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Regulation of GLUT4 glucose transporter trafficking

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Abstract

REGULATION OF GLUT4 GLUCOSE TRANSPORTER TRAFFICKING. Leah McNally and Jonathan Bogan, Departments of Internal Medicine and Cellular Biology, Yale University School of Medicine, New Haven CT.

In fat and muscle cells, insulin stimulates glucose uptake by causing the translocation of GLUT4 glucose transporters out of intracellular membranes and to the plasma membrane. Impaired GLUT4 translocation results in insulin resistance, and contributes to the pathogenesis of type 2 diabetes. Yet, how insulin signaling and protein trafficking pathways intersect remains poorly understood. In 3T3-L1 adipocytes, data support a model in which TUG ("tether, containing a UBX domain, for GLUT4) binds GLUT4 and retains it intracellularly in the absence of insulin. Insulin then signals the release GLUT4 from TUG, which mobilizes GLUT4 to the cell surface. How and where TUG retains GLUT4 intracellularly remains unknown. Previous data show that the TUG carboxyl terminus is required for intracellular retention of GLUT4, but is dispensable for binding to GLUT4 itself. Therefore we hypothesized that this region interacts with an unidentified, intracellular "anchoring" protein. Here, we tested if GCC185 may be this sought-after protein, to which TUG links GLUT4 in unstimulated cells. GCC185 is a Golgi matrix protein that captures vesicles arriving at the trans-Golgi network (TGN) from endocytic or biosynthetic pathways. It tethers the vesicles to the TGN membrane and promotes their fusion at the TGN. It has previously been suggested that GLUT4 may by retained by an intracellular cycle of fusion and budding at the TGN in unstimulated

cells. To test the hypothesis that TUG cooperates with GCC185 to facilitate such a cycle, we performed coimmunoprecipitation experiments. We found that TUG interacted with GCC185 in cotransfected 293 cells. Importantly, this interaction required the TUG carboxyl terminus, as predicted for the "anchoring" protein. The TUG-GCC185 interaction was confirmed in reciprocal coimmunoprecipitation experiments. Mutagenesis identified a particular residue in TUG that is likely involved in this interaction, which may be modified to control the binding of TUG and GCC185. A second project was prompted by the observation that Ubc9 is another protein that binds GLUT4 and promotes its accumulation in insulin-responsive storage vesicles. Because Ubc9 is a conjugating enzyme for the ubiquitin-like protein, SUMO, we tested the hypothesis that TUG is a target of SUMO modification. However, no data were obtained to support this hypothesis. In summary, our data show that GCC185 and TUG interact, and support a model in which GCC185 participates in targeting GLUT4 to vesicles that are mobilized acutely by insulin to control glucose uptake.

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Introduction

It has been estimated that by 2030, the number of people suffering from type 2 diabetes will reach some 366 million (1). It is also predicted that type 2 diabetes will increasingly become a worldwide phenomenon, with nations that traditionally had low disease rates (e.g. those in Africa and Southeast Asia) seeing a steep rise in disease prevalence over the coming years. Therefore, understanding the pathophysiology of the disease and identifying new targets for therapy, have become increasingly important.

Blood Glucose Homeostasis and the GLUT4 Transporter

Under physiological conditions, blood glucose concentrations are very tightly controlled, so that variation is minimized post-prandially or in times of fasting. The main hormone that regulates blood glucose concentration is insulin, which both suppresses hepatic glucose production and drives glucose uptake into adipose and muscle cells (2). Adipocytes and myocytes contain GLUT4 glucose transporters, which contain twelve membrane-spanning domains and, together with GLUT1, are the main glucose transporter present in these tissues. GLUT4 proteins are facilitative transporters that are inserted into the plasma membrane in response to insulin signaling (3, 4, 5). This distinguishes them from GLUT1 proteins, which are present at the plasma membrane even at the basal state. Their insertion into the membrane can occur rapidly because the transporters are targeted to and stored in specific, intracellular vesicles -termed GLUT4 storage vesicles (GSVs)- within unstimulated cells. After insulin has finished signaling, GLUT4 is endocytosed and sent back through the recycling pathway into GSVs, to await the next stimulus (6).

Thus, the process is reversible. The number of GLUT4 transporters present in the plasma membrane controls the overall rate of glucose uptake into fat and muscle cells. In addition, insulin appears to both increase exocytosis of GLUT4 and to slightly decrease its endocytosis (7).

Clearly, GLUT4 plays a key role in maintaining glucose homeostasis. One could hypothesize that defects in GLUT4 or its movement to the plasma membrane are at least partially responsible for the hyperglycemia of the insulin resistant and type 2 diabetic state. However, one of the first questions that was asked and answered in regards to this hyperglycemia was whether it was chiefly due to faulty movement of glucose across the plasma membrane (suggesting a role for GLUT4) or to faulty utilization of glucose once inside these cells. Studies utilizing NMR strongly supported the hypothesis that the problem lay in the transport of glucose into the cell from the bloodstream and not in its utilization within the cell (8, 9). Once this was established, the cause of this faulty glucose movement became the next and ongoing focus, with the role of GLUT4 translocation being a central theme (10, 11).

As reviewed above, GLUT4 transporters are the insulin-responsive channels through which glucose traverses the plasma membrane in mycocytes and adipocytes. Defects in the presence of GLUT4 at the plasma membrane are correlated with insulin resistance. In mouse models, decreased presence of GLUT4 at the plasma membrane through partial GLUT4 knockdown results in insulin resistance (12, 13, 14, 15). In addition, in humans with insulin resistance or type 2 diabetes, there is decreased GLUT4 at the plasma membrane in response to insulin (16). Thus it can be said that the translocation of GLUT4 to the plasma membrane in

response to insulin is not only defective in insulin resistance and type 2 diabetes, but also a major contributor to the pathophysiology. However, multiple mechanisms likely contribute to these defects. These include faulty insulin signal transduction (17, 18), down-regulation of GLUT4, and mislocalization of GLUT4 away from the GSV compartment (19, 20, 21).

GLUT4 Localization to GSVs

To understand the concept of GLUT4 mislocalization, what is known about the formation of GSVs should be reviewed. Even the very existence of GSVs took time to demonstrate. In the non-insulin stimulated state, GLUT4 is mainly localized to intracellular membrane compartments (22, 23). In fibroblasts (preadipocytes), exogenously expressed GLUT4 is thought to cycle between endosomes and the trans-Golgi network (TGN) (24, 25). However, in mature adipocytes, GLUT4 is largely excluded from endosomes. This has been shown in studies that used horse-radish peroxidase-conjugated transferrin to ablate compartments of the recycling pathway (24). In fibroblasts, this results in the destruction of the majority of GLUT4. However, when this is done in mature adipocytes, over 50% of GLUT4 remains unaffected, suggesting that it is sequestered in specialized compartments, namely GSVs.

The existence of GSVs is also supported by the finding that vesicles enriched in GLUT4, IRAP and VAMP2, but lacking common endosomal proteins such as transferrin and cellubrevin, have been isolated and appear to be insulin-responsive (26). It has been further shown that these small, specialized vesicles are not found in fibroblasts, and that the development of these vesicles correlates with adipocyte

maturation and insulin responsiveness. The production of GSVs in maturing adipocytes is linked to the expression of the cargo adaptor protein sortilin (27, 28, 29). Importantly, this work further demonstrated that exogenous expression of sortilin in 3T3-L1 preadipocytes resulted in GSV formation and that sortilin knockdown in 3T3-L1 adipocytes prevented the formation of GSVs. As adipocytes mature, GLUT4 can be seen to co-localize with TGN markers such as syntaxin 6 and 16. This suggests that at least some steps of GSV formation traffic through this region of the TGN. This theory is further supported by the finding that IRAP, an integral protein in GSVs, has sugar modifications consistent with its passage through the TGN (21).

Additional data supporting the existence of GSVs comes from the observation that the half-life of GLUT4 is significantly shorter in mature adipocytes than it is in fibroblasts. This suggests the GLUT4 has been sequestered from the general recycling pathway wherein it can be readily degraded by targeting to lysosomes. Interestingly, following stimulation with insulin, the rate of GLUT4 degradation increases, suggesting that insulin mobilizes GLUT4 from this sequestration compartment. Final evidence for the GSV compartment comes from the finding that there appears to be a dose dependent response of GLUT4 mobilization following insulin stimulation (30). In this study, low insulin doses resulted in only 10-20% of intracellular GLUT4 cycling through the plasma membrane, while high insulin doses resulted in ~ 70% of GLUT4 cycling though the plasma membrane. This graded response would fit with the presence of a sequestered GLUT4 pool (GSVs) that can be accessed to different extents in the presence of insulin. Taken all together, the

evidence points to the existence of GSVs whose formation likely involves steps at a sub-domain of the TGN.

Direct evidence for the mislocalization of GSVs in the insulin resistant state, type 2 diabetes and gestational diabetes comes from cell fractionation studies (19, 20, 21). In studies examining tissue from healthy subjects, GLUT4 is found in the light membrane (ie cell surface) compartment in adipocytes and mycocytes. However, in insulin resistant and diabetic states, it is found chiefly in the dense membrane (ie intracellular) compartment. One study also showed that IRAP's (one of the two protein known to associate with GSVs) distribution was similarly altered. These studies used biopsies from fasting individuals, suggesting that GLUT4 is not being compartmentalized properly within unstimulated adipose and muscle cells. These reports raise the possibility that unless GLUT4 is selectively trafficked to the GSV compartment, it will not be accessible to insulin signaling. Additional studies in diabetic patients have also highlighted the importance of proper GSV formation. For example, obese patients with increased TNF-alpha expression showed down-regulation of sortilin (31). As sortilin is necessary and sufficient for GSV formation in tissue culture models, this linkage of sortilin expression to an insulin resistant state, again emphasizes the importance of proper GLUT4 localization in the pathophysiology of type 2 diabetes.

A Role for TUG in GLUT4 Trafficking

Despite the apparent importance of GSV formation and GLUT4 localization, exactly how GLUT4 are targeted to and kept in GSVs, as well as how insulin mobilizes these vesicles to the cell surface, is not well understood. This area is the

main focus of work in the Bogan laboratory. In particular, work on a protein called TUG (tether, containing a UBX domain, for GLUT4) has shown promise in clarifying this issue. Data support the notion that in cultured 3T3-L1 adipocytes, TUG binds directly to GLUT4 and sequesters it specifically in GSVs, thus excluding it from the plasma membrane (32, 33, 34, 35). Overexpression of TUG appears to enhance targeting of GLUT4 to GSVs, and increases the insulin-responsive pool of GLUT4 proteins. Conversely, a decrease in TUG abundance (using RNAi) or inhibiting its action (using a truncated, dominant negative form, termed UBX C-terminal or UBX-Cter) prevents the accumulation of GLUT4 in GSVs. Truncation of the C-terminal of TUG (anywhere from amino acid 270 on) prevents TUG's ability to sequester GSVs away from the plasma membrane. Importantly, disruption of TUG action (using RNAi or the dominant negative form) results in targeting of GLUT4 to the cell surface, and mimics insulin action to a large extent. This fits with the idea that TUG functionally tethers GLUT4 to an intracellular anchoring site within unstimulated cells. In this proposed model the TUG in association with GLUT4 in GSVs binds to an anchoring site and thereby localizes the GSVs properly, in a configuration from which they can be mobilized, within cells. However, it should be noted that the association of TUG with GSVs and the anchoring site is not necessarily a static one. For example, GSVs may be allowed to move within the TGN sub-domain while remaining sequestered from the general recycling pathway. The molecular nature of this anchoring site is not fully understood. Nonetheless, data are consistent with the idea that insulin stimulates dissociation of a TUG-GLUT4 protein complex to release GLUT4 to the cell surface, and to enhance glucose uptake.

Present data from the Bogan laboratory support a model in which following insulin-stimulation, TUG is proteolytically processed and gives rise to a new ubiquitin-like modifier, termed TUGUL (for TUG ubiquitin-like). TUGUL joins a family of about 11 known ubiquitin-like modifiers (Ubls), including ubiquitin itself (36, 37, 38). These peptides are generally synthesized as part of larger, precursor proteins, which are cleaved in a site-specific manner to liberate the mature Ubl (as the N terminal cleavage product). The Ubl can then be attached covalently to target proteins or lipids. In the case of TUG, cleavage is a regulated event that occurs rapidly after insulin stimulation in 3T3-L1 adipocytes. TUG cleavage separates an N terminal region (TUGUL), which binds GLUT4, from C terminal regions, which bind the anchoring proteins. Preliminary data suggest that TUGUL moves with GLUT4 to the plasma membrane, and the C-terminal product remains behind and is degraded by the proteasome. This model predicts that subsequently endocytosed GLUT4 would require intact, newly-synthesized TUG protein for retention in GSVs of unstimulated cells. Most TUG resides in the cytosol, and it may be recruited to membranes containing endocytosed GLUT4 from this large reservoir.

In the sustained presence of insulin, endocytosed GLUT4 may not cycle through GSVs, and there need not be ongoing TUG processing and degradation. Thus, TUG is an essential compartment of a retention mechanism that is likely engaged only in the absence of insulin.

Ubc9 and GLUT4 Trafficking

Another protein that likely plays a crucial role in the targeting and storing of GLUT4 in GSVs is Ubc9, a SUMO conjugating enzyme (39, 40). Ubc9 binds to the

GLUT4 C terminus, and controls the accumulation of GLUT4 in GSVs in L6 myoblasts and 3T3-L1 adipocytes. Similar to ubiquitin conjugating enzymes, Ubc9 catalyzes the covalent attachment of a Ubl, SUMO, to target proteins. SUMO is 12 kD, and its attachment to target proteins modulates their activities or intracellular locations (41, 42, 43). The Ubc9 data suggest that SUMO modification ("SUMOylation") may function in GLUT4 trafficking. Of note, present data from the Bogan laboratory shows the C terminal remnant of TUG is observed in both 42 kDa and 54 kDa forms. The expected size of the cleavage product is 42 kDa, and the 54 kDa form may be a modified form of the 42 kDa cleavage product. One possibility, which was examined during the course of this work, was that the TUG C-terminal product is SUMOylated, and that this modification of the TUG C-terminal cleavage product may be involved in removing it from the anchoring site. This would vacate the anchoring site so that it is available for subsequent cycles of GLUT4 retention and release.

Usually, SUMO modifications are added to a lysine residue amidst a consensus site: aliphatic residue-lysine-any amino acid-glutamic acid (44). Several residues meeting these criteria can be found within TUG. However, as will be discussed, multiple experiments were not consistent with this idea of TUG SUMOylation. This raised the possibility of the putative TUG modifier being something other than SUMO.

In addition to SUMOylation, other common post-translational protein modifications include acetylation, methylation, ubiquitination, and ADP-ribosylation. Of note, these other modifications also frequently occur at lysines. In addition, previous work has shown that some lysines are alternatively acetylated

and SUMOylated, which made acetylation the logical modification to consider next (45, 46, 47, 48). Further, the UniProtKB online database showed that one of TUG's C-terminal residues was a site of acetylation (ref Q9BZE9**)**. A recent paper showed that lysine modification of the enzyme ornithine carbamoyltransferase decreased the affinity of this enzyme for one of its substrates. In addition, the acetylation of this enzyme was dependent on the extracellular glucose and amino acid availability (49). This resulted in the hypothesis that TUG acetylation may alter its affinity for a GSV anchoring protein, allowing GLUT4 to be released to the plasma membrane. In addition, this acetylation could be dependent on either extracellular glucose concentration, or the insulin signaling cascade. The idea that acetylation can be due to the concentration of metabolic products is also supported by work showing that deacetylation causes breakdown of NAD to produce nicotinamide and ADP-ribose. Build up of nicotinamide shifts the homeostasis to favor acetylation and build up of NAD favors deacetylation. Thus, if TUG's acetylation state alters its affinity for the anchor and this in turn affects the localization of GLUT4, the concentration of metabolic products- such as NAD- could have a role.

GCC185 as a Putative Anchor

A final question to consider is where GSVs are anchored within the cell. The membrane density work done in diabetic humans strongly suggested that proper GLUT4 localization played a key role in insulin-responsiveness. TUG is known to bind GLUT4 within its N-terminus and that deletion of the C-terminus of TUG results in its inability to retain GLUT4 intracellularly. This suggests that the C-terminal binds to an anchoring site for GSVs. Again this term "anchor" is used in the

functional sense and is not meant to imply a static interaction. Given that GSVs seem to at least traffic through the TGN, it is possible that a protein associated with the TGN is the anchor (50). One candidate for this anchor is the known tether Golgin Coiled-Coil protein 185 (GCC185). Golgin proteins are integral to the Golgi complex and frequently act as anchors for vesicles moving to and from this organelle.

Three reasons highlight GCC185 as a putative anchor. 1. This protein has been shown to localize to the TGN and to be a necessary tether for transport vesicles coming from endosomes (51). This model was best worked out in regards to the mannose-6-phosphate receptor (M6PR), a receptor for lysosomal enzymes that traffics between late endosomes/lysosomes and the TGN. After releasing its cargo to lysosomes, M6PR recycles back through the TGN, using GCC185 as its anchor. Without this interaction, M6PR's are mislocalized and instead found at random locations within in the cell. A similar model could be imagined for GLUT4 vesicles, which also must recycle back from the plasma membrane. Also of note, when GLUT4 colocalizes with syntaxin 6/16 at the TGN, this same subdomain is also labeled with M6PR (12, 22). This further emphasizes the idea that GCC185 may be an anchor for both the M6PR and for GLUT4. 2. GCC185 has been shown to interact with Rabs (52, 53, 54). Rabs are small GTPases that are active when GTP-bound and inactive when GDP-bound. They have diverse roles (54, 55). Those relevant to the topic at hand include vesicle budding, tethering, and fusion (57); vesicle motility when molecular motors are involved (58); and control of signaling pathways. Rabs are known to be involved in these steps in GLUT4 trafficking, and since GCC185 has been shown to associate with multiple Rabs, it is possible that it is during its association with

GCC185 that GSVs are formed and then later moved from their intracellular site to the plasma membrane (56, 59). This brings up reason 3: GCC185 anchors non-centrosomal microtubules. GLUT4 uses both actin and microtubules to arrive at the plasma membrane (60). In particular, insulin loads GLUT4 onto Kif5B microtubule motors to mobilize it from the perinuclear region, near the Golgi apparatus, and to target it to the cell periphery (61). It is possible that GSVs are loaded from their site in association with GCC185 onto these microtubules to traffic out to the plasma membrane in response to insulin.

Summary

Clarifying how GLUT4 is targeted and stored in its specific vesicles is key to understanding the cascade of events after insulin signaling. Data suggest TUG associates specifically with GLUT4 that is stored in GSVs, and that these GSVs may be functionally tethered at the TGN to await insulin signaling. Identification of the anchoring protein that TUG uses to hold GSVs in place as well as better characterization of how GSVs are mobilized to the plasma membrane will clarify how insulin acts to control GLUT4 targeting and glucose uptake.

Specific Aims:

The long-range goal of the project proposed here is to better understand GSV formation, localization, and insulin-induced translocation. As outlined above, it was hypothesized that TUG acts as a functional tether between GLUT4 within GSVs and an anchoring protein at the TGN (see Figure 1). It was hypothesized that this TGN anchor protein is GCC185. Additionally, it was hypothesized that TUG modification participates in a biochemical mechanism for the release of GSVs to the plasma membrane. This modification may occur at a lysine residue in the C-terminal of TUG and was considered to be either SUMOylation or acetylation. To test these hypotheses, here it is proposed:

1. To characterize interactions among GLUT4, GCC185, and TUG, and to learn if these proteins bind each other simultaneously as a complex in HEK293 and unstimulated 3T3-L1 adipocytes.
2. To learn if TUG is SUMOylated or acetylated, to define the site, and to study the functional role of this modification.

Accomplishment of these aims will clarify biochemical mechanisms that mediate the action of insulin to enhance glucose uptake. These mechanisms have high relevance for the pathogenesis of insulin resistance and diabetes.

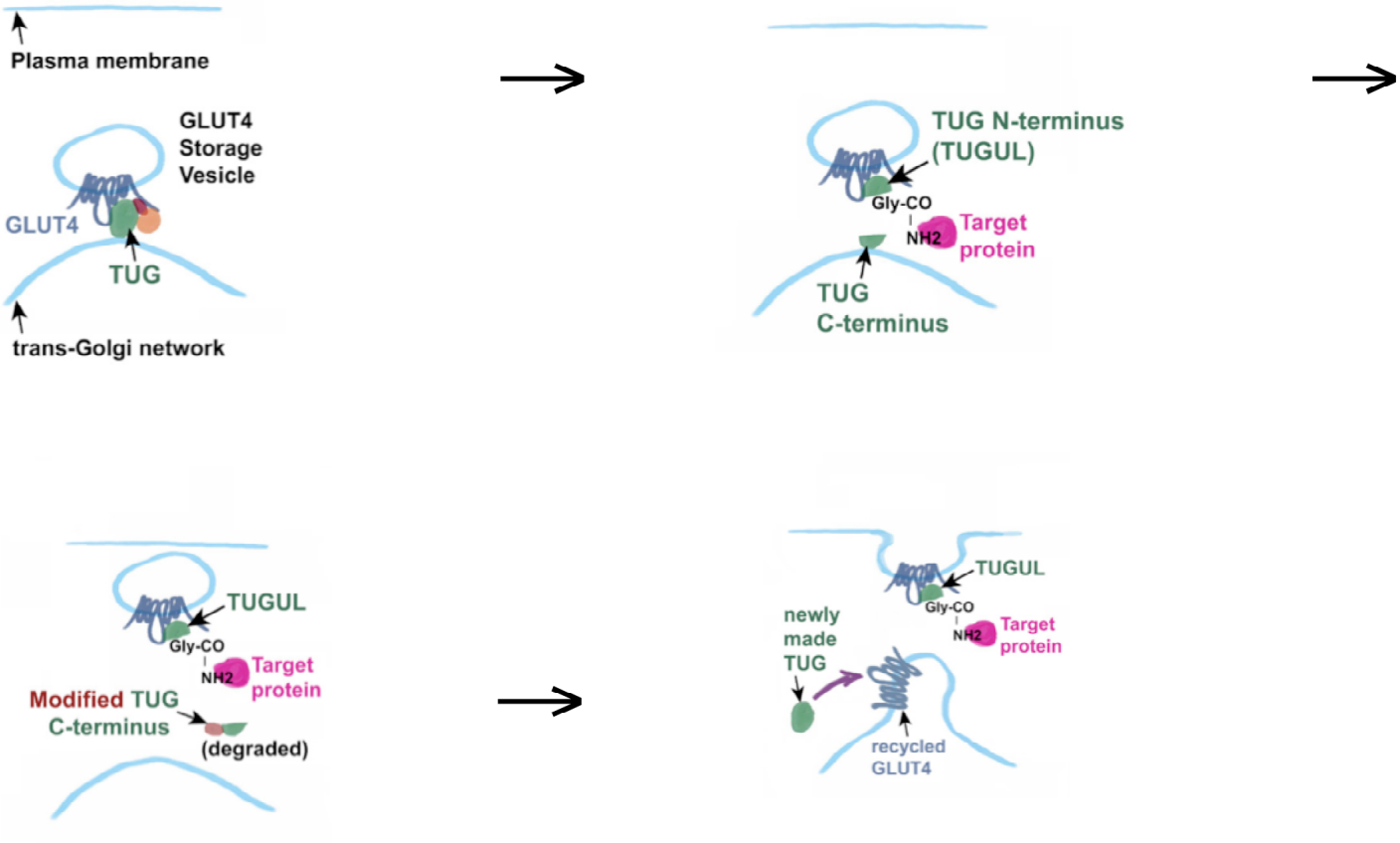

Figure 1. We hypothesize that TUG binds to both GLUT4 and an anchoring protein, possibly GCC185, at the TGN (not shown). Following insulin stimulus, TUG is cleaved, freeing GLUT4 to go to the plasma membrane. The C-terminal fragment of TUG is then modified, removed from the anchoring site, and degraded. This opens up the binding site at the anchoring protein for new TUG-GLUT4 complex to bind and for the cycle to begin anew in the basal state.

Methods:

Aim 1a. To test whether GCC185 binds to TUG as well as to GLUT4, initial experiments employed coimmunoprecipitation (Co-IP) from lysates of transiently transfected 293 cells. Transfection was carried out by treating plates of 293 cells grown in DMEM and 10% Bovine Growth Serum with a mixture of Lipofectamine, DNA, and DMEM. In general, one microgram of DNA, 15 microliters of Lipofectamine, and 250 microliters of DMEM were used for each transfected plate. These numbers were titrated up or down with the goal of having equal levels of protein expression of the transfected DNA in each plate (this was determined by comparing the prelysate protein levels). Cells were allowed to grow for 48-72 hours post-treatment and were then lysed on ice using ice-cold TNET (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% *Triton* X-100). This allowed for the investigation of non-covalent interactions.

A plasmid encoding GCC185 was acquired from the Pfeffer lab at Stanford University and was a myc-tagged construct. TUG was used either in the untagged form and pulled down with the specific antibody made in the Bogan lab, called L1C, or in the Flag-tagged form. GLUT4 was used either in the untagged form and blotted for with the specific antibody YU126, or was used in the V5 tagged form. These proteins were transfected in all possible combinations to probe for possible interactions. Co-IP's were carried out using affinity matrix beads to the various protein tags (ie FLAG, V5, myc) or using the specific antibody to the protein followed by protein G beads. Western blots were made with the pre-lysates and eluates. These samples were run out on 4-12% polyacrylamide gels and overnight dry gel

transfers were set up at a low voltage (2-4 Volts) to allow for complete transfer of GCC185, a large protein of 185 kDa. Membranes were developed using Pierce PICO ECL reagent. Images were acquired on film.

Aim 1b. To map the interaction between GCC185 and TUG, a series of truncations of both proteins were used to carry out coimmunoprecipitations in 293 cells. Again affinity matrix beads for the flag or myc tag were used. The TUG truncations already existed in the lab in the following forms: TUG UBX (amino acids 377-550), flag-TUG delta 18 (amino acids 1-532), and flag-L1N1 (TUG protein with amino acids 165-550). Two of the GCC185 truncation constructs were obtained from the Pfeffer lab and included delta C110 (amino acids 1-1574) and delta CC123 (amino acids 890-1684). Additional truncation constructs were created using PCR followed by Topo cloning, and were based on fragments that would fold into coiled-coils based on the paper by Hayes et al (53). The fragments included CC123 (amino acids 1-861), C110 (amino acids 1575-1684) and C343 (amino acids 1341-1684). All of the GCC185 constructs were tagged with myc.

Aim 1c. To assess whether TUG modification is necessary for interaction with the putative anchor protein GCC185. The lysine site most likely to be modified was located and mutated using PCR techniques. This construct was termed TUG K549R, and it mutated the penultimate lysine to an arginine. It was used in coimmunoprecipitations with the myc-GCC185 construct to assess for increased or decreased interaction.

Aim 2a. To assess whether TUG is a target of Ubc9-mediated SUMOylation. An outline of experiments to assess for protein SUMOylation can be found in an

overview by Ok-Kyong, Park-Sarge and Sarge (62). In the first experiment performed, SUMO-1, and SUMO-2 were tagged with the HA tag and then transiently transfected into 293 cells along with TUG. The HA tag was then immunoprecipitated and TUG was blotted for. Transfected cells were lysed in boiling .1% SDS to assess for covalent modifications with SUMO. In the second experiment, TUG was immunoprecipitated using the L1C antibody and protein G beads, and SUMO was blotted for. In the third experiment, purifications were carried out using affinity matrix beads containing a SUMO-interaction motif and then TUG was blotted for. In the fourth experiment, affinity matrix beads containing bound SUMO were used to pull down proteins to which SUMO normally bound. Beads containing bound ubiquitin, which is not believed to bind TUG, were used as a negative control. Again, TUG was blotted for. The TUG K549R construct was again used in experiments alongside wild type TUG to assess for any difference.

Aim 2b. To examine whether SUMO modifies TUG in 3T3-L1 adipocytes, cells stably expressing a GLUT4 reporter protein were used. This reporter contains seven myc epitope tags, as well as GFP fused in frame at the carboxy terminus. It has been validated extensively, and is expressed at about fivefold the abundance of endogenous GLUT4. The myc tags facilitate immunoprecipitation of GLUT4 and associated proteins, using an immobilized monoclonal antibody. In addition, stable 3T3-L1 cells have already been generated and characterized that contain both the GLUT4 reporter and overexpressed TUG, or the GLUT4 reporter and a dominant negative (UBX-Cter) TUG fragment. These three cell lines were then stably infected with the HA-SUMO 1and HA-SUMO 2 vector. Infections were achieved by first

transiently transfecting 293 cells with the desired DNA and the pCL vector system (63). The media from these 293 plates was then collected and added to the 3T3L1 plates. Successful uptake was confirmed using FACS and cells were then sorted to get 100% infection rates. These cells were then differentiated into mature adipocytes, treated in the absence of insulin or in the presence of insulin for 2, 5, or 10 minutes and then immunoprecipitated using the HA tag. Eluates were then immunoblotted to assess for the presence of associated TUG, and to determine if insulin affected this association. To concentrate the interaction of the desired proteins, this experiment was also done after making membranes and cytosol. In this experiment, three plates of the same type were combined, and lysates were spun in the Ultracentrifuge for 30 minutes at 100,000 rpm. The separated membranes and cytosol were then immunoprecipitated using the HA tag and TUG was blotted for.

Aim 2c. To examine whether acetylation is the modification of TUG, 293 cells were transiently transfected with TUG or TUG K549R. After 36 hours, these cells were treated with trichostatin A (TSA) at a concentration of 0.5 microMolar for 16-21 hours and with nicotinamide (NAM) at a concentration of 5 milliMolar for the last 6 hours. Both TSA and NAM are known to inhibit deacetylation. Cells were then lysed in boiling 0.1% SDS, triton-X100 was added to 1%, and TUG was immunoprecipitated using the L1C antibody. Eluates were then blotted using a commercial antibody to acetylated lysine.

Results

1a: *Determination of an interaction between GCC185 and TUG*. In transfected 293 cells, immunoprecipitation of the myc tag on GCC185 resulted in the simultaneous precipitation of TUG in the cell lines in which both TUG and GCC185 were transfected. In the lanes with only myc-GCC185 or only TUG, this effect was not seen (see Figure 2). The interaction of TUG and GCC185 was demonstrated even in lane 6 of Figure 2, where the transfection of the GCC185 construct occurred at a lower efficiency. The converse of this experiment was also done and showed that when the flag tag on TUG was immunoprecipitated, there was coimmunoprecipitation of GCC185. Again, this effect was only seen in the cell lines in which both flag-TUG and myc-GCC185 were expressed (see Figure 3). Both of these experiments were repeated on three separate occasions.

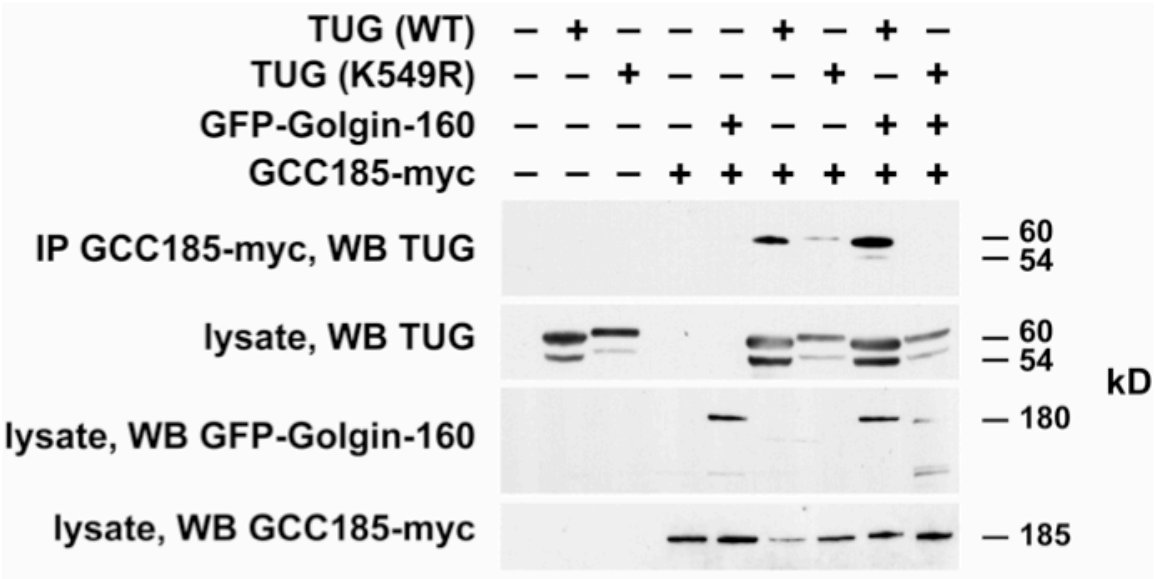

Figure 2. Immunoprecipitation of myc-GCC185 and Western Blot of TUG. In this experiment, 293 cells were transfected with combinations of TUG, TUG K549R mutant, GFP-Golgin-160, and myc-GCC185. Golgin-160, another large Golgi-resident protein was used to compare it to GCC185. The myc tag on GCC185 was immunoprecipitated and equal amounts of the various proteins were blotted for. TUG and TUG K549R were blotted for with anti-L1C, GCC185 with anti-myc, and GFP-golgin 160 with anti-GFP.

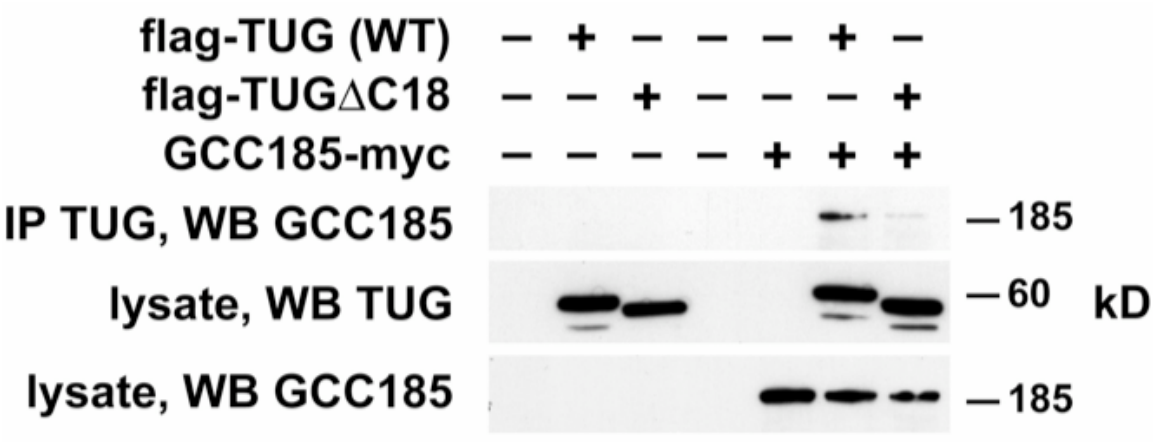

Figure 3. Immunoprecipitation of flag-TUG and Western Blot of myc GCC185. In the reciprocal experiment to the one in Figure 2, 293 cells were transfected with combinations of flag-TUG, flag-TUG delta18, and myc-GCC185. The flag tag was immunoprecipitated and equal amounts of the various proteins were blotted for. TUG and flag-TUG delta18 were blotted for with anti-flag and GCC185 with anti-myc.

Experiments were also done in which V5-GLUT4, myc-GCC185 and flag-TUG were expressed and the myc tag was immunoprecipitated. This was done with the goal of showing that TUG was a central protein binding both GLUT4 and GCC185, so that pulling down GCC185 would bring down TUG and the associated GLUT4 in complex. Unfortunately, the V5 -GLUT4 construct did not express in all of the plates in which it was transfected, so these results could not be interpreted (data not shown).

1b. *Mapping the interaction between TUG and GCC185*. In this experiment, flag-TUG, flag-L1N1 (TUG protein with amino acids 165-550), myc-delta C110 (GCC185 protein with amino acids 1-1574), and myc-delta CC123 (GCC185 protein with amino acids 890-1684) were transfected into 293 cells. The myc tag was immunoprecipitated, and the flag tag was blotted for. There was a specific band in the lanes with flag-TUG and myc-deltaCC123 (see figure 4). There was no band in

the lane with flag-TUG delta18 and myc-GCC185 (see Figure 3). The absence of a band in the lane with flag-TUG delta18 and myc-GCC185 was seen in two separate experiments. Unfortunately, the cells transfected with myc-delta C110 died on multiple occasions, so it was not possible to narrow down the site on GCC185 any further.

Figure 4. Immunoprecipitation of myc-delta CC123 and Western Blot of flag-TUG. 293 cells were transfected with combinations of flag-TUG and myc-delta CC123. The myc tag was immunoprecipitated and equal amounts of the various proteins were blotted for. TUG was blotted for with anti-flag and delta CC123 was blotted for with anti-myc.

1c. *Determining whether TUG modification is necessary for interaction with GCC185*. For the initial assessment of this, 293 cells were transfected with combinations of TUG, TUG K549R-whose penultimate lysine has been altered, with the purpose of making it unmodifiable- and myc-GCC185. When the myc tag was immunoprecipitated, and the eluate was blotted for TUG, it was seen that GCC185 and TUG came down together, but that TUGK549R and GCC185 did not come down together or came down significantly less (see Figure 2). The TUGK549R mutant was transfected at an efficiency that was about 50% less than that of the TUG construct, however the difference in the amount of TUGK549R and TUG in the eluate lanes is greater than 50%. This result was seen on two separate occasions.

2a. *Assessing TUG for SUMO-modification in 293 cells*. In multiple separate experiments, 293 cells were transfected with different combinations of HA-SUMO 1, HA-SUMO 2, and TUG. In some experiments the HA tag was immunoprecipitated and TUG was blotted for, and in other experiments TUG was immunoprecipitated and the HA tag was blotted for. In none of these experiments did the two proteins come down together. When affinity matrix beads containing SUMO-interacting motifs were used, no TUG came down. When affinity matrix beads containing bound SUMO were used, they did not bind to TUG, as no TUG came down in the eluate. Finally, when TUG and the mutant TUG K549R were transfected to see if SUMO interacted with one and not the other, no difference could be detected.

2b. *Assessing TUG for SUMO-modification in 3T3L1 adipocytes*. Six sets of 3T3L1 adipocytes were analyzed: the first expressed excess GLUT4 and HA-SUMO 1, the second expressed excess GLUT4, TUG, and HA-SUMO 1, the third expressed excess GLUT4, UBx-C terminal TUG fragment, and HA-SUMO 1, the fourth expressed excess GLUT4 and HA-SUMO 2, the fifth expressed GLUT4, TUG, and HA-SUMO 2, and the sixth expressed excess GLUT4, UBx-C terminal TUG fragment, and HA-SUMO 2. An insulin time course (0, 2, 5, and 10 minutes) was set up, the HA tag was immunoprecipitated, and TUG was blotted for in the eluates. In none of the plates was TUG seen to come down. In a similar experiment, membranes and cytosol were separated in the first 3 cell types and HA was again immunoprecipitated, this time without the insulin time course. When TUG was blotted for in the eluates, it was not seen to come down in any of the lanes.

2c. *Assessing TUG for acetylation in 293 cells*. 293 cells were transfected with either TUG or TUG K549R and then treated with either NAD and TSA (to prevent deacetylation) or with nothing. TUG was then immunoprecipitated in the cell lysates. In the prelysates, the plates treated with NAD and TSA had significantly more protein on the acetylated lysine blot as compared to the untreated plates. In the eluates blotted for acetylated lysine, there was a band in the TUG lane treated with NAD and TSA, but this band ran at 51 kDa as opposed to the normal level for TUG, which is 60 kDa. Thus, it was unclear what this band represented. Of note, another member of the laboratory (Charisse Orme) conducted an experiment in which she immunoprecipitated the flag-tags on both flag-tagged full length TUG and flag-tagged TUG delta18. She then blotted the eluates with an acetylated-lysine antibody (which detects acetylated-lysine residues). The immunoprecipitated protein was detected when flag-TUG was used, but not when flag-TUG delta18 was used (Figure 5). This suggests that TUG is acetylated and its C terminal 18 amino acids are required for this acetylation.

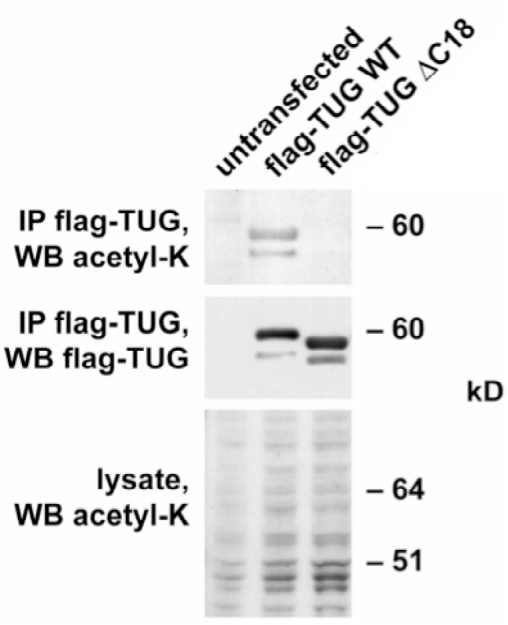

Figure 5. Courtesy of Charisse Orme. Immunoprecipitation of flag TUG and Western Blot of acetylated-lysine. 293 cells were transfected with combinations of flag-TUG and flag-TUG delta18. The flag tag was immunoprecipitated and equal amounts of the various proteins were blotted for. The acetylated-lysine antibody was used to probe for the presence of proteins with acetylated-lysine residues.

Discussion

At the outset of this project, the goal was to gain a better understanding of how GSVs form and where they localize within adipocytes and myocytes in the absence of insulin. Coimmunoprecipitation data from 293 cells suggest that GCC185 and TUG do in fact interact. This is supported by the fact that TUG can be pulled down when GCC185 is immunoprecipitated, with the reciprocal finding also being demonstrated. This data is limited by the fact that it was acquired from 293 cells, a cell line that is not insulin responsive. However, TUG was shown to sequester GLUT4 away from the cell surface in transfected 293 cells (32). Thus, although it is a promising result, it needs to be expanded upon with experiments in insulin-responsive cell lines, such as the 3T3L1 adipocytes. In such experiments, in addition to seeing an interaction between TUG and GCC185, one might also expect to see a loss of this interaction after stimulation of the cells with insulin. This would fit the hypothesized model in which TUG functionally tethers GSVs to an intracellular anchor, from which GSVs are released following insulin stimulation.

In regards to mapping the interaction between TUG and GCC185, the preliminary data suggest that the C terminus of TUG is necessary for this interaction, since the TUG delta18 construct does not appear to interact with GCC185. These data fit with previous TUG data, which showed that the N terminus of TUG interacted with GLUT4 and that the C terminus of TUG was necessary for it to sequester GSVs away from the plasma membrane. The finding that when TUG lacked its C terminus it was no longer able to sequester GSVs, is consistent with this new

finding that the C terminus of TUG is necessary to bind to GCC185, the putative anchor.

Furthermore, the fact that a fragment of TUG's C terminus, the UBX-Cter, appears to act as a dominant negative also fits this model. Presumably, this UBX-Cter fragment can bind to the anchoring site, and in so doing occupies the places where full length TUG would normally bind. Since the UBX-Cter fragment lacks the GLUT4 binding site, this would prevent TUG from being able to tether GSVs. This makes it important to test the hypothesis that the UBX-Cter fragment and GCC185 bind to one another. It also raises the need for functional data in an insulin-responsive cell line. For example, in the lab there is a 740++ 3T3L1 adipocyte cell line that has permanently knocked-down TUG. If the TUG delta18 construct were added back to this, it should be unable to rescue proper GSV localization, since it would presumably lack the anchor-binding site.

Mapping of the TUG binding site on GCC185 will also be important. The preliminary data suggest that TUG binds to the C-terminus of GCC185. Further narrowing of this interaction was not completed as plates transfected with the other fragments died.

It would also be crucial to demonstrate the co-localization of GCC185 and GLUT4 in an insulin responsive cell-line such as 3T3-L1 adipocytes. This was attempted using electroporation followed by cell staining but the cells did not survive. It seems likely that the harsh conditions imposed on the cells from the combination of these two methods resulted in this endpoint. As such, a cherry-GCC185 construct was created to be used alongside the already existing GFP-GLUT4

construct. By using these two constructs, electroporation would still be necessary, but the staining step could be skipped, since both constructs would fluoresce. In addition, some of these cells would have to be treated with insulin to show that this results in loss of co-localization. Alternatively, stable cell lines could be made expressing cherry-GCC185 and GFP-GLUT4, or transiently stable cells could be created with the two constructs using an adenovirus vector.

The data showing that the TUG K549R mutant significantly lessens the interaction between TUG and GCC185 is promising. This mutation was made based on the fact that this site seemed a likely site of modification and was seen in the UniProtKB database to be an acetylated residue. This mutation would prevent such acetylation from taking place by eliminating the lysine. It is therefore interesting that this mutant cannot interact with GCC185, and suggests that TUG acetylation is necessary for its association with GCC185. To assess the true significance of this finding, experiments would have to be set-up in an insulin responsive cell line. For example, if the 740++ cell line were used, the K549R mutant could be added back and it would be expected to be unable to localize GSVs to their appropriate intracellular location, since it would not bind to GCC185, the presumed anchoring site. A mutant of the UBX-Cter was also made with this K549R, and this could be tested in 3T3L1 adipocytes as well. The predicted outcome here would be that the mutant UBX-Cter would no longer be able to act as a dominant negative construct, since it presumably could no longer interact with GCC185.

Attempts to verify that acetylation occurs at this site yielded non-specific results. Hence, these experiments should first be re-attempted using a positive

control such as p-53, which is known to be acetylated. It is also possible to raise antibodies against specific acetylated lysine complexes, though this is an expensive and time-consuming endeavor. It would also be possible to look at the role of acetylation in 3T3L1 cells either by using deacetylase inhibitors, which might strengthen the interaction between GCC185 and TUG and hinder GSV translocation, or by promoting deacetylases, which might prevent the interaction between GCC185 and TUG.

As part of our initial hypothesis, we proposed that the 54 kDa fragments of TUG represented a modified form of processed TUG that allowed its removal from the anchoring site. We had hoped to characterize this modification. Given the variety of experiments done in both 293 and 3T3L1 adipocytes, along with the repetition of said experiments, it seems that SUMO is *not* the modifier of TUG. This may be consistent with the work from Liu et al., in which it was shown that the catalytically inactive form of UBC9 was just as effective at allowing GSV movement to the cell surface as the active form of UBC9 (40). Since the catalytic role of UBC9 is to facilitate SUMOylation, the observation that an inactive UBC9 still allows for GSV translocation suggests that SUMOylation does not play a role in the untethering of GSVs. Yet, because this work used an adenovirus to express Ubc9 transiently, and given that the 54 kD fragment is produced after TUG cleavage, it is formally possible that disrupting SUMOylation might not affect the initial cycle of GSV release. Nonetheless, it seems most likely that TUG SUMOylation is not involved in GSV translocation.

The nature of the modification causing the shift from a 42 kDa to a 54 kDa product remains unknown. One possibility is that this modification is ADP-ribosylation. Deacetylation and ADP-ribosylation have been shown to occur in concert and are both stimulated by NAD (64). If acetylation of TUG is needed for it to associate with GCC185, then TUG's deacetylation and subsequent ADP-ribosylation may allow it to dissociate. Further, the ADP-ribosylation could account for the 54 kDa band of TUG seen following insulin stimulation. This hypothesis deserves further investigation.

It should be stated that in experiments meant to represent an insulin-responsive cell model, 3T3L1 adipocytes were used to be consistent with other GLUT4 literature in which this is generally the model cell system. This is in part because myocyte cell lines have proved difficult to manipulate. It is presumed that the TUG and the anchor would play a similar role in mycocytes, since these cells are similarly responsive to insulin. However, this is something that would eventually have to be verified in a myocyte cell line. Additionally, GSV movement to the plasma membrane in mycocytes can also be stimulated by muscle contraction, and it is not clear whether this uses the same signal transduction pathway as insulin to release GSVs.

Although these results need to be developed further, they are exciting. Additional characterization of the TUG/GCC185 interaction may further support the idea that GCC185 is indeed the anchor to which the GSV-TUG complex binds. This would then provide a clearer sense of the proteins involved in the insulin-responsive complex. As previously reviewed, the formation and location of GSVs

play a key role in glucose homeostasis, making this valuable information. If GCC185 in fact plays this role, it would explain why GLUT4 and IRAP colocalize with TGN markers at various points in adipocyte maturation. The fact that GCC185 is associated with microtubules would also provide a transport mechanism whereby GSVs could move to the plasma membrane. In addition, the association of GCC185 with Rabs, would put at-hand the catalysts to allow this translocation.

It is also instructive to consider the fact that TUG is expressed in almost every cell type. This raises the possibility that TUG plays a similar functional tethering role in multiple cell types. If that is the case, the interaction of TUG and GCC185 may be conserved, with the TUG-associated vesicle being the variable. For this reason, other cell types with regulated secretion should be investigated to see if a similar set-up exists. Such cell types are multiple and would include gastric parietal (in which the H+/K+ pump is translocated to the cell surface), renal distal collecting tubules (in which aquaporin channels are translocated), and neurons (in which AMP-A glutamate receptors are translocated) for a start. Given the finely-controlled steps that must go into forming these secreted vesicles and then stimulating their release, it would make sense if the underlying proteins were conserved.

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