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Original Article

# Both STT3A- and STT3B-containing OST complexes are involved in the asparagine-linked glycosylation of human R-SPONDIN 3

**Chiung-Fang Chang[1], Hui-Fang Tsai[2, 3], Chu-Chyn Ou[4, 5], Jen-Ning Tsai[2, 3, *]**

[1] *Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan*
[2] *Department of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*
[3] *Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan*
[4] *School of Nutrition, Chung Shan Medical University, Taichung, Taiwan*
[5] *Department of Nutrition, Chung Shan Medical University Hospital, Taichung, Taiwan*

**Purpose:** R-spondins (RSPOs) play a pivotal role in stem cell development by potentiating Wnt signaling. We previously studied the biogenesis of RSPOs and found that asparagine-linked glycan (N-glycan) at N137 on RSPO1 affects intracellular stability and secretion efficiency. In the present study, we focused on RSPO3 as it has multiple N-linked glycan chains.
**Methods:** The N-glycosylation sites on RSPO3 were explored by site-directed mutagenesis and Western blot analysis. The identification of the N-glycosyltransferase(s) responsible for RSPO3 N-glycosylation was approached by specific siRNA knockdown.
**Results:** In addition to N137, two glycosylation sites at positions N36 and N194 were identified. Elimination of glycosylation at these sites led to opposing effects on RSPO3 secretion. While abrogation of N-glycosylation at N36 impaired its secretion, loss of N-glycan at N194 enhanced its secretion. These results highlight the differential roles of N-glycan at these two positions in the secretion of RSPO3. Finally, we showed that N137 is an STT3A-dependent site and N-glycosylation at N36 and N194 is STT3B-dependent using specific siRNA knockdown.
**Conclusion:** Based on the results, STT3A- and STT3B-associated OST complexes differentially glycosylate RSPO3 at multiple sites.

**Keywords:** R-SPONDIN 3, N-glycosylation, protein secretion, STT3A, STT3B

## 1. Introduction

R-spondins (roof plate-specific spondins, RSPOs) belong to a family of four secreted proteins known to enhance Wnt signaling activity. All RSPOs share a common structure that includes an N-terminal signal peptide (SP), two cysteine-rich furin-repeat domains (FR1 and FR2), a thrombospondin type 1 domain (TSR1), and a C-terminal region enriched with positively charged amino acids (CT). Recent studies have indicated that FRs of RSPOs are essential and sufficient to regulate the Wnt-potentiating effect [1-3].

RSPOs play a wide range of pleiotropic roles in physiological processes in vertebrates. For example, RSPO1 is best known as a growth factor for adult stem cells of the small intestine. As such, it has been of value in regenerative medicine. A recent study has also indicated that pericryptal myofibroblast-derived

* Corresponding Author: Jen-Ning Tsai
Address: No. 110, Sec. 1, Jianguo N. Rd., Taichung City, 40201, Taiwan.
Tel: +886-4-24730022 ext. 11720
E-mail: jeningts@csmu.edu.tw

RSPO3 is more potent than RSPO1 in stimulating canonical Wnt signaling and organoid growth in a mouse model [4].

Asparagine-linked (N-linked) glycosylation of proteins is one of the most common post-translational modification reactions in eukaryotic cells [5].

Almost half of all human proteins are glycoproteins and most contain N glycans. N-linked glycosylation of proteins is critical for their proper folding, assembly, and stability. It is also important for a wide range of biological processes, such as cell attachment to the extracellular matrix and protein-ligand interactions [5].

N-glycosylation occurs via oligosaccharyltransferase (OST) complex embedded in the rough endoplasmic reticulum [6]. OST catalyzes the transfer of preassembled oligosaccharides onto the asparagine residue of glycosylation acceptor sites or sequons (Asn-X-Thr/Ser, X≠Pro) of a newly synthesized protein [7-9]. Two human OST complexes distinguished by their association with either STT3A or STT3B catalytic subunit are responsible for N-linked glycosylation of proteins in the endoplasmic reticulum. The STT3A complex is associated with the translocon channel (Sec61 complex) that regulates co-translational N-glycosylation of nascent polypeptides as they enter the lumen of the ER [10-12]. The STT3B complex does not interact directly with the translocon channel but glycosylates acceptor sites that are skipped by the STT3A complex in post-translational mode [10,11,13].

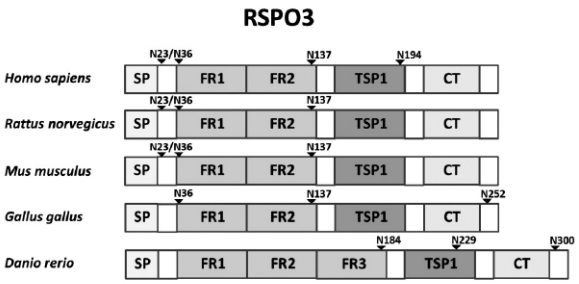


**Figure 1.** Location of sequons in vertebrate RSPO3 protein family. All RSPO proteins contain an N-terminal signal peptide (SP), two furin-repeat domains (FR1 and FR2), a thrombospondin type 1 domain (TSP1), and a C-terminal domain with several positively charged amino acids (CT). One exception is that teleost RSPO3 members contain three furin repeats. Putative sequons are denoted by triangles

In a previous study, we investigated the biogenesis of human RSPOs in HEK293T cells. Human RSPO1 and RSPO3 are both N-glycosylated at N137, a conserved site near the C-terminus of FR2 (Fig. 1). Prevention of N-glycosylation at these sites affects the accumulation of these RSPOs in media. In the present study, we identified two additional glycosylation sites at positions N36 and N194 on RSPO3. Elimination of glycosylation at these sites led to opposing effects on RSPO3 secretion. Finally, we showed that N137 is an STT3A-dependent site and N-glycosylation of N36 and N194 is STT3B-dependent using STT3 isoform-specific siRNA.

## 2. Materials and Methods

### 2.1. Plasmid construction and site-directed mutagenesis

The vectors expressing human RSPO3 with C-terminal HA-tag (pCS2-RSPO3-HA) were constructed as previously described [14]. To generate individual N-glycosylation mutants, the codon for Asn (N) in the consensus sequence for N-glycosylation was mutated to a codon for Gln (Q) using the QuickChange Lightning site-directed mutagenesis kit (Stratagene Inc., La Jolla, California, USA).

### 2.2. Cell culture and transfection

HEK293T cells were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FCS, Gibco) and incubated at 37°C with 5% $CO_2$ in humidified incubator. Before transfection, the culture medium was replaced with Gibco Opti-MEM reduced serum medium (Gibco Life Technologies, Grand Island, New York, USA) (containing 2% fetal calf serum). For transient expression of HA-tagged RSPO3 and its mutants, the cells were transfected using NTR II (T-pro Biotechnology Inc.). The conditioned media and the transfected cells were then collected and processed 48 hr post transfection.

### 2.3. siRNA treatment

HEK293T cells were seeded and grown for 24 h before transfection with siRNA and Lipofectamine 2000 in Opti-MEM (Gibco), based on the manufacturer's protocol (Invitrogen). Plasmid

**Table 1. Sequences of mutagenic primers, RT-qPCR primers, and siRNA**

| Name | Sequence |
|---|---|
| RSPO3 mutant N23Q | 5'-CTTCCCCGGGAGGCTTGTTGGCTGCCGATGTATT-3'<br>5'-AATACATCGGCAGCCAACAAGCCTCCCGGGGAAG-3' |
| RSPO3 mutant N36Q | 5'-CTTGGCAGCCTTGACTAACTTGAGGATGCATTCTTCGCTGG-3'<br>5'-CCAGCGAAGAATGCATCCTCAAGTTAGTCAAGGCTGCCAAG-3' |
| RSPO3 mutant N137Q | 5'-AGAAGGGTTGGAAGCCAACCAACATACTATGGAGTGTGTCA-3'<br>5'-TGACACACTCCATAGTATGTTGGTTGGCTTCCAACCCTTCT-3' |
| RSPO3 mutant N194Q | 5'-CTGTACACTTTCTTGTCTCTTGTGTTGGGGGACACAGGTTA-3'<br>5'-TAACCTGTGTCCCCCAACACAAGAGACAAGAAAGTGTACAG-3' |
| siRNA NC | 5'-UUCUCCGAACGUGUCACGUdTdT-3'<br>5'-ACGUGACACGUUCGGAGAAdTdT-3' |
| siRNA *STT3A* | 5'-GGCCGUUUCUCUCACCGGCdTdT-3'<br>5'-UCCGGUGAGAGAAACGGCCdTdT-3' |
| siRNA *STT3B* | 5'-GCUCUAUAUGCAAUCAGUAdTdT-3'<br>5'-CACUGAUUGCAUAUAGAGCdTdT-3' |
| RT-qPCR *GAPDH* | 5'-TGCACCACCAACTGCTTAGC-3'<br>5'-GGCATGGACTGTGGTCATGAG-3' |
| RT-qPCR *STT3A* | 5'-GTGTGGACCGTGAAGGTTCTC-3'<br>5'-ATCCTGACCAGCCAATGTTCTG-3' |
| RT-qPCR *STT3B* | 5'-AATCCACCTGTGGAGGACAGC-3'<br>5'-TGTGACCCAGGTACAGTGGAC-3' |

transfection was performed after 48 h of siRNA transfection (*siSTT3A*: 50 nM; *siSTT3B*: 60 nM) and cell lysates were collected 24 hr later. The siRNAs specific for *STT3A* and *STT3B* mRNAs (Table 1) have been previously characterized [10,11,13]. The siRNAs were custom synthesized by GenePharma (Shanghai, China).

## 2.4. Deglycosylation

Deglycosylation of the cell lysates with PNGase F (New England Biolabs, Ipswich, Massachusetts, USA) was carried out according to the manufacturer's instructions. Digestion with enzymes was performed at 37°C for 3 h. The digested samples were then denatured in SDS sample buffer and subjected to Western blotting.

## 2.5. Cell fractionation and Western Blot

To prepare the cell lysates, cells were washed once with PBS and incubated in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (Goal Bio Inc.) on ice for 10 min. After brief sonication, the cell homogenates were clarified at 10,000 x g at 4°C for 10 min and soluble cell lysate was collected. The protein contents of the cell lysates were analyzed on Bradford dye binding assay (Bio-Rad Inc., Hercules, CA, USA) with bovine serum albumin (Sigma, St. Louis, MO, USA) as the standard. Soluble cell lysates or media were denatured by the addition of 4x SDS sample buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 5% β-mercaptoethanol, 40% Glycerol, 0.04% Bromophenol Blue) in boiling water for 5 min. Denatured protein samples were then loaded onto 10% SDS-PAGE gel (Bio-Rad Mini-Protean Electrophoresis system, 10.1 cm x 7.3 cm with 1.5 mm spacer) and separated. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes (PVDF, Pall Corporation, Port Washington, NY, USA) by a MiniTrans-blot module (Bio-Rad). The membranes were blocked at room temperature for 1 h in blocking buffer (BlockPro™ blocking buffer, Visual Protein Biotechnology,

Taipei, Taiwan) and then incubated overnight with primary antibody (diluted in blocking buffer), with constant shaking at 4°C. After six washes in PBST (PBS containing 0.1% Tween 20) for 5 min each, the blots were further incubated with secondary antibody (diluted in blocking buffer) at room temperature for 1 h, washed six times in PBST for 5 min each, and developed using the T-pro LumiLong Chemiluminescence Detection kit (T-pro Biotech, New Taipei County, Taiwan) according to the manufacturer's protocol. The chemiluminescence signal was captured using ImageQuant™LAS 4000 (GE Health, Little Chalfont, UK) and densitometric quantification of the exposed images was carried out with MultiGauge software (Version 2.2, Fujifilm, Minato, Tokyo, Japan).

To quantitate the relative amounts of RSPO3 expressed in the media, the denatured samples containing cell lysates and conditioned media were run on the same gel using the following loading amounts: 2 μg for cell lysates and 15 μL for media. The loading conditions enabled relative signals to be quantitated on the same blot. Since the total amount of protein lysate and the total volume of conditioned media in each transfected cell were known, the relative level of RSPO3 in cell lysates and media could be obtained and the percentage of RSPO3 in media could be calculated by the following equation: the relative signals in media/ total signals (the relative signals in media and cell lysates). The antibodies used in this study included rabbit polyclonal anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1500), mouse monoclonal anti-α-tubulin antibody (Novus, Littleton, CO, USA, DM1A, 1:3000), goat anti-mouse antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, Baltimore, MD, USA, 1:3000), and goat anti-rabbit antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, 1:3000).

### 2.6. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted with Trizol (Invitrogen, Inc.) and treated with RNase-free DNase I (Promega, Inc.). cDNA was synthesized using GoScript™ Reverse Transcription System (Promega, Inc.) with oligo-dT primers. A portion of the synthesized cDNA was used as the template for subsequent qPCR with the specific primer pairs listed in Table 1. A real-time PCR assay was performed with Fast SYBR® Green Master Mix (Life Tech.) in a StepOne Real-Time PCR system (Life Tech.) according to the manufacturer's instructions. The data were then analyzed with StepOne software (V2.2) and presented as the averages of experiments performed in triplicate. They represented fold changes in target genes in siRNA-transfected cells relative to negative control-transfected cells. *GAPDH* was used as internal control for normalization during quantification.

### 2.7. Statistical analysis

There were three technical replicates for each experiment. Unpaired Student's *t*-test was utilized to determine statistical difference. For all figures, *p* values are defined as * $p< 0.05$, ** $p< 0.01$, and *** $p< 0.001$.

## 3. Results

### 3.1. RSPO3 is N-glycosylated at multiple sites in HEK293T cells

Our previous study indicated that RSPO3 is N-glycosylated at N137. Abrogation of N-glycosylation at this site affected its accumulation in media [14]. In this study, when HA-tagged human RSPO3 was expressed in HEK293T cells, multiple immunoreactive bands were detected in the cell lysates and conditioned media (Fig. 2A). To verify the properties of these multiple bands, the cell lysates containing expressed RSPO3 were treated with peptide N-glycosidase F (PNGase F), an enzyme that cleaves between the innermost GlcNAc and Asn residues of oligosaccharides from N-glycosylated proteins to release N-linked glycans [15]. Treatment with PNGase F markedly reduced the molecular masses of the reactive bands in cell lysates (Fig. 2A), indicating that these bands originated during N-glycosylation.

In addition to the conserved N137 sequon, there exist three other sequons in human RSPO3 (Fig. 1). To explore whether these sequons are also N-glycosylated in RSPO3, RSPO3 mutants (N23Q, N36Q and N194Q) were generated and transfected

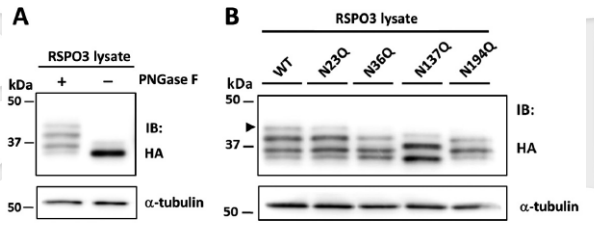


**Figure 2.** RSPO3 is N-glycosylated at multiple sites. (A) Human HA-tagged RSPO3-transfected HEK293T cell lysates were treated with (+) or without (-) PNGase F and subjected to Western blotting. (B) Cell lysates from HA-tagged human WT or mutant RSPO3-transfected HEK293T cells were analyzed for the expression of HA-tagged RSPO3 on Western blotting. Triangle denotes the band with the higher molecular mass missing from the cell lysates of N36Q, N137Q, and N194Q mutants. α-tubulin was used as a loading control on Western blotting.

into HEK293T cells. The results showed that at least one glycosylated band is missing in N36Q, N137Q, and N194Q when compared with wild type (WT) RSPO3. However, there were no significant differences in glycosylated patterns between WT and N23Q RSPO3 (Fig. 2B). This indicated that RSPO3 is N-glycosylated at positions N36, N137 [14], and N194 in HEK293T cells.

### 3.2. Abrogation of N-glycans at N36 and N194 leads to altered secretion efficiency of RSPO3

Our previous study indicated that mutation of N-glycosylation site at N137 of RSPO3 (N137Q mutant) significantly reduces the amount of protein that is secreted into media. To explore the effects of other glycosylated positions on the secretion of RSPO3, WT and mutant RSPO3 were transfected into cells and their relative percentages of expression in conditioned media were analyzed (as described in Materials and Methods). While mutation at N23 position of RSPO3 had little effect on secretion efficiency, prevention of N-glycosylation at N36 significantly reduced the percentage of mutant RSPO3 in media (Fig. 3A, B; WT: 28.1 + 1.2% vs N36Q: 8.0 + 0.8%). Intriguingly, abrogation of N-glycosylation at position N194 on RSPO3 enhanced the percentage of mutant RSPO3 in media, compared with that of WT RSPO3 (Fig. 3A, B; WT: 28.1 + 1.2% vs N194Q: 43.0 + 6.0%). Together, these

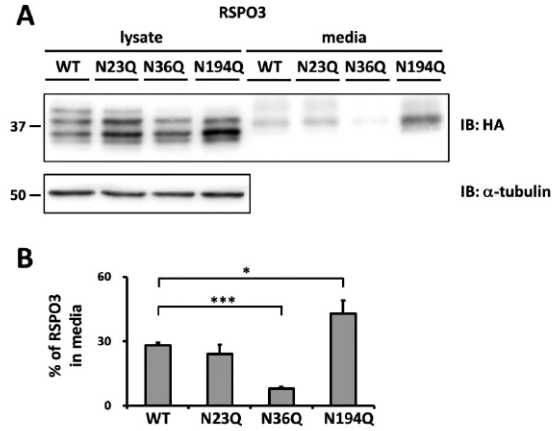


**Figure 3.** N-glycosylation at Asn36 enables efficient secretion while glycosylated Asn194 impairs secretion. (A) Human WT or mutant RSPO3 was transiently transfected into HEK293T cells. After 48 hr, the cell lysates and conditioned media were analyzed for HA-tagged RSPO3. α-tubulin was used as a loading control for the cell lysates. (B) Quantification of secretion efficiency for human WT or mutant RSPO3. Immunoblots were quantified densitometrically and percentage of secretion into media was calculated as described in Materials and Methods. Error bars represent the mean ± SD. Student's t-test was used to evaluate statistical significance (* $p$ <0.05; *** $p$ <0.001).

two additional N-glycosylation sites had opposing effects on secretion of RSPO3 into media. While N-glycosylation at N36 is required for efficient secretion, N-glycosylation at N194 inhibits secretion.

### 3.3. N-glycosylation of multiple sites on RSPO3 depends on both STT3A- and STT3B-associated OST complexes

Mammalian OST is composed of two different catalytic subunits, STT3A and STT3B, that act sequentially in protein co-translational and post-translational N-glycosylation [10]. To understand how multiple sites on RSPO3 become N-glycosylated, we investigated the involvement of both STT3 isoforms in this process using specific siRNAs against STT3 isoforms. The inhibitory effects of STT3A- and STT3B-specific siRNAs on the expressions of each mRNA in HEK293T cells were evaluated by RT-qPCR, which showed that STT3 isoforms are specifically depleted by their respective siRNAs, although the expressions of their counterparts are

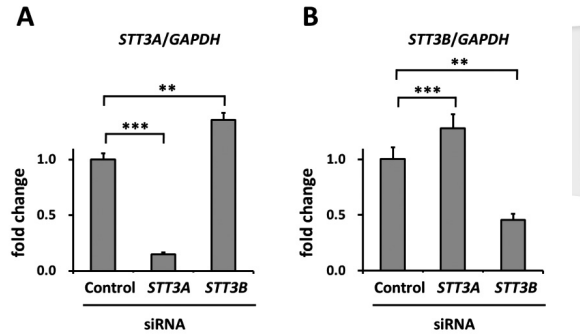


**Figure 4.** Effects of siRNA-mediated knockdown on the expressions of *STT3A* and *STT3B*. Endogenously expressed STT3A and STT3B mRNAs were knocked down in HEK293T cells by non-targeting siRNA (NC) or isoform-specific STT3 siRNAs. Relative *STT3A* (A) and *STT3B* mRNA (B) expression levels were determined on RT-qPCR analysis. Values were normalized to *GAPDH* mRNA levels. Student's t-test was used to evaluate statistical significance (**$p$ <0.01; *** $p$ <0.001).

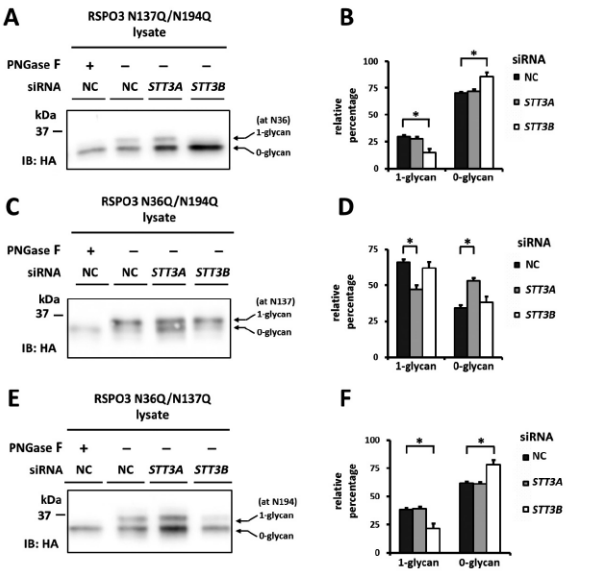


**Figure 5.** N-glycosylation of RSPO3 depends on both STT3A- and STT3B-associated OST complexes. (A, C, E) HEK293T cells were cultured for 24 hours prior to transfection with the indicated siRNA. Forty-eight hours after siRNA transfection, the cells were transfected with HA-tagged mutant RSPO3 plasmids, then harvested 24 hours later. The cell lysates were subjected to Western blotting. PNGase F digestion provided a mobility marker for nonglycosylated protein. (B, D, F) Immunoblots were quantified densitometrically and the relative percentages of glycosylated and non-glycosylated bands were calculated. Error bars represent the mean ± SD. Student's t-test was used to evaluate statistical significance (* $p$ <0.05).

partially enhanced during treatment (Fig. 4A, B). To facilitate the interpretation of the involvement of specific STT3 isoform in the glycosylation of multiple sites on RSPO3, we generated RSPO3 mutants, namely N137Q/N194Q, N36Q/N194Q, and N36Q/N137Q, each with only one glycan at N36, N137, and N194, respectively. The plasmid encoding each of the RSPO3 mutants was transfected into cells pretreated with specific siRNA against specific STT3 isoforms. In STT3A knockdown cells, the glycosylation patterns of N137Q/N194Q and N36Q/N137Q RSPO3 mutants were similar to those of NC siRNA transfected-cells. However, in STT3B knockdown cells, the glycosylated bands were diminished (Fig. 5A, B, E, F), suggesting that N-glycosylation at positions N36 and N194 is primarily mediated by STT3B isoform. In contrast, STT3B knockdown failed to reduce the amount of glycosylated protein at position N137 on N36Q/N194Q RSPO3, while STT3A knockdown impaired glycosylation (Fig. 5C, D). This suggests that N137 on RSPO3 is an STT3A-dependent site. Together, the data indicated that STT3A- and STT3B- associated OST complexes differentially glycosylate RSPO3 at multiple sites.

## 4. Discussion

In the present study, human RSPO3 was N-glycosylated at multiple sites in HEK293T cells. In addition to the previously identified N137, N-glycan is added at N36 and N194 on RSPO3. Intriguingly, the effects of N-glycosylation at these two positions differ. While prevention of N-glycosylation at position N36 inhibits the secretion of RSPO3, abrogation of N-glycosylation at position N194 enhances it. The involvement of STT3 isoform-associated OST complex was also investigated using specific siRNA knockdown. The results showed that while N137 is an STT3A-dependent site, N-glycosylation at N36 and N194 is associated with STT3B. These data suggested that specific STT3 isoforms preferentially contribute to the N-glycosylation of RSPO3 at different sites.

N-glycosylation of proteins in endoplasmic reticulum is critical for protein quality control [16]. We previously showed that addition of N-glycan at N137

of RSPO1 affects its intracellular stability [14]. The relative instability of unglycosylated N137 RSPO1 mutant also contributes to its reduced secretion into media. Since N36Q RSPO3 mutant also shows reduced secretion into media, the contribution of N-glycan at N36 to intracellular stability and its interaction with glycan at N137 merit further investigation.

Most N-glycosylation occurs co-translationally, mediated by STT3A-associated OST. There are few reports of post-translational N-glycosylation by STT3B-containing OST complex in mammalian cells [10,17]. In HEK293T cells, the percentage of glycosylated RSPO3 proteins at position N137 was higher than at positions N36 and 194 (Figure 5B, D, F, N137: 66%; N36: 29.7% and N194: 38.3%). This might be due to the fact that N-glycosylation of N137 depends on the co-translational modification of STT3A isoform, while N-glycosylation at N36 and N194 is mediated by the post-translational modification of STT3B isoform [10]. Compared with co-translational N-glycosylation by STT3A-associated complex, post-translational N-glycosylation by STT3B-associated OST is slower. Therefore, we speculated that N-glycosylation at N137 is more efficient than at N36 or N194 on RSPO3 in the steady state. This might also explain why RSPO3 mutant without N-glycosylation at N194 shows higher secretion efficiency than WT RSPO3.

All three N-glycosylation acceptor sites on RSPO3 are located within cysteine rich domains with disulfide bond pairings (Fig. 1) [12,18]. The nascent growing polypeptide ceases to be a substrate for OST in folded conformation. Thus, N-glycosylation and disulfide bond formation can be considered competing reactions during the translation process. In most glycoproteins, co-translational N-glycosylation by STT3A-associated OST allows efficient modification of acceptor sites in cysteine-rich protein domains before disulfide bond formation. STT3B-associated OST assembled with oxidoreductases glycosylates sequons that are skipped by the STT3A complex and that are proximal to cysteine residues in the nascent polypeptides [11]. Our data indicated that these two mechanisms cooperate to achieve efficient N-glycosylation of cysteine proximal acceptor sites within RSPO3.

In summary, we found that human RSPO3 is N-glycosylated at multiple sites in HEK293T cells. In addition to the previously studied N-glycan site N137, two additional glycosylation sites, N36 and N194, were identified on RSPO3. The N-glycans on these sites have opposing effects on the secretion efficiency of RSPO3. Finally, the two OST complexes, STT3A- and STT3B, cooperate to glycosylate the acceptor sites within cysteine-rich regions on RSPO3.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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