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Supplemental information

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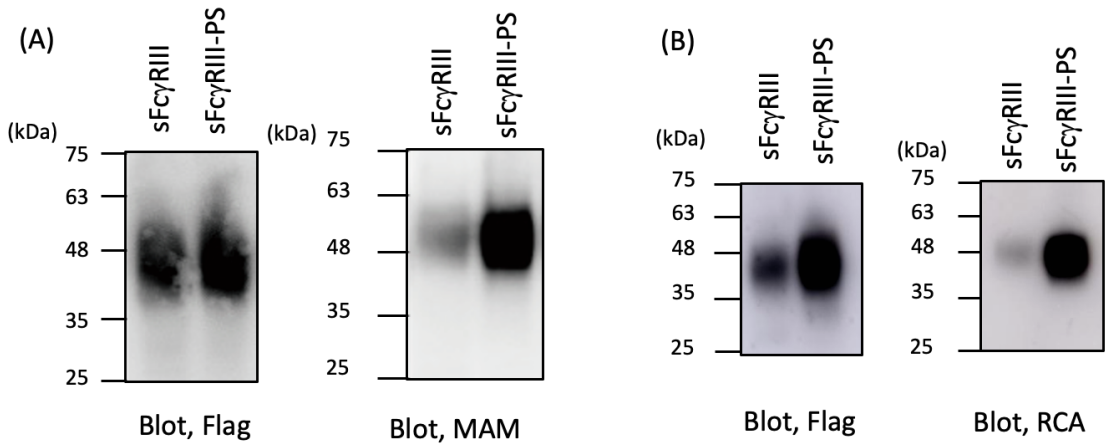

Figure S1. The effect of passport sequence on the galactosylation and sialylation levels of recombinant sFcγRIII.

(A) Tagging with the passport sequence increased the sialylation level of recombinant sFcγRIII produced in Expi293F cells. The sialylation level was determined through immunoblotting using anti-Flag antibody and lectin blotting using MAM. (B) Tagging with the passport sequence increased the galactosylation level of desialylated recombinant sFcγRIII produced in Expi293F cells. The galactosylation level was determined through immunoblotting using an anti-Flag antibody and lectin blotting using RCA.

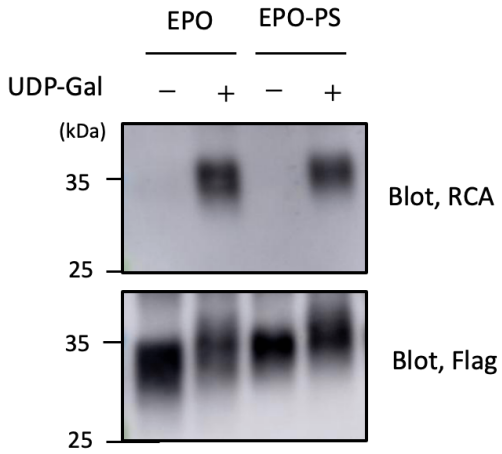

Figure S2. Assay to investigate the potential effect of PS on the *in vitro* galactosylation of EPO by recombinant B4GALT1.

Recombinant EPO-PS was produced in Expi293F cells and subjected to enzymatic treatment for de-sialylation and de-galactosylation. A portion of the de-galactosylated EPO-PS was treated with TEV protease to cleave the PS tag (EPO). EPO and EPO-PS were then used as acceptor substrates in an *in vitro* enzymatic reaction with recombinant B4GALT1. The galactosylation levels were determined by immunoblotting using an anti-Flag antibody and lectin blotting using RCA.

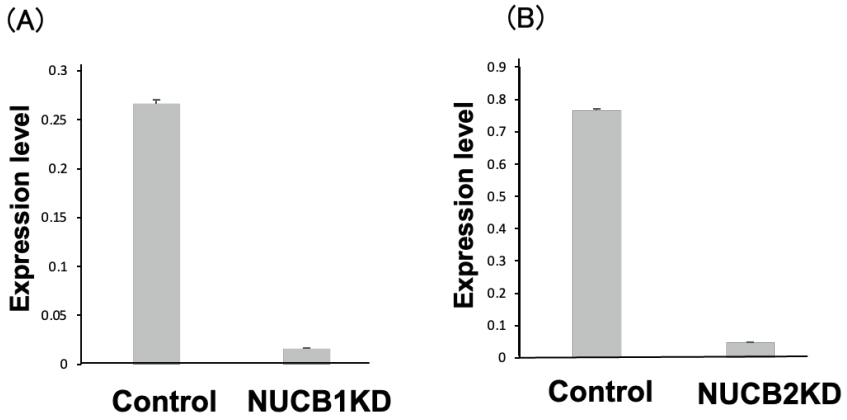

Figure S3. The efficiency of siRNA transfection.

The qRT-PCR revealed that (A) NUCB1 or (B) NUCB2 gene expression significantly decreased in Expi293F cells expressing EPO-PS under treatment with NUCB1 or NUCB2 siRNAs, respectively, compared to control siRNA

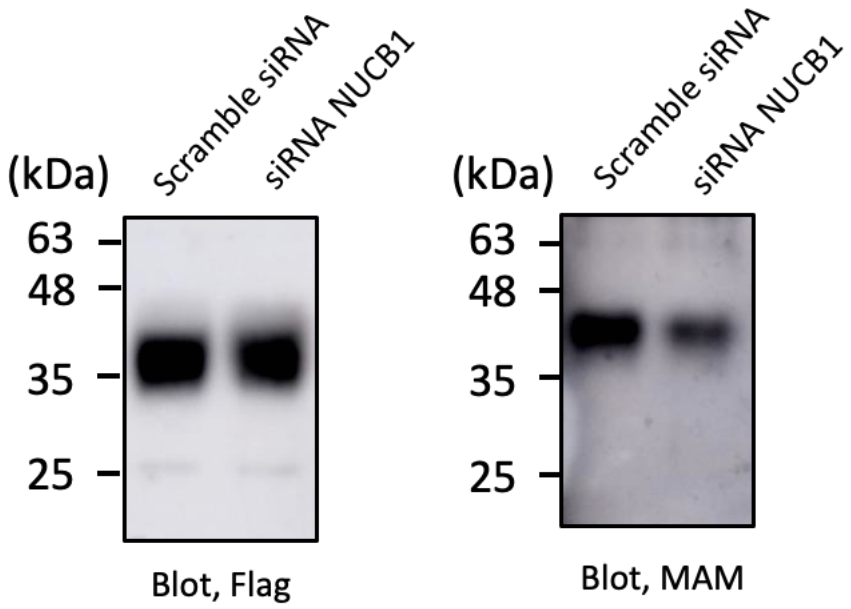

Figure S4. The depletion of NUCB1 had little to no effect on the expression levels of recombinant EPO-PS driven by the passport sequence.

Expi293F cells expressing EPO-PS were treated with control-siRNA and NUCB1-siRNA. After 24 h of cultivation, EPO and EPO-PS were purified from the culture medium using anti-Flag antibody-conjugated resin. Subsequently, the purified proteins were subjected to blotting with an anti-Flag antibody and MAM.

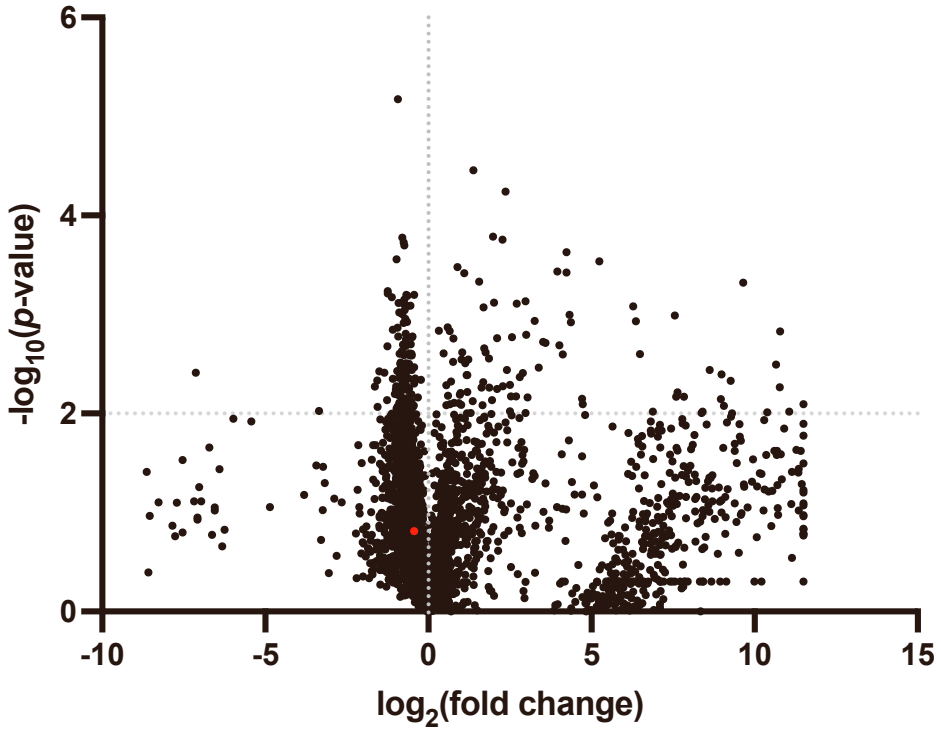

Figure S5. Volcano plot highlighting significantly enriched NUCB1-proximal proteins measured with LC-MS/MS.

Expi293F cells expressing NUCB1-TbN and NUCB1-TbC were incubated for 1 h with biotin to enrich biotinylated proteins in the secretory pathway. After pulldown with streptavidin-Sepharose, the tryptic digested samples were analyzed using MS. Red dot shows B4GALT1.

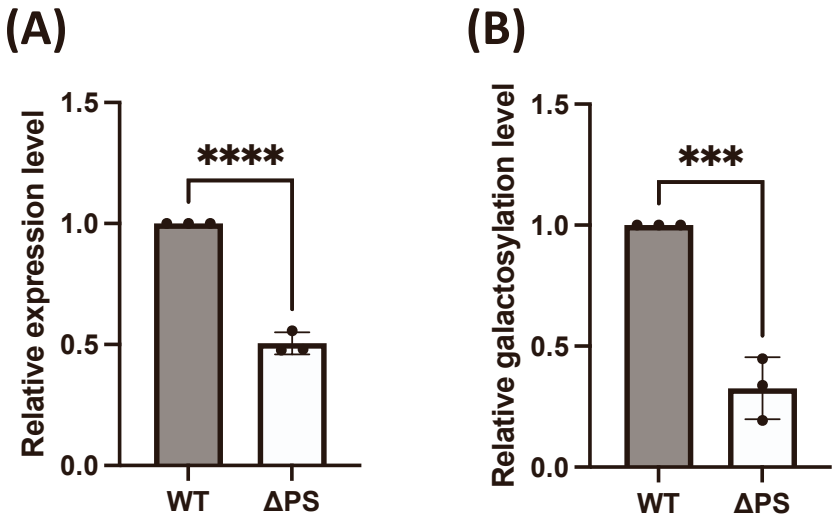

Figure S6. PS-dependent *N*-glycan maturation of recombinant FVIII produced in Expi293F cells.

(A) Relative expression and (B) glactosylation levels of FVIIIΔPS (ΔPS) to PS-harboring FVIII (WT) based on the density plot anti-Flag and RCA, respectively. By calculating the RCA/Flag ratios with these values, Figure 7B was produced. Error bars denote the SEM (n = 3 independent experiments). Statistical significance was assessed by unpaired t-test: ***$p < 0.001$, **** $p < 0.0001$

Table S1. List of plasmids used in this study

Plasmid name	Source	Benchling link
p3xFLAG-CMV9-EPO	[1]	
p3xFLAG-CMV9-EPO-PS	[1]	
p3xHA-CMV9-EPO	this study	https://benchling.com/s/seq-tgznJYRwle9x9NhSinGi?m=slm-N33Ifl7IPDC9F3Rnh9Rh
p3xFLAG-CMV9-sFcγRIIIa	[1]	
p3xFLAG-CMV9-sFcγRIIIa-PS	[1]	
p3xFLAG-CMV9-EPO-TEV-PS	this study	https://benchling.com/s/seq-NW6ysZ5qU8fgctknwjRy?m=slm-4dBY5EMMdcberQ7WVnJJ
p3xFLAG-CMV9-TurboID-EPO	this study	https://benchling.com/s/seq-IFZP9ylKjaHLULXGEtDR?m=slm-hVb5izqEfgWm0RRpcquA
p3xFLAG-CMV9-TurboID-EPO	this study	https://benchling.com/s/seq-N1BSRxAjxXQQkBbWZZop?m=slm-SKizLoccQLH7W1PT1Axa
NUCB1-HA	this study	https://benchling.com/s/seq-NpCTEDUOYRh4aGW1RseU?m=slm-Ds2uA3IJJYyWg6BeBKaa
pcold1-NUCB1	this study	https://benchling.com/s/seq-oVcLNda1X8z0XGHVjCzJ?m=slm-Up0tvm2C7wLCEZlQXpkM
pET28a Ub-FVIII fragment (Asn776–Asp838)	[1]	
NUCB1-EGFP	this study	https://benchling.com/s/seq-jvgviqj64x9HveN4hLs4?m=slm-CR5tIbr9x9oaQ4DetNk9
pCAGGS- B4GALT1-mScarlet-I	[2]	
pCAGGS-B4GALT1-TurboID	this study	https://benchling.com/s/seq-J8xckWrfZpVkttF5pFRI?m=slm-IoZ7Hwb8rW4Gc69AlLyJ

pCAGGS-B4GALT1-splitTurboID-N (B4GALT1-TbN)	this study	https://benchling.com/s/seq-AkuNI7U3xrabisBNYEZe?m=slm-bs2kPrfQPnzoVEhYY85S
pCAGGS-NUCB1-splitTurboID-N (NUCB1-TbN)	this study	https://benchling.com/s/seq-TUGS604RkdPznNlVkyLD?m=slm-oWTVR1sH4cY6MywDevL5
pCAGGS-NUCB1-splitTurboID-C (NUCB1-TbC)	this study	https://benchling.com/s/seq-9xMVVdXduZi0gWwEYipN?m=slm-AkhAd5JR8xFBsCx1IjV7
p3xFLAG-CMV9-FVIII	[1]	
p3xFLAG-CMV9-FVIIIΔPS	[1]	

References

1. Yagi, H., Yagi-Utsumi, M., Honda, R., Ohta, Y., Saito, T., Nishio, M., Ninagawa, S., Suzuki, K., Anzai, T., Kamiya, Y., Aoki, K., Nakanishi, M., Satoh, T., Kato, K.: Improved secretion of glycoproteins using an N-glycan-restricted passport sequence tag recognized by cargo receptor. Nat. Commun. 11, 1368 (2020)
2. Yagi, H., Tateo, S., Saito, T., Ohta, Y., Nishi, E., Obitsu, S., Suzuki, T., Seetaha, S., Hellec, C., Nakano, A., Tojima, T., Kato, K.: Deciphering the sub-Golgi localization of glycosyltransferases via 3D super-resolution imaging. Cell Struct. Funct. advpub, 24008 (2024)