OST4 is a subunit of the mammalian oligosaccharyltransferase required for efficient N-glycosylation

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Summary

The eukaryotic oligosaccharyltransferase (OST) is a membrane-embedded protein complex that catalyses the N-glycosylation of nascent polypeptides in the lumen of the endoplasmic reticulum (ER), a highly conserved biosynthetic process that enriches protein structure and function. All OSTs contain a homologue of the catalytic STT3 subunit, although in many cases this is assembled with several additional components that influence function. In S. cerevisiae, one such component is Ost4p, an extremely small membrane protein that appears to stabilise interactions between subunits of assembled OST complexes. OST4 has been identified as a putative human homologue, but to date neither its relationship to the OST complex, nor its role in protein N-glycosylation, have been directly addressed. Here, we establish that OST4 is assembled into native OST complexes containing either the catalytic STT3A or STT3B isoforms. Co-immunoprecipitation studies suggest that OST4 associates with both STT3 isoforms and with ribophorin I, an accessory subunit of mammalian OSTs. These presumptive interactions are perturbed by a single amino acid change in the transmembrane region of OST4. Using siRNA knockdowns, we show that OST4 depletion well-defined OST complexes are partially destabilised and a novel ribophorin I-containing subcomplex can be detected. Strikingly, cells depleted of either OST4 or STT3A show a remarkably similar defect in the N-glycosylation of endogenous prosaposin. We conclude that OST4 most likely promotes co-translational N-glycosylation by stabilising STT3A-containing OST isoforms.

Key words: Endoplasmic reticulum, Glycoprotein synthesis, Hypoglycosylation, Membrane protein complex, Ribophorin I, STT3 isoforms

Introduction

Asparagine-linked glycosylation (N-glycosylation) is one of the most common and important covalent modifications of proteins in eukaryotic cells. The central step of this process occurs in the lumen of the endoplasmic reticulum (ER), when a high-mannose oligosaccharide is transferred as one structural unit to asparagines, typically those present within Asn-X-Thr/Ser (where X≠Pro) sequons located in flexible and surface-exposed segments of nascent polypeptides (Kowarik et al., 2006; Lizak et al., 2011). In addition to facilitating interactions of polypeptides with components of the ER-associated quality control and degradation machineries (Molinari, 2007; Aebl et al., 2010), N-glycans can accelerate protein folding and increase thermodynamic stability (Shental-Bechor and Levy, 2008). Thus, N-glycosylation is essential to efficient protein folding and trafficking, and defects in this process lead to impaired biological function and disease (Schachter and Freeze, 2009; Mohorko et al., 2011).

In many eukaryotes, N-glycosylation is catalysed by a heterooligomeric membrane protein complex of the rough ER, the oligosaccharyltransferase (OST) (Kelleher and Gilmore, 2006). The OST complex associates with membrane-bound ribosomes at the Sec61 translocon (Chavan et al., 2005; Shibatani et al., 2005; Harada et al., 2009), thereby functioning in concert with co-translational protein translocation across, or integration into, the ER membrane (Ruiz-Canada et al., 2009). Although cotranslational N-glycosylation appears to be mediated primarily by the catalytic STT3A isoform in higher eukaryotes (Nilsson et al., 2003), they also possess an STT3B isoform, which N-glycosylates some sites skipped by STT3A and modifies obligatory post-translational sequons (Ruiz-Canada et al., 2009; Sato et al., 2012). STT3A and STT3B are assembled with a shared set of non-catalytic subunits to form two distinct populations of OST complexes (Kelleher et al., 2003; Roboti and High, 2012b). These two classes of the OST complex differ in terms of catalytic activity and selectivity towards donor substrates with different oligosaccharide structures (Kelleher et al., 2003); however, they can functionally complement each other to enable optimal protein N-glycosylation (Ruiz-Canada et al., 2009; Roboti and High, 2012a).

A range of different functions have been attributed to the non-catalytic OST subunits found in many eukaryotes. Hence, DAD1 and OST48, or their yeast equivalents, are required for the assembly of functional OSTs, and their perturbation destabilises OST complexes and causes severe hypoglycosylation phenotypes...
Evolutionary conservation of OST4 proteins

A BLAST search using the S. cerevisiae Ost4p sequence revealed OST4 homologues in diverse eukaryotes, encompassing animal, plant, fungal and protist kingdoms (Zubkov et al., 2004). Alignment of representative sequences showed that vertebrate OST4 proteins are 94% identical (Fig. 1). This high level of conservation is concentrated in the first 28 residues of the protein, whereas the remaining sequence is poorly conserved and of variable length (Fig. 1A; supplementary material Fig. S1). Even though the human OST4 sequence is substantially divergent from that of S. cerevisiae (36% overall identity; Fig. 1), the hydropathy plots are very similar and suggest that both proteins contain a single TM span (supplementary material Fig. S2). A predicted Nout-Cin orientation has been experimentally validated for yeast Ost4p (Kim et al., 2003); and for mammalian OST4 this topology is consistent with the positive-inside rule (von Heijne and Gavel, 1988) (Fig. 1A; supplementary material Fig. S1). The TM segment of yeast Ost4p extends from residues Leu10 to

![Fig. 1. Alignment of representative OST4 sequences.](image-url)

**Fig. 1. Alignment of representative OST4 sequences.** (A) OST4 amino acid sequences from vertebrates (human, dog, zebrafish and chicken), insects (D. melanogaster), nematodes (C. elegans), yeast (S. cerevisiae and S. pombe), plants (A. thaliana) and protists (D. discoideum) were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Residues that are identical, closely related and less conserved are denoted by shades of grey; the lighter the colour, the more conserved the residue. The single TM span of S. cerevisiae Ost4p is underlined. Crucial residues of the TM region that influence its interactions with Stt3p and/or Ost3p are shown in a red box. Note that a potential ER localisation motif of the KKXX type at the extreme C-terminus is highly conserved among OST4 proteins. (B) Similarity matrix showing % identity among the various sequences.
Val28 (Zubkov et al., 2004), suggesting that residues Phe10 to Val28 of human OST4 are most likely located within the membrane (Fig. 1A; supplementary material Fig. S2) (see also Gayen and Kang, 2011). At a functional level, the mutation of conserved residues located towards the cytosolic end of the S. cerevisiae Ost4p TM span (see Fig. 1A, red box) to Lys or Asp disrupted interactions within the Stt3p–Ost4p–Ost3p subcomplex, resulting in severely impaired OST activity in vitro (Kim et al., 2000; Kim et al., 2003).

OST4 resides in the ER and is assembled into discrete OST complexes

Our antibody raised against human OST4 proved ineffective for several applications including immunofluorescence microscopy and hence we were unable to localise the endogenous protein. We therefore transiently expressed a C-terminal FLAG-tagged human OST4 protein in COS-7 cells. Co-staining with anti-FLAG antibody and an antibody against the ER membrane protein calnexin showed substantial co-localisation of both the wild-type protein (supplementary material Fig. S3, OST4-FLAG panel) and a point mutant (supplementary material Fig. S3, V23K OST4-FLAG panel; see also below) with calnexin. Although it is entirely possible that this localisation reflects an ER retention phenotype that is specific to the exogenous OST4-FLAG, these results are consistent with OST-FLAG being correctly integrated into the ER membrane where it might associate with native OST complexes.

In contrast to yeast Ost4p, the association of the presumptive mammalian OST4 with OST complexes has not been convincingly demonstrated (Karaoglu et al., 1997; Spirig et al., 2005; Kelleher and Gilmore, 2006). Based on our previous observation that the levels of OST components are comparatively high in liver-derived HepG2 cells (Robotti and High, 2012b), we examined the incorporation of endogenous OST4 into native OST complexes by blue native (BN)-PAGE analysis of digitonin-solubilised HepG2 homogenates (Fig. 2A). Immunoblotting with antibodies specific for STT3A and STT3B was performed and, consistent with several previous reports (Wang and Dobberstein, 1999; Shibatani et al., 2005; Robotti and High, 2012b), STT3A was detected in two protein complexes of ~470 kDa and ~500 kDa (OSTC470 and OSTC500, respectively; Fig. 2A, lane 5), whereas STT3B could be observed only in a large, heterogeneous complex of ~520–580 kDa (OSTC550; Fig. 2A, lane 1). Immunoblotting with antibodies against two previously characterised OST subunits, ribophorin I and ribophorin II, revealed clear evidence for their presence in all three distinct OST isoforms (Fig. 2A, lanes 2 and 3). Interestingly, using our anti-OST4 antibody, we could also observe detectable levels of endogenous OST4 present in complexes that co-migrate with both STT3A- and STT3B-containing species (Fig. 2A, lane 4), consistent with the association of OST4 with three previously defined mammalian OST complexes (see Fig. 2B).

Fig. 2. OST4 assembles with core OST subunits into discrete complexes. (A) HepG2 homogenates were solubilised with digitonin, resolved by BN-PAGE and transferred to a PVDF membrane by immunoblotting. After transfer, the membrane was cut into individual strips, each representing a single lane from the original gel. Each strip was separately probed with a primary antibody recognising a known or potential subunit of the mammalian OST complex as follows: STT3B (lane 1), ribophorin I (Rib I, lane 2), ribophorin II (Rib II, lane 3), OST4 (lane 4) and STT3A (lane 5). The strips were then washed and individually probed with a fluorescent secondary antibody. Lastly, individual PVDF strips were reassembled in their original order and the fluorescent signals visualised by scanning the reassembled membrane using a Li-Cor Odyssey imager. The PVDF strips were scanned using two different sensitivity settings (low and high), and the image shown is a composite that combines data from the resulting low (lanes 1, 2, 3 and 5) and high (lane 4) sensitivity images. Some intervening strips present in the fully reassembled PVDF membrane are omitted from the final image shown. Protein complexes containing STT3A (OSTC470 and OSTC500) or STT3B (OSTC550) are indicated. (B) Scheme outlining the presumed subunit composition of different human OST complexes resolved by BN-PAGE based on this work, i.e. STT3A, STT3B, Rib I, Rib II and OST4. The subunits shown in parentheses are assigned here on the basis of our previous study (Robotti and High, 2012b), although the OST48 subunit (asterisk) is also analysed later in this study (cf. Fig. 5A). It is highly likely that additional OST subunits are also present in all three of the complexes that are outlined (Kelleher and Gilmore, 2006), but this proposal remains to be fully investigated.
OST4-FLAG binds to core OST subunits and these interactions are specifically disrupted by a TM domain mutation

To test the potential interaction of OST4 with core subunits of the mammalian OST complex suggested by our BN-PAGE analysis, we performed native co-immunoprecipitation experiments using digitonin-solubilised extracts of HeLa cells transiently expressing OST4-FLAG (Fig. 3). In parallel, we investigated the potential importance of conserved residues located within the C-terminal half of the putative OST4 TM domain by analysing cells expressing two OST4-FLAG point mutants, where Leu21 or Val23 was mutated to a Lys (Fig. 1A; cf. Kim et al., 2000). Although wild-type OST4-FLAG and the V23K variant were expressed at comparable levels, expression of the L21K mutant was poor (Fig. 3A, panels 1 and 2, cf. lanes 1–4 and Fig. 3B,C, panels 4 and 5). Immunoblotting with anti-FLAG antibody showed that each of the FLAG-tagged species was efficiently recovered following an anti-FLAG immunoprecipitation (Fig. 3A, panels 1 and 2), with recovery reflecting steady-state expression that was consequently very low for the L21K variant. We observed evidence of an increase in the level of endogenous ribophorin I associated with wild-type OST4-FLAG when compared to the signal recovered with a FLAG-gp78 control (Fig. 3A, panel 3, cf. lanes 5 and 8), and found this putative interaction is perturbed in cells expressing the V23K variant (Fig. 3A, panel 3, cf. lanes 5 and 7). This potential association between endogenous ribophorin I and wild-type OST4-FLAG (Fig. 3A, panel 3, cf. lanes 5 and 7) suggests that this ectopically expressed protein may be able to integrate into native OST complexes. In contrast, we could not detect any interaction between OST4-FLAG and components of the endogenous Sec61 complex under the same conditions (Fig. 3A, panel 4), supporting the specificity of the presumptive association with ribophorin I.

| A | OST4-FLAG + + + |
| L21K OST4-FLAG + + + |
| V23K OST4-FLAG + + + |
| --- | --- | --- |
| Input | IP: αFLAG | αFLAG-gp78 |

| B | OST4-FLAG + + + |
| L21K OST4-FLAG + + + |
| V23K OST4-FLAG + + + |
| --- | --- | --- |
| Input | IP: αFLAG | αFLAG-gp78 |

Fig. 3. Differential interactions of core OST subunits with overexpressed OST4-FLAG variants. (A–C) HeLa cells were (co-)transfected with expression plasmids for the proteins indicated and digitonin-solubilised cell lysates immunoprecipitated (IP) with mouse anti-FLAG. One quarter of the total lysate used for each immunoprecipitation reaction (Input) and the immunopurified proteins (IP: anti-FLAG) were analysed by immunoblotting (IB) with rabbit anti-FLAG (A–C), anti-ribophorin I (anti-Sec61β) (A), anti-STT3A (B) and anti-STT3B (C) antibodies. The asterisks indicate the positions of non-specific bands.
HeLa cells express relatively low levels of catalytic STT3A and STT3B, and we therefore investigated any potential interactions with OST4-FLAG using exogenously expressed proteins (Roboti and High, 2012b). Tellingly, a substantial amount of exogenous STT3A was co-precipitated with wild-type OST4-FLAG, but none was recovered with FLAG-gp78 (Fig. 3B, panel 3, cf. lanes 1 and 4). The V23K mutation of OST4-FLAG reduced the amount of associated STT3A to a fraction of that seen with the wild-type, whereas the lower levels of L21K expression and/or the effect of the mutation resulted in a barely detectable signal for associated STT3A (Fig. 3B, panel 3, cf. lanes 1–3). Likewise, with STT3B we also observe evidence for an association with wild-type OST4-FLAG when compared to the levels of material that are recovered with OST4-FLAG mutants or FLAG-gp78 (Fig. 3C, panel 3, cf. lanes 1–4). Consistent with our BN-PAGE analysis, these results suggest that OST4-FLAG can associate with both mammalian STT3 isoforms and the OST accessory subunit, ribophorin I. Notably, these presumptive interactions were abolished in cells expressing the V23K OST4-FLAG mutant, suggesting an important role for this residue/the TM region in the binding of OST4 to other OST subunits.

**OST4 expression stabilises STT3A levels**

To further investigate the association of OST4 with mammalian OST complexes, we used siRNA-mediated knockdown to analyse the effect of its depletion on steady-state levels of the catalytic STT3A and STT3B subunits, and the non-catalytic OST subunits, ribophorin I, OST48 and KCP2. Our anti-OST4 antibody is not well suited to quantitative immunoblotting using HeLa cell extracts, most likely due to a combination of low levels of endogenous OST4 and the biophysical properties of such a small hydrophobic protein (Karaoglu et al., 1997). We therefore used real-time PCR to quantify the relative levels of OST4 mRNA following its knockdown. Four distinct siRNAs targeting OST4 were tested, and cells transfected with three different 27-nucleotide siOST4 duplexes all showed a ~90% reduction in their OST4 mRNA level compared to cells transfected with a non-targeting control (Fig. 4A; see siOST4_{27(B)} and siOST4_{27(C)}). A
shorter, 21mer siRNA targeting OST4 resulted in >75% reduction in its mRNA level (Fig. 4A; see siOST4_{21}). For comparison, the treatment of HeLa cells with a 21mer siRNA targeting ribophorin I resulted in ~90% decrease in ribophorin I mRNA levels (Fig. 4A, siRib I); and this correlated with a >70% reduction in protein level as determined by quantitative immunoblotting (Fig. 4A, inset).

We next used the most effective of the three 27mer siRNAs (siOST4_{27}) to treat HeLa cells and analysed the effect upon the levels of specific OST subunits recovered in Triton X-100 solubilised material (Roboti and High, 2012a). OST4 depletion resulted in substantial reductions of both STT3A and KCP2 levels at steady state (Fig. 4B, cf. lanes 5 and 6; for quantification see Fig. 4C and Table 1). Likewise, the depletion of ribophorin I by ~70% resulted in a comparable reduction in the STT3A signal and an even greater reduction in KCP2 levels (Fig. 4B, cf. lanes 1 and 4; Fig. 4C and Table 1). The effects of STT3A and STT3B depletion reflected previous reports (Ruiz-Canada et al., 2009; Roboti and High, 2012a), including a substantial reduction in KCP2 levels following a knockdown of STT3A (Fig. 4B, cf. lanes 1 and 2; Fig. 4C and Table 1). Furthermore, by using an affinity-purified anti-OST4 antibody, we were able to obtain qualitative evidence for reductions in the levels of OST4 protein following the siRNA-mediated depletion of STT3A, ribophorin I and OST4 (supplementary material Fig. S4). Collectively, these data are consistent with a model where OST4 preferentially stabilises the STT3A isoform and its interacting partner KCP2 (Roboti and High, 2012a; Roboti and High, 2012b). Likewise, our results also suggest that STT3A and/or ribophorin I stabilise OST4.

To address the possibility that these reductions in levels of OST subunits may reflect changes in the detergent solubility of specific components rather than the actual amount of protein (Roboti and High, 2012a), we repeated our analysis using whole cell lysates solubilised directly into SDS-PAGE sample buffer. As before, the OST4 knockdown led to a substantial reduction in STT3A levels (supplementary material Fig. S5), confirming a visible reduction in the solubility of OST components following ribophorin I depletion we observe, we cannot rule out the possibility that an alteration in the solubility of OST components following ribophorin I depletion contributes to the reduction in detectable OST complexes following BN-PAGE (cf. supplementary material Fig. S5), albeit the behaviour of the Sec61 complex appears unaffected (Fig. 3C, see Sec61 complexes).

Immunoblotting for ribophorin I further validated the conclusions outlined above, confirming a visible reduction in the levels of OSTC470, OSTC500 and OSTC550 after OST4 depletion (Fig. 5B, cf. lanes 1 and 3; see also Fig. 2B). Intriguingly, the anti-ribophorin I antibody detected faster migrating complexes of ~150 kDa in STT3A-, STT3B- and OST4-knockdown cells (RibIC; Fig. 5B, cf. lanes 2, 4 and 5). These ribophorin I-containing species are not present in control cells (Fig. 5B, cf. lanes 1 and 3), suggesting that they are specific ribophorin I-containing subcomplexes resulting from perturbations of native OST complexes. Immunoblotting with anti-Sec61β revealed that OST subunit depletion did not alter the

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**Table 1. OST subunit levels in siRNA-treated HeLa cells**

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Protein</th>
<th>STT3A (%)</th>
<th>STT3B (%)</th>
<th>Rib 1 (%)</th>
<th>OST48 (%)</th>
<th>KCP2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>siSTT3A</td>
<td>27 ± 6</td>
<td>93 ± 5</td>
<td>83 ± 3</td>
<td>64 ± 5</td>
<td>16 ± 3</td>
<td></td>
</tr>
<tr>
<td>siSTT3B</td>
<td>110 ± 12</td>
<td>30 ± 4</td>
<td>93 ± 3</td>
<td>70 ± 7</td>
<td>97 ± 9</td>
<td></td>
</tr>
<tr>
<td>siRib I</td>
<td>40 ± 10</td>
<td>71 ± 7</td>
<td>29 ± 5</td>
<td>57 ± 7</td>
<td>19 ± 3</td>
<td></td>
</tr>
<tr>
<td>siOST427</td>
<td>32 ± 7</td>
<td>99 ± 9</td>
<td>107 ± 6</td>
<td>85 ± 4</td>
<td>46 ± 13</td>
<td></td>
</tr>
</tbody>
</table>

The levels of individual proteins after the various siRNA treatments indicated are expressed relative to those determined for non-targeting controls that were set at 100%. *This apparent reduction of STT3A in Rib I-depleted cells was not observed when samples were solubilised directly into SDS-PAGE sample buffer (see supplementary material Fig. S5).
levels or mobility of OST proximal Sec61 translocon complexes (Fig. 5C, see Sec61C and Sec61C9), confirming the distinct identities of native Sec61 translocons and OST complexes (Wang and Dobberstein, 1999; Ménetret et al., 2005). Together, these data demonstrate that whereas depletions of STT3A and STT3B specifically disrupt the formation of OST complexes containing only the target STT3 isoform, depletion of OST4 perturbs both STT3A- and STT3B-containing complexes (see Fig. 2B).

OST4 is required for the efficient N-glycosylation of endogenous prosaposin

As OST4 appears to be important for the structural integrity of native OST complexes, we lastly investigated its potential contribution to N-glycosylation using a well-established assay for the N-glycosylation of endogenous prosaposin (pSAP) in HeLa cells (Fig. 6A) (see Ruiz-Canada et al., 2009; Roboti and High, 2012a). The early stages of pSAP biosynthesis were analysed by pulse-labeling, and fully-glycosylated pSAP species were readily apparent after immunoprecipitation from control cells (pSAP-5; Fig. 6B, cf. lanes 2 and 6). In contrast, tunicamycin treatment completely blocked pSAP N-glycosylation (pSAP-0; Fig. 6B, cf. lanes 1, 2 and 6), STT3A depletion substantially decreased the efficiency of pSAP modification, and STT3B knockdown had no significant effect (Fig. 6B, cf. lanes 2–4) (Ruiz-Canada et al., 2009; Roboti and High, 2012a). Intriguingly, depletion of either OST4 or ribophorin I each resulted in a significant hypoglycosylation phenotype, that was both qualitatively and quantitatively comparable to the defect observed upon the depletion of the catalytic STT3A isoform (see Fig. 6B).

In order to confirm that this hypoglycosylation of pSAP was a consequence of OST4 depletion, we studied the effect of all four of the independent siRNAs that had resulted in a substantial reduction in OST4 mRNA levels (cf. Fig. 4A). In every case, we found that the treatment of HeLa cells with these siRNAs led to a clear defect in pSAP N-glycosylation (supplementary material Fig. S6). The formation of hypoglycosylated pSAP species in siSTT3A-, siRibI- and siOST4- treated cells each reflects the absence of up to two of the normal five N-linked glycans appended to the protein (Fig. 6B,C). When compared to the effect of tunicamycin treatment, the comparatively mild hypoglycosylation of pSAP in STT3A-depleted cells suggests that despite a detectable impairment in STT3A-containing native OST complexes (see Fig. 5, cf. lanes 3 and 4), residual OST activity is sufficient to facilitate a substantial level of N-glycosylation (Ruiz-Canada et al., 2009; Roboti and High, 2012a).

Following OST subunit knockdowns, we find no evidence for X-box binding protein-1 (XBP1) mRNA splicing (supplementary
material Fig. S7), indicative of an ER stress response via the inositol-requiring protein-1 pathway (Ron and Walter, 2007). Although we cannot exclude a stress response via an alternative route (Ron and Walter, 2007; Roboti et al., 2009), this observation supports a model where a combination of the residual OST complexes/components remaining after siRNA mediated knockdowns, combined with functional redundancy between STT3A- and STT3B-containing OST isoforms, can accommodate much of the normal demand for protein N-glycosylation in HeLa cells (Ruiz-Canada et al., 2009; Roboti and High, 2012a). Overall, these results clearly indicate that OST4 and ribophorin I both modulate the efficiency of pSAP N-glycosylation. Since the N-glycosylation of pSAP appears to be primarily a co-translational event (Ruiz-Canada et al., 2009), and the depletion of STT3A, OST4 and ribophorin I each induce a similar degree of pSAP hypoglycosylation (Fig. 6A,B), we conclude that the effects of siOST4$_{27}$ and siRib I treatments most likely reflect the destabilisation of the STT3A-containing OST isoforms resulting from the depletion of these subunits (cf. Fig. 6C).

**Discussion**

Whilst several biochemical and genetic studies of yeast Ost4p have been performed since its first discovery, the associations of its putative mammalian homologue, OST4, and any potential role it may play during protein N-glycosylation have not previously been addressed.

**Physical interactions of OST4 with bona fide OST subunits**

The purification of enzymatically active, detergent-solubilised mammalian OST complexes established the existence of distinct OST isoforms containing ribophorin I, ribophorin II, OST48 and DAD1 together with either STT3A or STT3B (Kelleher et al., 2003). Likewise, native gel electrophoresis studies demonstrated that STT3A is stably assembled into two distinct native oligomeric complexes, OSTC470 and OSTC500, whereas STT3B is present in a larger complex, OSTC550 (Roboti and High, 2012b). All three of these species contain ribophorin I, ribophorin II, OST48, DAD1 and DC2 (Shibatani et al., 2005), whereas KCP2 seems to be preferentially incorporated into OSTC500 (Roboti and High, 2012b; Roboti and High, 2012a). In this study, we show that endogenous OST4 co-migrates with all three of the major OST complexes detectable following BN-PAGE and immunoblotting (see Fig. 2A,B) thereby providing the first indication that OST4 is a true and ubiquitous constituent of these mammalian OST complexes, as provisionally suggested by Kelleher and Gilmore (Kelleher and Gilmore, 2006). Epitope-tagged versions of OST subunits have been successfully used for previous studies (Yan et al., 2003; Chavan et al., 2006; Roboti and High, 2012b), and we have employed a C-terminal FLAG tag to study the interacting partners of human OST4. Exogenously expressed OST4-FLAG could be immunoisolated in digitonin-stable complexes with exogenous STT3A and STT3B, and may
also associate with endogenous ribophorin I. These data suggest that in mammals there is a close, and potentially direct, association between OST4 and both isoforms of the enzymatic STT3 subunit that are present, a situation that mirrors the interaction between OST4 and Stt3p in *S. cerevisiae* (Karaglu et al., 1997; Zubkov et al., 2004). Ribophorin I homologues are closely associated with several OST subunits (Kelleher et al., 1992; Kelleher et al., 2003; Yan et al., 2003; Yan et al., 2005), and hence the potential association of OST4 with ribophorin I that we observe may be direct or involve one or more intermediary components.

NMR studies of both yeast Ost4p (Zubkov et al., 2004) and, more recently, human OST4 (Gayen and Kang, 2011) indicate that >50% of the protein is embedded in the ER membrane, suggesting that its TM domain has an important function(s). To investigate the role of the OST4 TM region (cf. Fig. 1), we made two point mutations, one of which, Leu21 to Lys, was not stably expressed (Kim et al., 2000; Kim et al., 2003). In contrast, a Val23 to Lys mutant was exogenously expressed at similar levels to the FLAG-tagged wild-type protein and displayed substantial defects in its association with STT3A, STT3B and ribophorin I. These data are compatible with a model where the C-terminal portion of human the OST4 TM region plays a key role in mediating its direct interaction with the two mammalian STT3 isoforms, and adds yet further weight to previous studies mapping *S. cerevisiae* Ost4p contacts (Kim et al., 2000; Kim et al., 2003; Zubkov et al., 2004).

**OST4 stabilises OST isoforms**

A key objective of this study was to investigate the role of OST4 during protein N-glycosylation. The portion of OST4 exposed to the ER lumen is small, whereas the distance of the OST active site from the luminal face of the ER membrane is estimated to be >30 Å (Nilsson and von Heijne, 1993). When combined with structural information about membrane-inserted forms of the yeast OST (Li et al., 2008) and its bacterial equivalent (Lizak et al., 2011), there is no strong indication that OST4 is likely to contribute directly to the catalysis of protein N-glycosylation. Furthermore, there is good evidence that yeast Ost4p acts as a scaffold protein to stabilise the association of either one of two non-catalytic subunits Ost3p or Ost6p with native OST complexes (Spirig et al., 2005), a role akin to that suggested for some of the small subunits of the mitochondrial TOM complex (Dekker et al., 1998; Sherman et al., 2005). We find that an siRNA-mediated knockdown of human OST4 substantially reduces steady-state levels of STT3A without noticeably affecting STT3B (Fig. 4; Table 1). Conversely, our provisional analysis indicates that OST4 levels are dependent upon STT3A expression (supplementary material Fig. S4). Taken together, these data strongly support a close association between STT3A and OST4 that influences the stability of both components.

Using BN-PAGE we investigated the effect of OST4 depletion upon the stability of OST complexes, and observed qualitative reductions in the level of all three major OST species, including OSTC500 and OSTC470 that we previously defined as STT3A-containing complexes (Roboti and High, 2012b; Roboti and High, 2012a). The most striking effect of OST4 depletion upon the species detectable by BN-PAGE was observed when we analysed for ribophorin I-containing complexes. The depletion of either OST4 or STT3A resulted in the appearance in the absence 150 kDa OST-derived component that contains ribophorin I, but not detectable levels of OST48. Thus, the depletion of OST4 and STT3A have a similar effect on OST stability, namely a reduction in the levels of the ~500 kDa and ~470 kDa complexes presumed to represent functional species (Roboti and High, 2012a), and a corresponding increase in a novel ribophorin I-containing subcomplex that lacks either of the two STT3 isoforms and OST48 (Fig. 5). This phenotype is distinct from that observed following Ost4p knockout in yeast, where the ribophorin I homologue Ost1p remained present in two higher molecular weight species, denoted complex I and complex II, that also contained the catalytic Stt3p subunit, the OST48 homologue Wbp1 and several other OST subunits (Spirig et al., 2005). Loss of yeast Ost4p results in the absence of one of two non-catalytic subunits, Ost3p or Ost6p, that correlates with a conversion of OST complex I to OST complex II (Spirig et al., 2005). Unfortunately, we are unable to analyse the fate of any of the mammalian equivalents of Ost3p and Ost6p (Kelleher et al., 2003) in the BN-PAGE system due to a lack of suitable antibodies. Our data tentatively suggest that a similar ribophorin I-containing subcomplex may also arise following STT3B depletion; however, substantial additional studies will be required to convincingly address this issue.

**OST4 depletion perturbs protein N-glycosylation**

This study provides the first analysis of mammalian OST4 function using a siRNA-mediated knockdown approach that has previously been successfully used to address the contributions of both catalytic and non-catalytic OST subunits (Wilson et al., 2008; Ruiz-Canada et al., 2009; Roboti and High, 2012a). We provide evidence that OST4 levels can influence protein N-glycosylation efficiency, in that cells depleted of OST4 using four independent siRNAs all show a clear defect in the N-glycosylation of PSAP (supplementary material Fig. S6). In fact, the hypoglycosylation phenotype resulting from the OST4 knockdown is directly comparable to that observed in cells depleted of the catalytic STT3A subunit (Fig. 6B) (Ruiz-Canada et al., 2009; Roboti and High, 2012a). Loss of Ost4p results in the hypoglycosylation of several yeast glycoprotein precursors (Chi et al., 1996; Spirig et al., 2005), and we conclude that the principle effect of OST4 depletion upon pSAP N-glycosylation (see Fig. 6C) occurs via a destabilisation of the OST complex and the resulting perturbation of the normal interactions between its subunits.

In order to provide a comparator for the effects of OST4 depletion on OST complex stability and function, we carried out parallel experiments using ribophorin I depletion. Although the effect of ribophorin I depletion upon steady-state levels of specific OST subunits and protein N-glycosylation has been previously studied (Wilson and High, 2007; Wilson et al., 2008; Ruiz-Canada et al., 2009), the effects of its knockdown upon OST complex stability, as assessed using BN-PAGE or the N-glycosylation of endogenous PSAP in HeLa cells, had not been established. A knockdown in ribophorin I of >70% at the protein level lead to a qualitative reduction in the level of OST4 and a more complex effect on STT3A levels that may reflect changes in its solubility in non-ionic detergents. Independent of its precise effects on STT3A levels or OST complex stability and assembly, at a functional level ribophorin I depletion had a robust and significant effect on N-glycosylation. In fact, the outcome of ribophorin I depletion upon pSAP N-glycosylation was strikingly similar to that following STT3A and OST4 knockdowns (this study) and OST48 and DAD1 depletion (Roboti and High, 2012a).
In contrast, STT3B depletion has no detectable effect on pSAP N-glycosylation as previously reported (Ruiz-Canada et al., 2009; Roboti and High, 2012a). In terms of pSAP modification, to date only KCP2 depletion has been found to result in an ‘intermediate’ hypoglycosylation phenotype, supporting a model where KCP2 is a peripheral component and hence its loss does not destabilise STT3A-containing OST complexes per se (Roboti and High, 2012a). In contrast, it appears that the loss of any one of several other non-catalytic subunits, specifically OST4 (this study), OST48 and DAD1 (Roboti and High, 2012a) and more tentatively ribophorin I (this study) result in wide-ranging perturbations of OST complex stability. In the case of HeLa cells, these perturbations in OST complex stability typically correlate with a reduction in the cellular capacity for the N-glycosylation of the endogenous precursor pSAP (cf. Fig. 6C) that is directly comparable to a knockdown of the ‘co-translational’ STT3A catalytic subunit alone (this study; see also Roboti and High, 2012a). To date, it has not yet been possible to deplete a ‘core’ non-catalytic subunit of the mammalian OST without destabilising OST complex(es) as a whole. Hence, although it has been postulated that increasing the number of different subunits that make up an OST complex may enhance the range and complexity of the substrates that it can N-glycosylate (Schwarz and Aebi, 2011; Roboti and High, 2012a), in practice this hypothesis remains to be unambiguously tested in a mammalian system.

Materials and Methods

DNA and siRNA constructs

Human OST4 CDNA sequence was optimised with proprietary OptimumGene algorithm (GenScript, Piscataway, NJ, USA) for efficient expression in human cells, and the optimised gene was inserted into the HindIII-EcoRI sites of a pcDNA3.1(+) vector (Invitrogen). The stop codon was PCR amplified out of human OST4 coding sequence using primers that included a C-terminal FLAG tag to create OST4-FLAG. Using pcDNA3.1(+)OST4-FLAG as a template, QuikChange site-directed mutagenesis (Agilent Technologies, Stockport, UK) was performed to generate L21K or V23K OST4-FLAG. All constructs were confirmed by DNA sequencing. The plasmid encoding STT3A was purchased from OriGene Technologies (Rockville, MD, USA). Plasmids encoding FLAG-gp78 and mouse STT3B-FLAG were kindly provided by Ivan Robert Nabi (University of British Columbia, Vancouver, Canada) and Claude Perreault (University of Montreal, Quebec, Canada), respectively. The natural stop codon of the mouse STT3B gene was introduced by QuikChange mutagenesis, thereby silencing the expression of the C-terminal FLAG epitope (Roboti and High, 2012b). siRNA duplexes (supplementary material Table S1) to deplete STT3A, STT3B and ribophorin I are as previously described (Ruiz-Canada et al., 2009) and, together with the 21-mer duplex targeting OST4 (supplementary material Table S1) and the AllStars non-targeting siRNA control, were from QiAGEN. Complementary single-stranded 27mer oligonucleotides for depleting OST4 (supplementary material Table S1), and the Trilencer-27 siRNA duplex negative control, were from OriGene. Additional siOST42 was custom synthesised as complementary single-stranded 27mers by Integrated DNA Technology (Coralville, Iowa, USA) and subsequently annealed in our laboratory.

Cell culture and transfections

HeLa, COS-7 and HepG2 cells were grown in DMEM containing 10% foetal bovine serum and 2 mM L-glutamine at 37°C and 5% CO2. DNA transfections were performed using Lipofectamine 2000 (Invitrogen) for 20–24 hours according to the manufacturer’s guidelines. For siRNA transfections, circa 2.6×106 to 5×106 HeLa cells were plated in 6- or 10-cm dishes 24 hours before transfection with 100 nM siRNA duplexes using Oligofectamine (Invitrogen) following the manufacturer’s instructions. After 6 hours, the transfection medium was replaced by complete growth medium and the cells were harvested either 48 hours (RT-PCR analysis) or 72 hours (immunoblotting and radiolabelling analysis) post-transfection. Immunofluorescence was performed with transiently transfected COS-7 cells as previously described (Roboti and High, 2012b).

Immunoblotting

Triton X-100-soluble extracts or whole-cell lysates in SDS-PAGE sample buffer were prepared as described previously (Robotti and High, 2012a) and analysed by infrared immunoblotting according to standard procedures (Roboti and High, 2012b). Polyclonal antibodies specific for STT3A, STT3B, ribophorin I, ribophorin II, OST48 and KCP2 were as previously described (Wilson and High, 2007; Roboti and High, 2012b). Rabbit polyclonal antibodies recognising OST4 were custom-made by Cambridge Research Biochemicals (Cleveland, UK) and polyclonal anti-FLAG was purchased from Sigma. Antibodies recognising α-tubulin and Sec61β were from Keith Gull (University of Oxford, UK) and Bernhard Dobberstein (University of Heidelberg, Germany), respectively. Mouse anti-actin monoclonal antibody (clone C4) was from MP Biomedicals (Illkirch, France). Fluorescently labelled secondary antibodies were purchased from LI-COR Biosciences (Cambridge, UK). The fluorescent bands were visualised and quantified on the Odyssey Infrared Imager (LI-COR Biosciences) using the software provided by the manufacturer.

BN-PAGE analysis

BN-PAGE analysis of digoxigenin-solubilised HepG2 homogenates was as described previously (Roboti and High, 2012b). BN-PAGE analysis of OST complexes in siRNA-treated HeLa cells was performed using the NativePAGE Novex Bis-Tris gel system (Invitrogen). Approximately 45 mg of sedimented cells were resuspended in 1× native sample buffer (50 mM Bis-Tris, 16 mM HCl, 50 mM NaCl, 10% glycerol, 0.001% ponceau S, pH 7.2) supplemented with 1% digitonin and 1% protease inhibitor cocktail (Sigma). Following incubation on ice for 30 minutes, insoluble material was removed by centrifugation at 16,000×g for 10 minutes at 4°C and the supernatant was mixed with Coomassie blue-G250 to a final concentration of 0.25%. Samples equivalent to less than 1.25 mg dry cell pellet were run on a 4–16% Novex Bis-Tris gel at 4°C for 60 minutes at 150 V, and gel running was then continued at 250 V until complete. The protein complexes separated by BN-PAGE were transferred by wet transfer onto PVDF membrane (Millipore) at 70 V for 70 minutes. Membrane were destained with methanol prior to blocking overnight and subsequent immunodetection by infrared immunoblotting as described above. Native protein standard (GE Healthcare) was used as the marker.

Co-immunoprecipitation analysis

Cells were solubilised at 4°C for 1 hour with IP buffer (10 mM Tris-HCl pH 7.5, 140 mM NaCl, 1 mM EDTA) supplemented with a protease inhibitor cocktail and 2% digitonin (Merck Chemicals). Non-solubilised material was removed by centrifugation at 16,000×g for 10 minutes at 4°C. A quarter of the post-nuclear supernatant was denatured in SDS-sample buffer (input), while the rest was incubated at 4°C for 1 hour with 10 μl of Panabrin to ‘preclear’ the lysate. Immunoprecipitations were performed by incubating preclarified lysates with monoclonal anti-FLAG and 20 μl Protein A-Sepharose beads at 4°C for 4 hours. Beads were washed three times with IP buffer containing 0.1% digitonin and denatured with sample buffer at 37°C for 1 hour. Samples were analysed by SDS-PAGE and western blotting.

Real-time PCR and XBPI mRNA splicing

Total RNA was isolated from siRNA-treated HeLa cells using RNeasy kit (Qiagen) and reverse-transcribed into cDNA using Supergo-IT and AMV reverse transcriptase (Roche). Real-time PCR amplifications were performed in triplicate or duplicate on cDNA equivalent to 50 ng RNA in 25 μl reaction volume containing 1× SYBR green PCR reagent (Eurogentec) and 100 nM forward and reverse primers (see supplementary material Table S2). Total RNA from HeLa cells after knockdowns or 2 hours of treatment with 10 μM DTT was reverse-transcribed into cDNA, and a segment of XBPI mRNA then amplified using appropriate primers (see supplementary material Table S2). Spliced and unspliced XBPI products were resolved by electrophoresis on a 2% agarose gel and visualised with ethidium bromide staining under UV light. Parallel amplifications of GAPDH cDNA were performed (see supplementary material Table S2) to verify equal protein loading.

Metabolic labelling and immunoprecipitation

Cells were starved in a methionine- and cysteine-free DMEM ( Gibco) supplemented with 2 mM L-glutamine for 20 minutes at 37°C and then incubated in fresh starvation medium containing 22 μCi/ml [35S]Met/Cys protein labelling mix (PerkinElmer; specific activity >1,000 Ci/mmol) for 10 minutes. Tunicamycin (Calbiochem) was added at a final concentration of 10 μg/ml ~20 hours prior to the starvation and was also included throughout the starvation and pulse. Cells were washed twice with PBS and solubilised with Triton X-100 lysis buffer. Lyophilised lysates were denatured at 1% SDS at 37°C for 30 minutes, then diluted fivefold with Triton X-100 ss buffer containing 8 mM cold Met/Cys and 1 mM PMSF, and pre-cleared by incubation with pansorbin (Calbiochem) at 4°C for 1 hour. The pre-cleared lysates were incubated overnight with antibodies specific for saposin D (Konrad Sandhoff, University of Bonn, Germany) and immune complexes were captured using Protein-A-Sepharose beads (GenScript, USA). Beads were washed three times with Triton X-100 buffer before eluting proteins with SDS-PAGE sample buffer. Samples were denatured at 37°C for


Supplementary Figure Legends

**Fig. S1. Amino acid sequence alignment of OST4 from diverse eukaryotes.** Sequence alignment of various OST4 homologues was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Residue conservation values were obtained using Jalview (Waterhouse et al., 2009), and a histogram showing the degree of conservation of each residue is depicted below the sequence alignment. The conservation of residues is shown by shades of grey/yellow; the lighter the colour, the more conserved the residue.

**Fig. S2. Comparison of yeast and human OST4 transmembrane regions.** (A) Amino acid sequences of human OST4 and *S. cerevisiae* Ost4p. (B) Hydropathy analysis for both human OST4 and *S. cerevisiae* Ost4p was performed using the TMPred program (Hofmann and Stoffel, 1993). Peaks > 500 indicate TM-spanning domains. (C) Prediction of the free energy difference, \( \Delta G_{\text{pred}} \), for insertion of human OST4 and *S. cerevisiae* Ost4p into the ER membrane was performed using \( \Delta G \) predictor server (http://dgpred.cbr.su.se/) (Hessa et al., 2007). The graphs show predicted \( \Delta G \) values against position in sequence for different helix lengths (L = 19–25). The more negative the predicted \( \Delta G \) value of a sequence, the more efficient it can be inserted in the ER membrane. The \( \Delta G_{\text{pred}} \) value after setting residues 10 to 28 of the human OST4 as the TM span (cf. Fig. 1A) is +0.27, whilst the 19 residue-span of human OST4 with the lowest \( \Delta G_{\text{pred}} \) value (-0.52) is residues 7 to 25. It should be noted that the lowest \( \Delta G_{\text{pred}} \) value is for the optimal TM span that undergoes ER translocon-mediated membrane integration, and the subsequent assembly of a membrane protein into an oligomeric complex can lead to subsequent alterations in its TM-spanning region (Kauko et al., 2010; Öjemalm et al., 2013).

**Fig. S3. Subcellular localisation of exogenously expressed OST4-FLAG in COS-7 cells.** Immunofluorescence microscopy of COS-7 cells expressing either wild-type OST4-FLAG or V23K OST4-FLAG and labelled with the anti-FLAG antibody (red) and an antibody against the ER marker calnexin (green). Scale bars: 10 µm.
**Fig. S4. Depletion of STT3A or ribophorin I destabilises OST4.** HeLa cells were transfected with siRNAs targeting STT3A, STT3B, ribophorin I and OST4, or with non-targeting siRNAs (nt, nt_{27}) as a control. Triton X-100-soluble cell extracts were prepared 72 h post-transfection and analysed by immunoblotting with anti-OST4.

**Fig. S5. Effect of siRNA-mediated knockdowns on protein levels.** The stability and detergent-extractability of OST subunits were analysed by direct solubilisation of siRNA-treated HeLa cells in SDS-PAGE sample buffer, followed by infrared immunoblotting for the proteins indicated. The graph shows the quantification results from this analysis. Fluorescent signals were quantified, normalised to the corresponding γ-tubulin signals, and expressed as a percentage of the signal in control cells.

**Fig. S6. Four independent siRNAs targeting OST4 all result in hypoglycosylation of endogenous prosaposin in HeLa cells.** HeLa cells were treated with one of four different siRNAs targeting OST mRNA (cf. Figure 4A of main text), or an appropriate control siRNA (nt or nt_{27}), as indicated, and radiolabelled pSAP species were detected by immunoprecipitation and phosphorimaging. The authentically N-glycosylated form of pSAP (pSAP-5), and faster migrating species that lack one or more N-linked glycans (pSAP<5 (xCHO)) are indicated (see also Roboti and High, 2012a).

**Fig. S7. siRNA-mediated OST subunit depletion does not induce XBP1 mRNA splicing.** Total RNA was isolated from HeLa cells after 2 h of treatment with 10 mM DTT or after 48 h of transfection with the indicated siRNAs, and XBP1 mRNA splicing was determined by RT-PCR. Unspliced (u) and spliced (s) XBP1 mRNA products are indicated. GAPDH served as a loading control.
**Figure S2**

**A**

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**B**

![Graph showing hydrophobic score with amino acid number on the x-axis and hydrophobic score on the y-axis. The graph compares S. cerevisiae Ost4p and Human OST4.]

**C**

**S. cerevisiae OST4**

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**Human OST4**

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![Graph showing predicted G (kcal/mol) with sequence position (of center of helix) on the x-axis and predicted G (kcal/mol) on the y-axis. The graph compares S. cerevisiae Ost4p and Human OST4.]

**Note:** The graphs in Figure S2 illustrate the comparison between human and yeast OST4 proteins in terms of their predicted transmembrane (TM) helices and hydrophobic scores. The sequence alignments and predicted helices highlight differences in the predicted transmembrane regions.
Figure S4

IB: αOST4

IB: αtubulin

OST4
Figure S5

IB: αSTT3A
IB: αSTT3B
IB: αRib I
IB: αOST48
IB: αtubulin

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Figure S7

The figure shows a gel electrophoresis experiment with bands labeled as XBP1u and XBP1s. The samples are labeled as nt27, siOST427, nt, siSTT3A, siSTT3B, siRibL, + DTT, and - DTT. The bands are compared to GAPDH for reference.
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