metabolism of key metabolic substrates, including glycogen. Glycophagy has emerged as a noncanonical pathway of glycogen flux, and recent findings reveal a role for glycophagy in mediating physiological carbohydrate metabolism. The field of glycophagy research is at an early stage, yet already glycophagy involvement in metabolic stress pathologies is apparent. The current state of evidence suggests that specific glycophagy protein machinery involves Stbd1 as the glycophagy "receptor," Gabarapl1 as the "Atg8 partner" autophagosome protein and acid α-glucosidase as the lysosomal glycogen degradation enzyme. Additional protein mediators may emerge with future research focus. Given the challenges of translating autophagy therapeutics to clinical application, highly specific interventions targeting glycophagy may yield valuable outcomes in several disease contexts.

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Abbreviations—The abbreviations used are: AIM, Atg8-interacting motif; ER, endoplasmic reticulum; Gaa, acid α-glucosidase; GDE, glycogen-debranching enzyme; HA, hemagglutinin; SR, sarcoplasmic reticulum.

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