

Starch Binding Domain-containing Protein 1 Plays a Dominant Role in Glycogen Transport to Lysosomes in Liver

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Running title: *Stbd1* dominates glycogen transport to lysosomes in liver

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ABSTRACT

A small portion of cellular glycogen is transported to and degraded in lysosomes by acid alpha-glucosidase (GAA) in mammals, but why and how glycogen is transported to the lysosomes are unclear. Stbd1 has recently been proposed to participate in glycogen trafficking to lysosomes. However, our previous study demonstrated that knockdown of Stbd1 in GAA knockout mice did not alter lysosomal glycogen storage in skeletal muscles. To further determine whether Stbd1 participates in glycogen transport to lysosomes, we generated GAA/Stbd1 double knockout mice. In fasted double knockout mice, glycogen accumulation in skeletal and cardiac muscles was not affected, but glycogen content in liver was reduced by nearly 73% at age of 3 months and by 60% at 13 months compared with GAA knockout mice, indicating that the transport of glycogen to lysosomes was suppressed in liver by the loss of Stbd1. Exogenous expression of human Stbd1 in dKO mice restored the liver lysosomal glycogen content to the level of GAA knockout mice, so did a mutant lacking the Atg8 family interacting motif (AIM), and another mutant that contains only the N-terminal 24 hydrophobic segment and the C-terminal CBM20 starch binding domain interlinked by an HA-tag. Our results demonstrate that Stbd1 plays a dominant role in glycogen transport to lysosomes in liver and the N-terminal transmembrane region and the C-terminal CBM20 domain are critical for this function.

Liver and muscle are the two primary sites for glycogen metabolism in mammals. Glycogen is synthesized and mostly degraded in the cytoplasm but a small percentage of glycogen is transported into the lysosomes and hydrolyzed by the enzyme acid alpha-glucosidase (GAA) (1-3). Deficiency of GAA causes Pompe disease (glycogen storage disease type II), resulting in a progressive lysosomal glycogen accumulation and dysfunction in cardiac and skeletal muscle and other tissues (4-6). Enzyme replacement therapy with recombinant human GAA is the only available therapy for this disorder with remarkable success, but also with significant limitations like high titer antibody formation and difficulties in delivering the therapeutic protein to skeletal muscle tissues (7-11). A conceivable alternative way to treat Pompe disease would be to block the transport of cytoplasmic glycogen to the lysosomes. Thus understanding how glycogen enters the lysosome is a critical step in finding new therapeutic targets for Pompe disease. It has been demonstrated that conventional autophagy (macroautophagy) plays an important role in the transport of glycogen to lysosomes in skeletal muscle (12). Specifically, knockout of an autophagy key gene Atg7 resulted in a decrease of glycogen content by 50-60% in skeletal muscles of GAA knockout mice (12). This suggests other routes exist for glycogen transport to lysosomes.

In recent studies, starch binding domain-containing protein 1 (Stbd1) has gained attention for its potential involvement in glycogen transport. The Stbd1 protein contains a putative N-terminal

transmembrane region, an Atg8 interacting motif (AIM), and a C-terminal CBM20 carbohydrate-binding domain (13-15). The observation that it binds to glycogen and interacts with the autophagy protein GABARAPL1 in cultured cells led to speculations that Stbd1 may function as a cargo binding protein that delivers glycogen to lysosomes in a specific autophagic pathway (13,14). Contradictorily, our previous study undermined this proposition, as significant knockdown of Stbd1 in skeletal muscle of GAA knockout mice (GKO) did not affect lysosomal glycogen storage in the tissue (16). In this study we generated Stbd1/GAA double knockout (dKO) mice to explore the role of Stbd1 in glycogen transport to lysosomes, as accumulated lysosomal glycogen is an easily measurable indicator of glycogen transport to this organelle.

RESULTS

Stbd1 plays a dominate role in transporting glycogen to lysosomes in liver, but not in heart and skeletal muscles—The lack of GAA in GKO and dKO mice enables us to monitor glycogen transport to lysosomes, because the glycogen cannot be degraded once inside lysosomes and glycogen build-up/storage can readily be observed/quantified. To assess whether the loss of Stbd1 affects glycogen transport to lysosomes, three-month-old male GKO and dKO mice were subjected to overnight fasting and glycogen contents were measured in different tissues. Age- and gender-matched Stbd1 knockout (SKO) mice and their littermate wild-type (WT) mice were used as controls.

As shown in Fig. 1A, both SKO and WT mice had very low level of glycogen in heart, skeletal and liver. The GKO mice showed high levels of lysosomal glycogen storage in these tissues as seen in the GAA knockout ($6^{neo}/6^{neo}$) mice (17). There were no significant differences in glycogen contents in cardiac and skeletal muscles between the dKO mice and GKO mice. Surprisingly liver glycogen content in the dKO mice (11.8 ± 3.9 $\mu\text{mol glucose/g}$) was only 27% of that in the GKO mice (43.7 ± 4.6 $\mu\text{mol glucose/g}$). Consistent with this result, PAS staining of liver sections showed strong glycogen staining in the GKO mice and markedly weaker staining in the dKO mice. No obvious PAS-positive glycogen was observed in the livers of WT and SKO mice (Fig.

1B). Our data indicate that Stbd1 plays a significant role in glycogen transport to the lysosomes in liver, but not in skeletal muscle and heart.

The fact that liver glycogen level in dKO mice was still significantly higher than in WT (Fig 1A & 1B) indicates that a small amount of glycogen was still transported to lysosomes in the absence of Stbd1. To determine the impact of the Stbd1-independent pathway(s) on lysosomal glycogen accumulation over time, we examined liver glycogen levels in old dKO mice at 13 months of ages. As shown in Fig. 1C, fasting liver glycogen content in the 13-month-old dKO mice (20.0 ± 5.8 $\mu\text{mol glucose/g}$) was significantly higher than that of the 3-month-old dKO mice (11.8 ± 3.9 $\mu\text{mol glucose/g}$), but it was still far below the level in age-matched GKO mice (50.6 ± 8.1 $\mu\text{mol glucose/g}$). This demonstrates that other minor route(s) has limited contribution to the transport of glycogen into lysosomes, and Stbd1-mediated pathway plays a dominate role in this process.

To exclude the possibility that the reduced lysosomal glycogen accumulation in dKO mouse liver was due to suppression of glycogen synthesis in the absence of Stbd1, we compared liver glycogen contents in WT, GKO and dKO mice at age of three months at fed state. No significant difference in liver glycogen contents was found among these genotypes (Fig. 1D). Glycogen level in GKO mice was seemingly higher possibly due to an extra glycogen pool trapped in the lysosomes. We also examined the expression of the two key enzymes for glycogen synthesis, glycogen synthase 2 (GS2) and glycogen branching enzyme (GBE1). There was no difference in expression of either enzyme between GKO and dKO livers as determined by Western blotting (Fig. S1). Thus, Stbd1 knockout does not affect liver glycogen synthesis.

The N-terminal transmembrane region and the C-terminal CBM20 domain are critical for glycogen transport to lysosomes in liver—Stbd1 has an N-terminal hydrophobic transmembrane region, a putative leucine zipper, an identified Atg8-family interaction motif (AIM), and a C-terminal carbohydrate binding module (CBM20) (13,14,18). To determine critical elements in Stbd1 for glycogen transport to lysosomes, we constructed a series of AAV vectors to express WT human Stbd1 and its mutants in dKO mice (Fig. 2A). AAV transduction efficiencies for all constructs are

comparable as real-time PCR results revealed similar viral copy numbers in livers of AAV-injected dKO mice (Fig. S2). Western blots demonstrated that the AAV vectors expressed GFP (negative control), Stbd1, and its mutants in liver of injected dKO mice (Fig. 2B). Protein level of the Δ N24 mutant was extremely low and only weakly detectable with longer exposure possibly due to the instability of this mutant protein in liver cells (Fig. 2B).

Exogenous expression of human Stbd1 restored glycogen transport to lysosome in liver as indicated by the rebound of liver glycogen storage in the treated dKO mice to a similar level of GKO mice (Fig. 2C). This confirms that the much less liver lysosomal glycogen storage in dKO mice was a direct result of Stbd1 knockout.

To test whether the interaction between Stbd1 and GABARAPL1 is required for glycogen transport to lysosomes, we generated an Atg8 family interacting motif (AIM) mutant, AIMm (W203A/V206A; Fig. 2A) that loses the GABARAPL1 binding ability (14). The AIMm mutant restored lysosomal glycogen storage in liver as potently as wild-type Stbd1 (Fig. 2C). This indicates that the Stbd1-GABARAPL1 interaction is not required for glycogen transport to lysosome in liver.

The Δ N24 mutant failed to restore glycogen storage in liver (Fig. 2C). However, the extremely low expression level of this mutant prevents us from drawing a conclusion on the necessity of the N-terminus in glycogen transport. It seems that this N-terminal transmembrane region is important for the stability of the protein in liver, because the same construct could express the protein in comparable amount to other constructs in 293T cells (data not shown).

Lastly we constructed a mutant (Δ 26-259) that contains only the N-terminal transmembrane region and the C-terminal CBM20 domain linked by an HA-tag (Fig. 2A). Surprisingly, this mutant functioned as potently as the full length Stbd1 to restore lysosomal glycogen storage in the liver of dKO mice (Fig. 2C). This demonstrated that the intervening region (aa26-aa259) is dispensable for glycogen transport to lysosomes in liver.

Stbd1 Knockout does not block autophagy activation in mouse liver—Autophagy has been shown to be involved in glycogen trafficking to lysosomes in fast-twitch skeletal muscle (12). To

check whether the loss of Stbd1 affects autophagy activation in liver, we checked the autophagy marker LC3 in dKO and GKO mice under both fed and fasted conditions. As shown in Fig. 2D, LC3-II level in fasted liver was dramatically higher than that in fed liver of both GKO and dKO mice, indicating induction of autophagy by fasting in both genotypes. In other words, the activation of autophagy was not blocked by the Stbd1 knockout. In addition, there was no obvious difference in expression of other autophagy-related genes like P62, Beclin 1 and Atg8L1 in fasted livers between the dKO and GKO mice (Fig. S1). On the other hand, fasting-induced activation of autophagy in liver was not accompanied by obvious change in the level of Stbd1 protein in GKO mice (Fig. 2D). Our results suggest loss of Stbd1 does not affect conventional autophagy in liver.

Taken together, our data demonstrate the Stbd1 plays a dominate role in transporting glycogen to lysosomes in liver, and that the N-terminal transmembrane region and the C-terminal CBM20 domain are critical in this process.

DISCUSSION

Cellular glycogen is mostly degraded in the cytoplasm but a small portion is transported into the lysosomes and hydrolyzed by GAA (1-3). To understand the mechanism of how glycogen is transported into lysosome is a critical step for the exploration of new therapeutic targets for Pompe disease considering the significant limitations of ERT (7-9). It has been demonstrated that autophagy plays a partial role in the transport of glycogen to lysosomes in skeletal muscle of GAA-KO mice (12), which suggests other route(s) also exist for this process.

Recent studies led to a proposition that Stbd1 functions as a cargo receptor for delivery of glycogen to lysosomes in a specific autophagic pathway (termed “glycophagy”), and the interactions between Stbd1 and GABARAPL1 might be critical in this process (13,14). This provoked a great expectation that Stbd1 might be a new therapeutic of Pompe disease. Contradictorily, our previous report demonstrated that significant knockdown of Stbd1 in skeletal muscle of GAA knockout mice did not reduce lysosomal glycogen accumulation, suggesting that Stbd1 may not be involved in lysosomal glycogen trafficking at least in this tissue (16). In an effort to clarify this

controversy, we generated *Stbd1*/GAA double knockout mice. These mice serve as an ideal system for investigating the role of *Stbd1* in transporting glycogen into lysosomes in various tissues because glycogen accumulation due to the lack of GAA is a reliable and quantifiable indicator of glycogen transport into lysosomes.

In agreement with our previous report (16), we found that loss of *Stbd1* in dKO mice did not affect lysosomal glycogen accumulation in cardiac and skeletal muscles (Fig. 1A), indicating that *Stbd1* may not be involved in glycogen transport to lysosomes in these tissues. But strikingly, we found that lysosomal glycogen storage in liver of dKO mice was 73% lower than that of the GKO mice, suggesting that *Stbd1* is a major but not the sole mediator for glycogen transport into lysosomes.

Similar to our findings of *Stbd1*'s function in liver, Raben *et al.* demonstrated that autophagy plays a critical role in muscle in transporting glycogen to lysosomes. They reported that suppression of autophagy reduced lysosomal glycogen content by 50-60% in skeletal muscles of GAA-KO mice (12). These results demonstrate that different tissues could have distinct major pathways for glycogen transport to lysosomes, and in the same tissue multiple routes exist. Since liver and muscle are very different in terms of the regulation of glycogen metabolism and the utilization of glycogen storage (3), it is not surprising that different pathways play major roles in transporting glycogen to lysosomes in the two tissues. Although our data clearly demonstrated that *Stbd1* plays a dominant role in glycogen transport to lysosomes in liver, we cannot exclude the possibility that it may play a minor role in muscle.

Stbd1 knockout did not block the activation of autophagy in liver, as a similar elevation of LC3-II abundance was observed in the GKO and dKO mice when fasted (Fig. 2D), and had no effect on the expression of autophagy-related proteins P62, Beclin1, and GABARAPL1 (Fig. S1). On the other hand, fasting-induced activation of autophagy in GKO liver was not accompanied by apparent change in the level of *Stbd1* protein (Fig. 2D). These results indicate that the reduced lysosomal glycogen accumulation in dKO mouse liver was not due to blockage of the conventional autophagy. Whether the conventional autophagy contributed to the transport of the small amount of

lysosomal glycogen in the dKO liver, in the absence of *Stbd1*, remained undetermined.

The *Stbd1*-GABARAPL1 interaction is not required for *Stbd1*-mediated glycogen transport to lysosome because the *Stbd1* mutant with the GABARAPL1 binding motif mutation (AIMm) was able to replenish the lysosomal glycogen content in liver of dKO mice (Fig. 2C). Furthermore, the mutant missing the entire intervening region ($\Delta 26-259$) also successfully restored the liver lysosomal glycogen content in dKO mice (Fig. 2C), demonstrating that only the N-terminal hydrophobic transmembrane region and the C-terminal CBM20 are critical for carrying out this function. Our data support the notion that *Stbd1* may function as an anchor to bring glycogen to an intracellular membrane (13). GABARAPL1 is implicated in several different cellular processes including autophagy and is the most highly expressed gene in the central nervous system among the GABARAP family members (19-21). This *Stbd1*-GABARAPL1 interaction may play important roles in other tissues especially the central nervous system.

This is the first report to demonstrate the function of *Stbd1* in mice. Our results demonstrate that *Stbd1* dominates glycogen transport to lysosomes in liver, but not in muscles. It is interesting to further investigate the mechanisms by which *Stbd1* participates in this process. As *Stbd1* is also highly expressed in skeletal muscle and heart, it would be interesting to uncover the function of this protein in these tissues. Our study suggests that the mechanism of glycogen transport to lysosomes in liver is distinct from that in muscles, thus different treatment schemes might be developed for different target tissues in Pompe disease.

EXPERIMENTAL PROCEDURES

Generation of GAA/*Stbd1* double knockout mice—We first generated *Stbd1* knockout (SKO, *Stbd1*^{-/-}) mice on C57BL/6J genetic background by replacing exon 2 of *Stbd1* with a *neo* cassette (detailed information will be published separately). The *Stbd1* knockout mice were backcrossed to C57BL/6 for 5 generations. The SKO mice were crossed with the GAA-KO (6^{neo}/6^{neo}) mice (17) to generate *GAA*^{-/-}/*Stbd1*^{+/-} mice, which were then used as breeders to produce *Stbd1*/GAA double knockout (dKO, *GAA*^{-/-}/*Stbd1*^{-/-}) mice along with littermate GAA knockout (GKO,

GAA^{-/-}/*Stbd1*^{+/+} mice for this study as described (22).

Antibodies and Immunoblotting—Tissues were homogenized in cold RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, P8340). Proteins were resolved by SDS-PAGE and blotted to PVDF membranes. Primary antibodies used: LC3B (L7543) and β -actin (A3854) from Sigma-Aldrich, HA (sc-7392) from Santa Cruz Biotechnology, GFP (A6455) from Molecular Probes, and *Stbd1* (11842-1-AP) from Proteintech. Rabbit anti-mouse *Stbd1* is a gift from Dr. Peter J. Roach, Indiana University (13).

Construction and packaging of AAV vectors for expression of wild-type and mutant *Stbd1*—The scAAV-miR26a-eGFP vector was a gift from Joshua Mendell (Addgene plasmid # 21894) (23). The pri-miR26a sequence was removed to generate AAV-GFP vector that served as backbone and negative control vector. Human *Stbd1* cDNA and its mutants were cloned into the AAV-GFP vector to replace GFP. The *Stbd1* mutants were generated as described by Jiang et al (13,14) unless described otherwise. All constructs were verified by DNA sequencing. The resulting

AAV vectors were packaged as AAV2/9 in HEK 293T cells using standard phosphate-mediated transfection and purified using iodixanol gradient ultracentrifugation (24-26).

Animal injection and tissue collection—AAV vectors were intravenously (tail vein) injected into 2-month-old male dKO mice at a dose of 5×10^{11} viral genomes (vg) per mouse. Tissues were collected 30 days later following overnight fasting. All animal procedures were done in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Tissue glycogen content measurement and PAS staining—Tissue glycogen content was quantified using an enzymatic method as described previously (27). Periodic acid-Schiff (PAS) staining of glycogen was performed on formalin-fixed tissues at the Research Histology Laboratory at Duke University.

Statistical analysis—Values in the graphs are presented as mean \pm SD. Statistical significance between two groups was determined by unpaired, equal valence, two-tailed t-test.

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Conflict of interest: The authors declare that they have no conflicts of interest with the content of this article.

Author contributions: TS and HY conducted most of the experiments, analyzed the results, and wrote most of the paper. CY managed the mouse colonies. PSK and BS conceived the idea for the work. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

*T. Sun and H. Yi contributed equally to this work.

The abbreviations used are: Stbd1, starch binding domain-containing protein 1; AAV, adeno-associated virus; GSD, glycogen storage disease; GAA, acid α -glucosidase; LC3, microtubule-associated protein light chain 3; CMB20, carbohydrate-binding module family 20.

FIGURE LEGENDS

Figure 1. *Stbd1* knockout greatly decreased lysosomal glycogen accumulation in liver, but not in cardiac and skeletal muscles. (A) Glycogen contents in heart, skeletal muscle (gastrocnemius) and liver of 3-month-old mice. WT, wild-type; SKO, *Stbd1*-knockout; GKO, GAA-knockout; dKO, GAA/*Stbd1* double knockout. All mice were fasted overnight. (B) Representative PAS staining of liver sections from mice in A. Scale bar = 200 μ m. (C) Fasting liver glycogen content in GKO and dKO mice at ages of 3 and 13 months. Data for 3-month-old mice are the same as in A. (D) Liver glycogen contents in mice fed *ad lib*. Data in A, C, and D are shown as Mean \pm SD; n=4-7; *p<0.01, **p<0.0001.

Figure 2. Critical elements in *Stbd1* protein for glycogen transport into lysosomes in liver. (A) Schematic diagram of human *Stbd1* and its mutants constructed into an AAV vector. An HA-tag was inserted to replace amino acids 26 to 259 in the mutant Δ 26-259. (B) Representative Western blots showing expression of *Stbd1* constructs in liver of dKO mice one month after AAV injection. The anti-*Stbd1* antibody was from Proteintech. Mutant Δ 26-259 can be detected with an anti-HA antibody but not with the *Stbd1* antibody. Expression of mutant Δ N24 was extremely low, so a separate blot is shown to compare its expression with the full-length *Stbd1* by overexposure. (C) Restoration of lysosomal glycogen accumulation in liver of dKO mice by expressing *Stbd1* constructs. WT, GKO, and dKO indicate 3 genotypes; “dKO mice +AAV” indicates dKO mice expressing indicated proteins. Data are presented as Mean \pm SD. n \geq 4. *p<0.01, **p<0.0001; N.S., not significantly different from dKO mice (p > 0.05). (D) Western blots showing the increase in LC3-II in liver of dKO mice induced by overnight fasting. The shifting to the LC3-II form after overnight fasting indicates that autophagy was induced in both genotypes. The *Stbd1* antibody was from P.J. Roach.

Figure 1

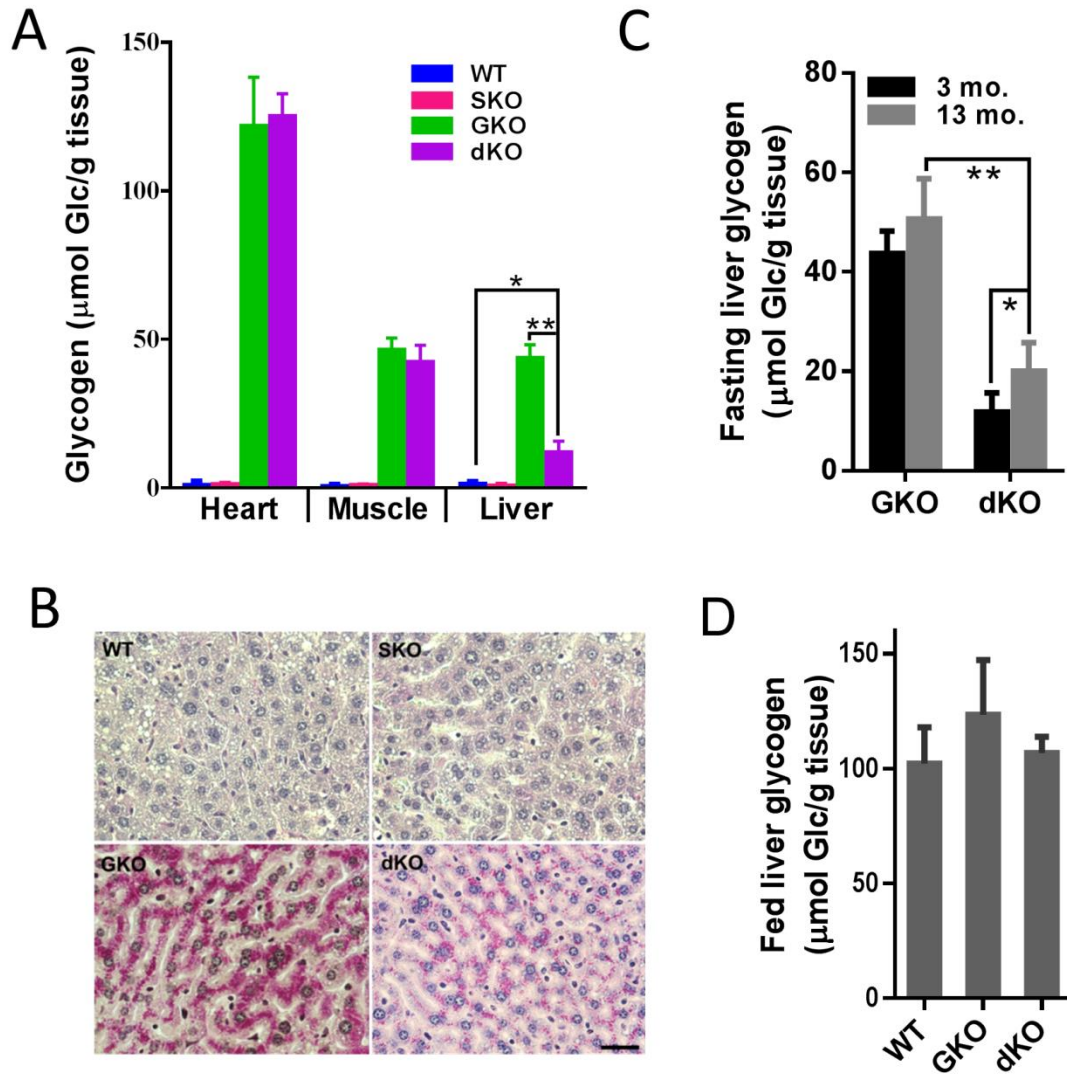
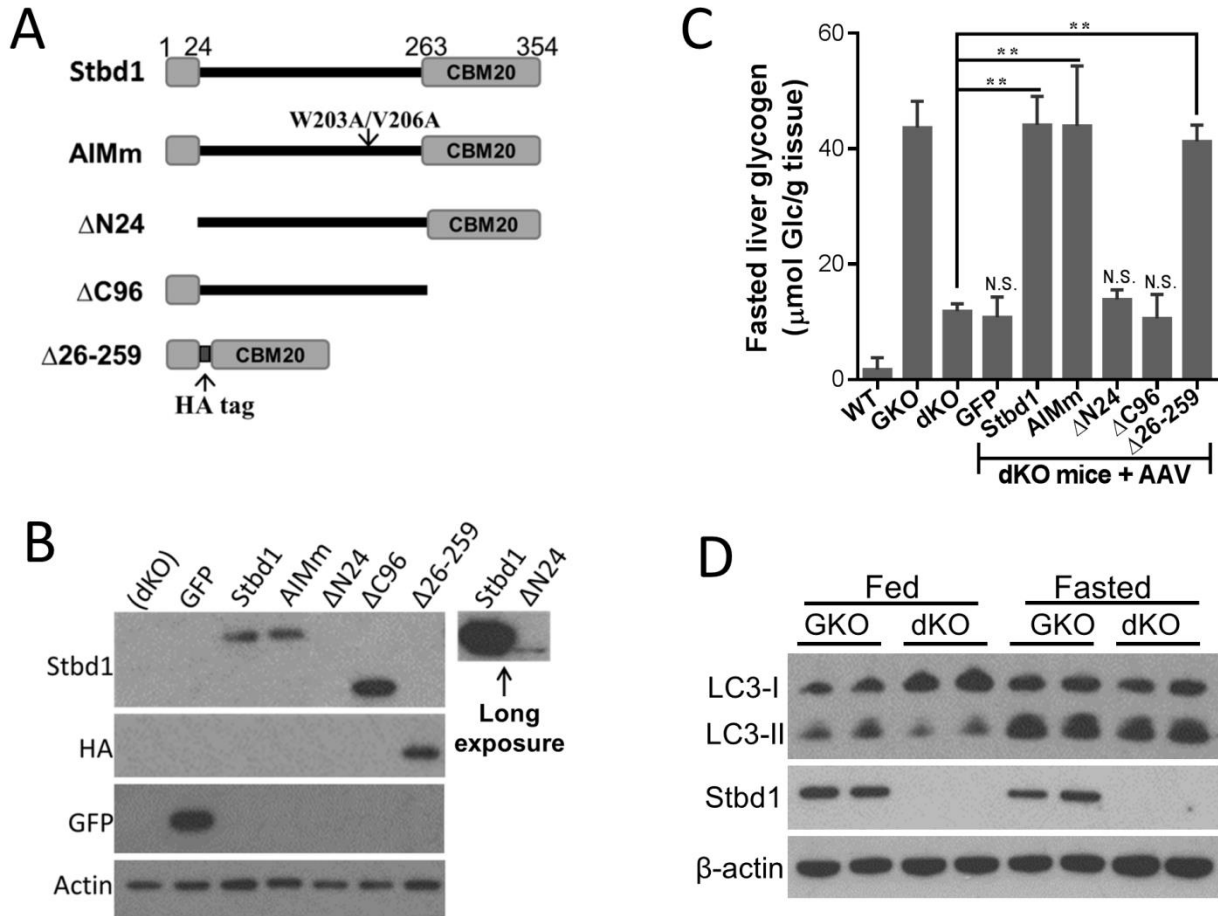


Figure 2



Starch Binding Domain-containing Protein 1 Plays a Dominant Role in Glycogen Transport to Lysosomes in Liver

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