Fluctuation in O-GlcNAcylation inactivates STIM1 to reduce store-operated calcium ion entry via down-regulation of Ser\(^{621}\) phosphorylation

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Stromal interaction molecule 1 (STIM1) plays a pivotal role in store-operated Ca\(^{2+}\) entry (SOCE), an essential mechanism in cellular calcium signaling and in maintaining cellular calcium balance. Because O-GlcNAcylation plays pivotal roles in various cellular function, we examined the effect of fluctuation in STIM1 O-GlcNAcylation on SOCE activity. We found that both increase and decrease in STIM1 O-GlcNAcylation impaired SOCE activity. To determine the molecular basis, we established STIM1-knockout HEK293 (STIM1-KO-HEK) cells using the CRISPR/Cas9 system and transfected STIM1 WT (STIM1-KO-WT-HEK), S621A (STIM1-KO-S621A-HEK), or T626A (STIM1-KO-T626A-HEK) cells. Using these cells, we examined the possible O-GlcNAcylation sites of STIM1 to determine whether the sites were O-GlcNAcylated. Co-immunoprecipitation analysis revealed that Ser\(^{621}\) and Thr\(^{626}\) were O-GlcNAcylated and that Thr\(^{626}\) was O-GlcNAcylated in the steady state but Ser\(^{621}\) was not. The SOCE activity in STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells was lower than that in STIM1-KO-WT-HEK cells because of reduced phosphorylation at Ser\(^{621}\). Treatment with the O-GlcNAcase inhibitor ThiameT G or O-GlcNAc transferase (OGT) transfection, which increases O-GlcNAc, reduced SOCE activity, whereas treatment with the OGT inhibitor ST045849 or siOGT transfection, which decreases O-GlcNAcylation, also reduced SOCE activity. Decrease in SOCE activity due to increase and decrease in O-GlcNAcylation was attributable to reduced phosphorylation at Ser\(^{621}\). These data suggest that both decrease in O-GlcNAcylation at Thr\(^{626}\) and increase in O-GlcNAcylation at Ser\(^{621}\) in STIM1 lead to impairment of SOCE activity through decrease in Ser\(^{621}\) phosphorylation. Targeting STIM1 O-GlcNAcylation could provide a promising treatment option for the related diseases, such as neurodegenerative diseases.

O-Linked N-acetylglucosamine (O-GlcNAc) modification (O-GlcNAcylation) is a common posttranslational modification in numerous cytoplasmic and nuclear proteins (1–3). Extracellular glucose enters the hexosamine biosynthesis pathway, leading to the production of UDP-GlcNAc, which serves as a substrate for O-GlcNAcylation (4). O-GlcNAcylation is controlled by O-GlcNac transferase (OGT) and O-GlcNAcase (OGA) and occurs on the Ser/Thr residues of proteins (5).

Fluctuation in O-GlcNAcylation is involved in various diseases (6). Abnormal increase in O-GlcNAcylation has been implicated in diabetes mellitus (7–9), neurodegenerative diseases (10, 11), heart failure (12, 13), and cancer (14, 15). However, the role of O-GlcNAcylation in Alzheimer’s disease (AD) or Parkinson’s disease is controversial. Abnormal increase in O-GlcNAcylation slows neurodegeneration and stabilizes tau against aggregation (16). On the other hand, abnormal decrease in O-GlcNAcylation has been observed in the AD brain, accompanying hyperphosphorylation of tau (17). O-GlcNAcylation blocks the aggregation and toxicity of the protein α-synuclein associated with Parkinson’s disease (18). On the other hand, excessive O-GlcNAcylation is detrimental to neurons because it leads to the inhibition of autophagy and increase in α-synuclein accumulation (19).

Despite the accumulation of knowledge regarding the role of O-GlcNAcylation in these diseases, the functional effects of O-GlcNAcylation remain poorly understood. Therefore, obtaining further data on the O-GlcNAcylation of diabetes-related and neuroregulatory molecules may have important implications in developing therapeutic strategies for these diseases.

Stromal interaction molecule 1 (STIM1), one of the main sensors for Ca\(^{2+}\) in the endoplasmic reticulum (ER), is a critical regulator of store-operated calcium entry (SOCE) (20–23). SOCE is the process by which the emptying of ER Ca\(^{2+}\) stores causes influx of Ca\(^{2+}\) across the plasma membrane, and it plays pivotal roles in several physiological and pathological conditions, including neuronal excitability (24), hypoxic/ischemic neuronal injury (25), cardiac hypertrophy (26), proliferation of vascular smooth muscle cells (27), and carcinogenesis (28). STIM1 oligomerizes upon sensing Ca\(^{2+}\) depletion within the ER and is translocated to the ER–plasma membrane junction (29, 30). STIM1 oligomerization activates SOCE via binding to store-sensitive calcium channels, such as Orai1 (31), and transient receptor potential channels (TRPCs) (32) present on the plasma membrane. STIM1 contains EF-hand and sterile α motif (SAM; EF-SAMs) domains in its N terminus as a Ca\(^{2+}\)-sensing region, which is crucial for promoting SOCE activity (33). Recently, extracellular signal–regulated kinase 1/2 (ERK1/2) was found to phosphorylate STIM1 at Ser\(^{575}\), Ser\(^{608}\), and...
Ser^621 residues during Ca^{2+} depletion in the ER, which is also crucial for regulating SOCE activity (34). STIM1 has been found to be a microtubule plus-end–tracking protein and is directly associated with terminal-binding protein 1 (EB1), which plays a key role in regulating cellular Ca^{2+} homeostasis via modulating SOCE activity (35–37). O-GlcNAcylation of STIM1 impairs SOCE activity, resulting in impaired Ca^{2+} homeostasis in rat neonatal cardiomyocytes (38). Therefore, in the current study, we aimed to investigate the involvement of O-GlcNAcylation of STIM1 in the phosphorylation, oligomer formation, interaction with EB1 and Orai1, and SOCE activity.

**Results**

**Both glucose depletion and high glucose induced decrease in SOCE activity**

We measured SOCE activity using HEK293 cells to determine whether the glucose concentration affected this activity. HEK293 cells were treated with different glucose concentrations (low (0 g/liter), normal (1 g/liter), or high (4.5 g/liter)) for 48 h, and SOCE activity was measured via treatment with 1 μM TG injection. The peak SOCE activity in HEK293 cells after Ca^{2+} addition was evaluated from the experiments, as shown in A. Dots, data points. The data were analyzed by using one-way ANOVA followed by Tukey’s test (n=30–40 cells). *, p < 0.05. C, O-GlcNAc and STIM1 expression levels for HEK293 cells treated with different glucose concentrations. O-GlcNAcylation of endogenous STIM1 for HEK293 cells treated with different glucose concentrations was examined using immunoprecipitation assays. α-Tubulin served as the loading control. D, relative abundance of O-GlcNAcylated STIM1 was quantified as shown in C. The data were analyzed using one-way ANOVA followed by Tukey’s test. Data are represented as mean ± S.D. values (error bars) (n=3). *, p < 0.05. TG, a SOCE inducer (a SERCA inhibitor).

Fluctuation in STIM1 O-GlcNAcylation reduced SOCE activity

For in-depth analysis of the role of O-GlcNAcylation of STIM1 in SOCE activity, we generated STIM1-knockout HEK293 (STIM1-KO-HEK) cells by using CRISPR/Cas9 gene-editing technology. STIM1-KO-HEK cells showed almost no SOCE activity, but this activity was rescued by transient
Inactivation of STIM1 by fluctuation in O-GlcNAcylation

Figure 2. Dual role played by STIM1 O-GlcNAcylation in SOCE activity. A, C, E, and G. STIM1-KO-HEK cells transfected with the STIM1-mKATE-WT plasmid were treated with 10 μM TMG (gray line) or DMSO (black line) as a solvent control (A), 50 μM OGT inhibitor ST045849 (gray line) or DMSO (black line) (C), the OGT plasmid (gray line) or mock control (black line) (E), or siOGT (gray line) or siControl (black line) (G) for 24–48 h before the experiments. Subsequently, the SOCE activity was measured. B, D, F, and H, peak SOCE activity in HEK293 cells after Ca^{2+} addition, which was evaluated from the results shown in A, C, E, and G, respectively. Dots, data points. The data were analyzed using the F-test followed by Student’s t test. Data are represented as mean ± S.D. values (error bars) (n = 30–40 cells). *, p < 0.05.

STIM1-WT overexpression (Fig. S1, A and B). STIM1-KO-HEK cells transfected with the STIM1-mKATE-WT plasmid (STIM1-KO-WT-HEK cells) showed decreased SOCE activity after Thiamet G (TMG) treatment (Fig. 2, A and B). STIM1-KO-WT-HEK cells showed decreased SOCE activity upon treatment with the OGT inhibitor ST045849 (Fig. 2, C and D) and on cotransfection with OGT (Fig. 2, E and F) or with siOGT (Fig. 2, G and H). Furthermore, STIM1-KO-WT-HEK cells showed decreased SOCE activity upon treatment with siOGA (Fig. S2, A and B). As expected, O-GlcNAcylation of STIM1 increased after TMG treatment and decreased after ST045849 treatment (Fig. S3, A and B). These data indicate that fluctuation in STIM1 O-GlcNAcylation reduces SOCE activity.

Fluctuation in STIM1 O-GlcNAcylation reduced phosphorylation at Ser^{621}

Phosphorylation of STIM1 at the ERK1/2 target sites Ser^{575}, Ser^{608}, and Ser^{621} plays a pivotal role in SOCE activity (36). To evaluate the effect of the fluctuation in STIM1 O-GlcNAcylation on phosphorylation at these sites, Western blotting analyses were performed using antibodies specific to phosphorylated STIM1. To examine the effect of increased O-GlcNAcylation on phosphorylation, STIM1-KO-WT-HEK cells cotransfected with OGT were treated with 1 μM TG for 30 min. STIM1 phosphorylation at Ser^{621} in siOGT-transfected cells was markedly lower than that in siControl-transfected cells, whereas STIM1 phosphorylation at Ser^{575} and Ser^{608} did not change significantly (Fig. 3, A and B). The overall gene-silencing efficiency of nearly 90% was detected by Western blotting analysis (Fig. 3E). Furthermore, STIM1 phosphorylation at Ser^{621} in siOGA-transfected cells was markedly lower than that in siControl-transfected cells, whereas STIM1 phosphorylation at Ser^{575} and Ser^{608} did not change significantly (Fig. S2, C and D). An siOGA gene-silencing efficiency of about 58% was detected by Western blotting analyses (Fig. S2E). These data indicate that fluctuation in O-GlcNAcylation of STIM1 reduced Ser^{621} phosphorylation.

Ser^{621} and Thr^{626} were O-GlcNAcylated, but only Thr^{626}, and not Ser^{621}, was O-GlcNAcylated in the steady state

We focused on the potential O-GlcNAcylation sites within the Ser/Pro-rich domain of STIM1 because STIM1-dependent SOCE activity is accomplished by phosphorylation of STIM1 at ERK1/2 target sites within the Ser/Pro-rich domain of STIM1 (36). The computational prediction program YinOYang 1.2 was used to identify potential O-GlcNAcylation sites (41). The program provided two potential O-GlcNAcylation sites (Ser^{621} and Thr^{626}); YinOYang threshold >0.5; Fig. 4A). To confirm whether Ser^{621} and/or Thr^{626} sites are O-GlcNAcylated, we performed a co-immunoprecipitation assay using S621A and/or T626A mutants. The overall O-GlcNAcylation in T626A and S621A/T626A reduced considerably with or without siOGT treatment, and O-GlcNAcylation decreased in WT and S621A after siOGT treatment (Fig. 4, B and C). Moreover, O-GlcNAcylation increased in WT and T626A after TMG.
Inactivation of STIM1 by fluctuation in O-GlcNAcylation

Figure 3. Effects of OGT overexpression or OGT deficiency on the phosphorylation of STIM1 at ERK1/2 target sites. A, STIM1-KO-HEK cells cotransfected with mock/STIM1-mKATE-WT or OGT/STIM1-mKATE-WT plasmids were treated with 1 μM TG for 30 min. The cell lysates were subjected to Western blotting analyses with antibodies against OGT, O-GlcNAc, phospho-Ser575-STIM1, phospho-Ser608-STIM1, phospho-Ser621-STIM1, and total-STIM1. α-Tubulin served as the loading control. B, relative abundance of STIM1 phosphorylation at Ser575, Ser608, and Ser621 in relation to total STIM1 was quantified as shown in A, and the control levels (Mock) were set at 1.0. Dots, data points. The data were analyzed using one-way ANOVA, followed by Tukey’s test. Data are represented as mean ± S.D. values (error bars) (n = 3). *, p < 0.05. C, STIM1-KO-HEK cells cotransfected with siControl/STIM1-mKATE-WT or siOGT/STIM1-mKATE-WT plasmids were treated with 1 μM TG for 30 min. The cell lysates were subjected to Western blotting analyses using antibodies against OGT, O-GlcNAc, phospho-Ser575-STIM1, phospho-Ser608-STIM1, phospho-Ser621-STIM1, and total-STIM1. α-Tubulin served as the loading control. D, relative abundance of STIM1 phosphorylation at Ser575, Ser608, and Ser621 in relation to total STIM1 was quantified as shown in C, and the control levels (siControl) were set at 1.0. Dots, data points. The data were analyzed using one-way ANOVA, followed by Tukey’s test. Data are represented as mean ± S.D. values (error bars) (n = 3). *, p < 0.05. E, relative abundance of OGT in siOGT-transfected cells was quantified after normalization to the loading control, α-tubulin, as shown in A, and the control levels (siControl) were set at 1.0. The data were analyzed using the F-test followed by Student’s t-test. Data are represented as mean ± S.D. values (error bars) (n = 3). *, p < 0.05.

SOCE activity decreased in WT after TMG treatment, whereas S621A and S621A/T626A did not show changes (Fig. 4, D and E). These data suggest that Thr626 is O-GlcNAcylated in the steady state and that the O-GlcNAcylation is down-regulated with decrease in O-GlcNAcylation. Ser621 is not O-GlcNAcylated in the steady state, and the O-GlcNAcylation is up-regulated with increase in O-GlcNAcylation.

SOCE activity decreased on decrease in STIM1 O-GlcNAcylation at Thr626 via decrease in Ser621 phosphorylation

The phosphorylation of STIM1 at Ser621 markedly decreased in T626A compared with WT, as well as S621A and S621A/T626A in the steady state (Fig. 4, D and F). The phosphorylation decreased in WT after TMG treatment, whereas S621A, T626A, and S621A/T626A did not show changes after TMG treatment (Fig. 4, D and F). Given that T626A lacks O-GlcNAcylatation at Thr626, O-GlcNAcylation might be involved in the phosphorylation at Ser621. To evaluate the effect of STIM1 O-GlcNAcylation at Thr626 on SOCE activity, STIM1-KO-HEK cells transfected with S621A (STIM1-KO-S621A-HEK cells) or T626A (STIM1-KO-T626A-HEK cells) were prepared, and SOCE activity was measured. Both STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells showed significantly lower SOCE activity than STIM1-KO-WT-HEK cells (Fig. 5, A and B). Moreover, the SOCE activity of STIM1-KO-T626A-HEK cells was not significantly affected by TMG treatment (Fig. 5, C...
To determine whether O-GlcNAcylation of STIM1 at Thr626 regulates SOCE activity by decreasing the phosphorylation of STIM1 at Ser621, we introduced phosphomimetic mutations (S575E, S608E, and S621E) in the Ser/Pro-rich domain of STIM1 in the T626A mutants and transfected them into STIM1-KO-HEK (STIM1-KO-S575E/T626A-HEK, STIM1-KO-S608E/T626A-HEK, and STIM1-KO-S621E/T626A-HEK, respectively) cells. There were no significant differences in expression levels among these mutants (Fig. S4, A and B). STIM1-KO-S621E/T626A-HEK cells showed significantly higher SOCE activity than STIM1-KO-T626A-HEK cells, whereas STIM1-KO-S575E/T626A-HEK and STIM1-KO-S608E/T626A-HEK cells did not show significant changes, indicating that the phosphorylated mimetic STIM1 at Ser621 (S621E) overcomes the effect of T626A on SOCE activity (Fig. 5, E and F). These data suggest that decrease in STIM1 O-
Inactivation of STIM1 by fluctuation in O-GlcNAcylation

GlcNAcylation at Thr$^{626}$ lowers SOCE activity by decreasing the phosphorylation of STIM1 at Ser$^{621}$. Puncta formation decreased in both STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells. STIM1 forms oligomers and interacts with and activates the calcium ion channel Orai1 (42–44). STIM1 oligomer formation can be observed as puncta formation with confocal microscopy. To evaluate the effect of increased O-GlcNAcylation at Thr$^{626}$ on puncta formation, STIM1-KO-WT-HEK, STIM1-KO-S621A-HEK, and STIM1-KO-T626A-HEK cells were incubated with or without 10 μM TMG at 37 °C for 48 h. Puncta formation increased after TG treatment in STIM1-KO-WT-HEK cells; however, it was eliminated by TMG pretreatment (Fig. 6, A). STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells showed no puncta formation even after TG treatment (Fig. 6, B and C). These results suggest that the STIM1 activity was mainly triggered by the phosphorylation of STIM1 at Ser$^{621}$, which was negatively regulated by O-GlcNAcylation of STIM1 at Thr$^{626}$.

Puncta formation decreased in both STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells

STIM1 forms oligomers and interacts with and activates the calcium ion channel Orai1 (42–44). STIM1 oligomer formation can be observed as puncta formation with confocal microscopy. To evaluate the effect of increased O-GlcNAcylation at Thr$^{626}$ on puncta formation, STIM1-KO-WT-HEK, STIM1-KO-S621A-HEK, and STIM1-KO-T626A-HEK cells were incubated with or without 10 μM TMG at 37 °C for 48 h. Puncta formation increased after TG treatment in STIM1-KO-WT-HEK cells; however, it was eliminated by TMG pretreatment (Fig. 6A). STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells showed no puncta formation even after TG treatment (Fig. 6, B and C). These results suggest that the STIM1 activity was mainly triggered by the phosphorylation of STIM1 at Ser$^{621}$, which was negatively regulated by O-GlcNAcylation of STIM1 at Thr$^{626}$.

O-GlcNAcylation of STIM1 may be involved in its dissociation from EB1 and interaction with Orai1 via STIM1 phosphorylation at Ser$^{621}$

Phosphorylation of STIM1 at ERK1/2 target sites triggers its dissociation from EB1 during Ca$^{2+}$ store depletion and increases its interaction with Orai1 (36). STIM1-KO-WT-HEK, STIM1-KO-S621A-HEK, and STIM1-KO-T626A-HEK cells were incubated with or without 10 μM TMG at 37 °C, and the effect of O-GlcNAcylation of STIM1 at Ser$^{621}$ and Thr$^{626}$ on the interaction with EB1 was examined with a co-immunoprecipitation assay. The dissociation of STIM1 from EB1 after TG treatment was reduced by TMG pretreatment in STIM1-KO-WT-HEK cells (Fig. 7, A and B). Conversely, STIM1 did not dissociate from EB1 after TG treatment in STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells. STIM1 activates SOCE via interaction with Orai1 (31);
therefore, we examined the effect of O-GlcNAcylation of STIM1 at Ser\textsuperscript{621} and Thr\textsuperscript{626} on interaction with Orai1. The increased interaction between STIM1 and Orai1 after TG treatment was reduced by TMG pretreatment in STIM1-KO-WT-HEK cells (Fig. 7, A and C). Conversely, interaction with Orai1 did not significantly increase after TG treatment in STIM1-KO-S621A-HEK and STIM1-KO-T626A-HEK cells. These data suggest that O-GlcNAcylation of STIM1 at Thr\textsuperscript{626} plays a pivotal role in TG-stimulated STIM1 dissociation from EB1 and the increased interaction with Orai1, probably through regulation of Ser\textsuperscript{621} phosphorylation. To prove that O-GlcNAc does not modify the other protein kinases and cellular pathways that indirectly affect STIM1 phosphorylation, we examined whether ERK, upstream of STIM phospho-Ser\textsuperscript{621}, was O-GlcNAcylated and affected the phosphorylation. ERK was not O-GlcNAcylated (data not shown), and the activity (phosphorylated ERK) was not changed after TMG and ST045849 treatment in HEK293 cells (Fig. S5, A and B), indicating that the upstream signaling pathway may not indirectly affect STIM1 Ser\textsuperscript{621} phosphorylation.

Discussion

In the current study, we examined the effect of STIM1 O-GlcNAcylation on SOCE activity. Both increase and decrease in O-GlcNAcylation resulted in impaired activity, implying that fluctuation in O-GlcNAcylation regulates the function (Fig. 8). We had expected that STIM1 O-GlcNAcylation would either increase or decrease at the same Ser/Thr residue, and therefore we performed further in-depth analysis to determine how both increase and decrease in O-GlcNAcylation caused the same effect (i.e. STIM1 inactivation). We found two sites of O-GlcNAcylation (i.e. Ser\textsuperscript{621} and Thr\textsuperscript{626}). Our results showed that Thr\textsuperscript{626} was O-GlcNAcylated in the steady state, whereas Ser\textsuperscript{621} was not, suggesting that O-GlcNAcylation of Thr\textsuperscript{626} was easier than that of Ser\textsuperscript{621}. When overall O-GlcNAcylated decreased, the O-GlcNAcylated Thr\textsuperscript{626} was reduced, resulting in a decrease in SOCE activity. Therefore, it is thought that this Thr\textsuperscript{626} O-GlcNAcylation in the steady state is important for STIM1 function. When overall O-GlcNAcylation increased, the intact Ser\textsuperscript{621} in the steady state was O-GlcNAcylated, leading to a decrease in SOCE activity. Both decrease in O-GlcNAcylation at Thr\textsuperscript{626} and increase in O-GlcNAcylation at Ser\textsuperscript{621} decreased SOCE activity through reduction of phosphorylation at Ser\textsuperscript{621}.

Reduced O-GlcNAcylation of STIM1 at Thr\textsuperscript{626} lowered SOCE activity by decreasing the phosphorylation of STIM1 at Ser\textsuperscript{621}. We speculated that O-GlcNAcylation of Ser\textsuperscript{621} competes with its phosphorylation. We could not obtain direct evidence to show how O-GlcNAcylation of Thr\textsuperscript{626} affects phosphorylation of Ser\textsuperscript{621} and are planning to investigate this further in our future studies.

A previous study had shown that SOCE activity is triggered via STIM1 phosphorylation at the Ser\textsuperscript{575}, Ser\textsuperscript{608}, and Ser\textsuperscript{621} residues (34). However, we found that decrease in STIM1 phosphorylation at Ser\textsuperscript{621}, but not at Ser\textsuperscript{575} or Ser\textsuperscript{608}, led to its dissociation from EB1 and subsequent interaction with Orai1 to decrease SOCE activity, indicating that STIM1 phosphorylation at Ser\textsuperscript{621} could be sufficient for increasing SOCE activity. Phosphorylation at Ser\textsuperscript{621} might play the most important role in STIM1 activity and should be investigated further in future

Figure 6. Effects of non-O-GlcNAcylation of the STIM1 Ser/Pro-rich domain on puncta formation of STIM1. A, B, and C, STIM1-KO-HEK cells transfected with STIM1-mKate-WT, STIM1-mKate-S621A, or STIM1-mKate-T626A plasmids were incubated at 37 °C for 48 h—with or without 10 \mu M TMG—and the cells were treated with 1 \mu M TG for 10 min, fixed, and stained with anti-STIM1 antibody. Representative images of the cells are shown (n = 10–15 cells). Scale bar, 20 \mu m.
studies, along with the effect of STIM1 phosphorylation at Ser575 and Ser608 on STIM1 activity.

STIM1 abnormality is closely related to the etiology of various diseases; for instance, STIM1 malfunction is linked to neurodegeneration and heart disease (26, 45). Familial AD-associated presenilin 1 mutants promote γ-secretase cleavage of STIM1, leading to impairment of SOCE (46). Cardiac STIM1 silencing impairs adaptive hypertrophy and promotes heart failure through inactivation of mTORC2/Akt signaling (26). Targeting STIM1 itself might not be a promising therapy for these diseases, because the STIM1 activator is not available at the moment. Given that the fluctuation in STIM1 O-GlcNAcylation regulates STIM1 function, this modification could be involved in various diseases, such as AD and heart failure. TMG increases O-GlcNAcylation, although it is not specific for STIM1. Therefore, targeting STIM1 O-GlcNAcylation could provide a promising treatment option for these diseases if the specific modulator for STIM1 O-GlcNAcylation can be found.

Experimental procedures

Reagents and antibodies

The OGA inhibitor TMG, the OGT inhibitor ST045849, and doxycycline (DOX) were purchased from Carbosynth (Compton, UK), TimTec (Newark, DE, USA), and Tokyo Chemical Industry (D4116, Tokyo, Japan), respectively. The SERCA inhibitor TG, the nonionic surfactant Pluronic-F127, and the prototypic inhibitor of organic anion transport, probenecid, were obtained from Sigma–Aldrich. The calcium-sensitive dye Fura-2-AM was purchased from Dojindo (Kumamoto, Japan). Anti-STIM1 (catalog no. MAB3602, Abnova (Taipei, Taiwan)), anti-O-GlcNAc (catalog no. NB300-524 (RL-2), Novus (Centennial, CO, USA)), anti-EB1 (catalog no. sc-15347, Santa Cruz Biotechnology, Inc. (Dallas, TX, USA)), anti-Orai1 (catalog no. SAB3500127, Sigma–Aldrich), and anti-α-tubulin (catalog no. PM054, MBL (Nagoya, Japan)) antibodies were used. Antibodies against phospho-Ser575-STIM1, phospho-Ser608-STIM1, and phospho-Ser621-STIM1, and phospho-Ser621-STIM1 were raised against phosphopeptides

Figure 7. Effects of non-O-GlcNAcylation of the STIM1 Ser/Pro-rich domain on interaction of STIM1 with EB1 and Orai1. A, STIM1-KO cells transfected with the STIM1-mKATE-WT, STIM1-mKATE-S621A, or STIM1-mKATE-T626A plasmids were grown with 5 μM TMG for 24 h and treated with 1 μM TG for another 30 min. The cell lysates were subjected to co-immunoprecipitation with the anti-STIM1 antibody. The EB1, STIM1, and O-GlcNAc expression levels in the cell lysates and the EB1, Orai1, and STIM1 expression levels in immunoprecipitates were then determined by Western blotting analyses. α-Tubulin served as the loading control. B, relative abundance of co-precipitated EB1 was quantified as shown in A. Dots, data points. The data were analyzed using one-way ANOVA followed by Tukey’s test. Data are represented as mean ± S.D. values (error bars) (n = 3), *, p < 0.05. C, relative abundance of co-precipitated Orai1 was quantified as shown in A. Dots, data points. The data were analyzed using one-way ANOVA followed by Tukey’s test. Data are represented as mean ± S.D. values (error bars) (n = 3), *, p < 0.05.
corresponding to appropriate regions of the mouse STIM1, respectively (36).

**Cell culture**

HEK293 cells were obtained from the JCRB (Japanese Collection of Research Bioresources) Cell Bank (National Institute of Health Sciences, Kanagawa, Japan) and cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere of air/CO₂ at 37 °C. HEK293 cells were treated with different glucose concentrations (low (0 g/liter), normal (1 g/liter), or high (4.5 g/liter)), as shown in Fig. 1. Otherwise, Dulbecco’s modified Eagle’s medium containing 1 g/liter glucose was used.

**Generation of STIM1-KO-HEK cells**

The guide pair (sense 5’-CACCAGATGACAGACCG-GAGTCAT and antisense 5’-AAACTGAGGTGATTATGGC-GAGTC) was identified using the CRISPR.mit.edu-Zhang Lab web tool (https://zlab.bio/guide-design-resources). This pair targets exon 6 of the STIM1 (NM_003156, locus (ENSE00003492515)). The antisense dsDNA guide and the sense guide were cloned into eSPCAS9 1.1 constructs using BbsI restriction enzyme (New England Biolabs). Cells were cotransfected with 7 μg of guide RNA construct and 3 μg of pCW-hyPBase by electroporation in a 10-cm dish. Transfected cells were selected using blasticidin (10 μg/ml) for 48 h, and individual clones were analyzed by immunoblotting and sequencing. Genomic DNA was isolated, and the target site was amplified by PCR (primers: forward, 5’-AGCTTACTGTG-ATAAGTGATGCGAAGTGTTGCT; reverse, 5’-CTCCTGCTCAGTCCGCTGTAAC). Sequencing of exon 6 in STIM1-KO-HEK cells revealed a bp 627–631 deletion of STIM1, confirming the successful KO of the STIM1 locus.

**Expression vectors, siRNAs, and transfection**

Mouse STIM1 (NCBI accession number: NM_009287.5) was cloned into PB-TET-CF-Bridge-2A-mKATE (kindly provided
Inactivation of STIM1 by fluctuation in O-GlcNAcylation

by Dr. Cody Kime; Retinal Regeneration, RIKEN Center for Biosystems Dynamics Research, Kobe, Japan) inducible by DOX (STIM1-mKATE). Cotransfection of STIM1-mKATE with OGT siRNA or OGA siRNA was performed using the TransIT-TKO reagent (Mirus Corp., Madison, WI) according to the manufacturer’s instructions. The targeting sequences of the OGT siRNA were as follows: sense, 5’-GCAACAAACCUCAGACCACAU TT-3’; antisense, 3’-TTCCUGUUUUGGACUGUGUA-5’. The targeting sequences of the OGA siRNA were as follows: sense, 5’-GCAACAAACCUGACCACAU TT-3’; antisense, 3’-TTCCUGUUUUGGACUGUGUA-5’.

Immunoblotting and co-immunoprecipitation

DOX-treated cells were lysed in SDS-PAGE sample buffer and boiled for 10 min at 100 °C. After SDS-PAGE, the proteins were transferred to a polyvinylidene fluoride membrane and boiled for 10 min at 100 °C. After SDS-PAGE, the proteins eluted with SDS-PAGE sample buffer were subjected to immunoblotting with anti-STIM1 (1:3000 dilution), anti-phospho-Ser575-STIM1 with nonphosphorylation with anti-STIM1 (1:3000 dilution), anti-(Merck Millipore, Billerica, MA, USA) and subjected to immunoblotting with anti-STIM1 antibody (Figs. 1C, 4 (B and D), and 7A) conjugated with SureBeads Magnetic Beads (Bio-Rad). The proteins eluted with SDS-PAGE sample buffer were subjected to SDS-PAGE.

Confocal microscopy

Cells on glass-bottomed dishes were fixed with 4% paraformaldehyde for 10 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. After the cells were subjected to blocking in PBS with 10% BSA, they were treated with anti-STIM1 antibody (1:500 dilution) for 60 min, followed by treatment with Alexa Fluor 488–labeled secondary antibody (1:400 dilution). The fluorescently labeled cells were visualized using confocal microscopy (SP8; Leica, Wetzlar, Germany).

SOCE measurement

Cells on glass-bottomed dishes were incubated with 2 mM Fura-2-AM along with 0.025% Pluronic-F127 and 12.5 mM probenecid for 50 min at 37 °C in loading buffer. Subsequently, the buffer was replaced with Ca2+-free loading buffer. Depletion of Ca2+ stores was triggered by adding 1 μM TG to the dishes. The composition of the Ca2+-free loading buffer was as follows: 20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.34 mM Na2HPO4, 0.44 mM KH2PO4, 4.17 mM NaHCO3, and 0.8 mM Mg2+. SOCE was measured by monitoring the increase in [Ca2+], after the addition of 2 mM CaCl2 to the TG-containing medium. The [Ca2+]i was calculated by monitoring the F340 nm/F380 nm excitation ratio with an emission wavelength of 510 nm (Nikon, Japan). Because this ratio correlates with intracellular Ca2+ concentration, this method is generally used for the measurement of SOCE activity. Digital images were recorded with a Hamamatsu C9100 EM-CCD camera (Hamamatsu, Japan) with the Metafluor software. All measurements were performed at 37 °C (MATS-505RA20, Tokai Hit, Japan).

Statistical analyses

The data are expressed as the means ± S.D. for more than three determinations. Statistical analyses were performed using Student’s t test (Figs. 2 (B, D, F, and H), 3E, and 5D and Fig. S2 (B, D, and E)) or one-way analysis of variance (ANOVA) followed by Tukey’s test (Figs. 1 (B and D), 3 (B and D), 4 (C, E, and F), 5 (B and F), and 7 (B and C) and Figs. S1 (B and D), S3B, and S5B) or one-way ANOVA followed by Dunnett’s test (Fig. S4B). p values <0.05 were considered significant.

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: OGT, O-GlcNAc transferase; OGA, O-GlcNAcase; AD, Alzheimer’s disease; ER, endoplasmic reticulum; SOCE, store-operated Ca2+ entry; TRPC, transient receptor potential channels; ERK, extracellular signal–regulated kinase; SERCA, sarco(endo)plasmic reticulum Ca2+-ATPase; TG, thapsigargin; KO, knockout; TMG, Thiamet G; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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