File S4

GPI anchoring

This Supporting File contains additional information related to **Biosynthesis of Wall Components Along the Secretory Pathway, *GPI anchoring***. The subheadings used in the main text are retained, and new subheadings are underlined. Literature cited in this File but not In the main text is listed at the end of the File.

Assembly of the GPI precursor and its attachment to protein in the ER:

Steps on the cytoplasmic face of ER membrane:

Gpi3. Gpi3 is a member of GT Family 4 and has an EX_7E motif conserved in a range of glycosyltransferases (Coutinho *et al*. 2003). Mutational analyses indicate that the glutamates are be important for function of Gpi3 and certain EX_7E motif glycosyltransferases, although the comparative importance of the two glutamates varies between different transferases (Kostova *et al*. 2003). However, in the case of Alg2, the EX_7E motif is not important for protein function (Kämpf et al. 2009).

Formation of GlcNAc-PI by GPI-GnT. The acyl chains of the PI species that receive are the same length as those in other membrane phospholipids (Sipos *et al*. 1997). Evidence that GlcNAc transfer occurs at the cytoplasmic face of the ER membrane is that i) the catalytic domain of Gpi3's human orthologue faces the cytoplasm (Watanabe *et al*. 1996; Tiede *et al*. 2000), and ii) GlcNAc-PI can be labeled with membrane topological probes on the cytoplasmic side of the mammalian ER membrane (Vidugiriene and Menon, 1993).

Significance of Ras2 regulation of GPI-GnT. A clue to the significance of Ras2 regulation of GPI-GnT came from the observation that conditional mutants in GPI-GnT subunits show the phenotype of hyperactive Ras mutants, filamentous growth and invasion of agar. This led to the suggestion that Ras2-mediated modulation of GPI synthesis may be involved in the cell wall and morphogenetic changes that occur in the dimorphic transition to filamentous growth (Sobering *et al*. 2003; 2004).

Location of GlcNAc-PI de-N-acetylation. The de-acetylase reaction likely occurs at the cytoplasmic face of the ER membrane, because the bulk of Gpi12's mammalian orthologue is cytoplasmic, and because newly synthesized GlcN-PI is accessible on the cytoplasmic face of intact ER vesicles (Vidugiriene and Menon, 1993).

Transmembrane translocation of GlcN-PI. GlcN-PI is the precursor species most likely to be translocated to the lumenal side of the ER membrane. Flipping of GlcN-PI as well as GlcNAc-PI has been reconstituted in rat liver microsomes, but the protein involved has not been identified, and the possibility has been raised that GlcN-PI translocation may be mediated by a generic ER phospholipid flippase (Vishwakarma and Menon, 2006).

Lumenal steps in GPI assembly:

Inositol acylation. The acyl chain transferred to GlcN-(acyl)PI *in vivo* is likely palmitate, although a range of different acyl chains can be transferred from their corresponding CoA derivatives *in vitro* (Costello and Orlean, 1992; Franzot and Doering, 1999). Because mutants blocked in formation of all mannosylated GPIs accumulated inositol-acylated GlcN-PI (Orlean, 1990; Costello and Orlean, 1992), and because mannosylated GPI intermediates lacking an inositol acyl chain have not been reported, it is likely that inositol acylation precedes mannosylation *in vivo*. Gwt1, the acyltransferase, is likely to be catalytic because its affinity-purified mammalian orthologue transfers palmitate from palmitoyl CoA to a dioctanoyl analogue of GlcN-PI (Murakami *et al*. 2003). The protein has 13 transmembrane domains (Murakami *et al*. 2003; Sagane *et al*. 2011), and amino acid residues critical for function all face the lumen, indicating acyl transfer is a lumenal event (Sagane *et al*. 2011), although it is not yet known how acyl CoAs enter the ER lumen. Despite Gwt1's multispanning topology, the possibility that this inositol acyltransferase is also a GlcN-PI transporter is unlikely, because non-acylated, mannosylated GPIs can be formed in cell lines deficient in Gwt1's mammalian orthologue (Murakami *et al*. 2003).

GPI Man-T-I. The α1,4-Man-T Gpi14 shows greatest similarity to Alg3, is predicted to have 12 transmembrane segments (Oriol *et al*. 2002), and is assigned to GT Family 50. Two additional proteins, Arv1 and Pbn1, are involved in the GPI-Man-T-I step along with Gpi14. *arv1Δ* cells grow at 30°C but not at 37°C, and are delayed in ER to Golgi transport of GPI-anchored proteins, and accumulate GlcN-(acyl)PI *in vitro* (though not *in vivo*) (Kajiwara *et al*. 2008). Further, their temperature sensitivity is suppressed by overexpression the genes for most of the subunits of GPI-GnT, suggesting a functional link between *ARV1* and GPI assembly (Kajiwara *et al*. 2008). However, *arv1Δ* cells were not defective in Dol-P-Man synthase activity or in N-glycosylation, nor were mild detergent-treated *arv1Δ* membranes defective in GPI-Man-T-I activity, suggesting that Arv1 is not a Dol-P-Man flippase or directly involved in mannosyltransfer, and leading to the proposal that Arv1 is involved in delivering GlcN-(acyl)PI to GPI-Man-T-I (Kajiwara *et al*. 2008). Essential Pbn1 has been implicated at the GPI-Man-T-I step in yeast because expression of both *GPI14* and *PBN1* is necessary to complement mammalian cell lines defective in Pbn1's mammalian homologue Pig-X, and likewise, co-expression of *PIG-X* and the gene for Gpi14's mammalian homologue, *PIG-M*, partially rescues the lethality of *gpi14Δ* (Ashida *et al*. 2005; Kim *et al*. 2007). Repression of *PBN1* expression leads to accumulation of some of the ER form of the GPI protein Gas1, a phenotype seen in GPI precursor assembly mutants (Subramanian *et al*. 2006). However, it has not been reported whether *pbn1* mutants accumulate the predicted GPI intermediate GlcN-(acyl)PI. Because Pbn1 is also involved in processing a number of non-GPI proteins that pass though the ER to the vacuole, the vacuolar membrane, and the plasma membrane, it must have additional functions in the ER (Subramanian *et al*. 2006).

GPI Man-T-II. Unlike the other Dol-P-Man-utilizing transferases of the GPI assembly and dolichol pathways, the α1,6-Man-T Gpi18 is predicted to have 8 transmembrane domains (Fabre *et al*. 2005; Kang *et al*. 2005). This protein and its orthologues have been assigned to GT Family 76.

GPI Man-T-III and IV. These two α1,2-Man-T, together with their homologues in the dolichol pathway, Alg9 and Alg12, are predicted to have 12 transmembrane domains and are assigned to GT Family 22 (Oriol *et al*. 2002). Overexpression of *GPI10* does not rescue the lethal *smp3Δ* null mutation, and *vice versa*, indicating that the two α1,2-Man-T have very strict acceptor specificities (Grimme *et al*. 2001).

Phosphoethanolamine addition: origin of Etn-P from Ptd-Etn. There is good evidence that the Etn-Ps, at least those on Man-1 and Man3, originate from Ptd-Etn. Yeast mutants unable to make CDP-Etn or CDP-Cho from exogenously supplied Etn, but still capable of making Ptd-Etn by decarboxylation of Ptd-Ser, do not incorporate [^{3}H]Etn into protein-bound GPIs or into a Man_2-GPI precursor that otherwise receives Etn-P on Man-1. However, radioactivity supplied as [^{3}H]Ser is incorporated into the Man_2-GPI after formation and decarboxylation of Ptd-[^{3}H]Ser (Menon and Stevens, 1992; Imhoff *et al*. 2000). The importance of Ptd-Ser decarboxylation for GPI anchoring is underscored by the finding that the combination of a conditional *gpi13* mutation, defective in the EtnP-T-III, with *psd1Δ* and *psd2Δ*, nulls in the two Ptd-Ser decarboxylase genes, are inviable (Toh-e and Oguchi, 2002). Direct transfer of Etn-P from Ptd-Etn to a GPI remains to be demonstrated *in vitro*.

Phosphoethanolamine addition: importance of the alkaline phosphatase domain of Mcd4, Gpi7, and Gpi13. These three proteins all have a large lumenal loop of some 400 amino acids that contains sequences characteristic of the alkaline phosphatase superfamily (Gaynor *et al*. 1999; Benachour *et al*. 1999, Galperin and Jedrzejas, 2001), consistent with involvement in formation or cleavage of a phosphodiester. This domain is important for function, because the $G^{227}E$ substitution that results in temperature-sensitive growth and a conditional block in GPI precursor assembly in the *mcd4-174* mutant (Gaynor *et al*. 1999) lies in one of the two metal-binding sites in alkaline phosphatase family members (Galperin and Jedrzejas, 2001). The metal is commonly zinc, and *in vitro* Etn-P addition from an endogenous donor is zinc dependent (Sevlever *et al*. 2001) and Zn^{2+} suppresses the temperature sensitivity of a *gpi13* allele.

Phosphoethanolamine addition: Man_2-GPI may be Mcd4's preferred substrate. Three sets of findings suggest that Mcd4 may act preferentially on Man_2-GPI: i) treatment of wild type cells with the terpenoid lactone YW3548, which inhibits addition of Etn-P to Man-1, leads to accumulation of Man_2-GPI (Sütterlin *et al*. 1997, 1998), ii) Man_2-GPI is the most abundant of the accumulating GPIs in *mcd4-174*, and iii) Man_2-GPI is the largest GPI formed *in vitro* by *mcd4* membranes (Zhu *et al*. 2006).

Phosphoethanolamine addition: importance of the Etn-P added to Man-1 by Mcd4 and additional possible functions for Mcd4. The finding that *mcd4* mutants accumulate unmodified Man_2-GPI suggests that the presence of Etn-P on Man-1 is important for GPI-Man-T-III to add the third Man. The requirement, though, is not absolute because *mcd4Δ* cells can be partially rescued by overexpression of Gpi10 (Wiedman *et al*. 2007). In addition to enhancing the efficiency of mannosylation by Gpi10, the Etn-P moiety on Man-1 may be important for additional reasons. *mcd4Δ* cells expressing human or trypanosomal Gpi10 orthologues, Man-T known to mannosylate Man_2-GPIs lacking Etn-P on Man-1 efficiently, still grow slowly (Zhu *et al*. 2006; Wiedman *et al*. 2007). Further, *mcd4Δ* cells expressing trypanosomal Gpi10 are retarded in export of GPI-proteins from the ER, unable to remodel their GPI lipid moiety to ceramide, and are defective in selection of axial budding sites (Zhu *et al*. 2006). How the presence of Etn-P on Man-1 influences these processes is not yet known.

Mutations in *MCD4* also impact cellular processes that are not directly connected with GPI biosynthesis. Cells expressing the Mcd4-P^{301}L variant, but not G^{227}E, are defective in the transport of Ptd-Ser to the Golgi and vacuole for decarboxylation, but unaffected in GPI anchoring suggesting an additional role for Mcd4 in transport dependent Ptd-Ser metabolism (Storey *et al*. 2001). Further, yeast overexpressing Mcd4 (as well as Gpi7 and Gpi13) release ATP into the medium, and Golgi vesicles from the Mcd4 overexpressers were enriched in that protein and showed elevated levels of ATP uptake (Zhong *et al*. 2003). It was suggested that Mcd4 normally mediates symport of ATP and Ptd-Etn into the ER lumen, and that overexpression of the protein leads ATP to accumulate in secretory vesicles, which eventually fuse with the plasma membrane (Zhong *et al*. 2003).

Phosphoethanolamine addition to Man-2 and its possible functions. GPI-Etn-P-II consists of catalytic Gpi7 and non-catalytic Gpi11. *Both gpi7Δ* and temperature-sensitive *gpi11Δ* disruptants complemented by the human Gpi11 orthologue *PIG-F* accumulate a Man_4-GPI bearing Etn-P on Man-1 and Man-3 but missing one on Man-2 (Benachour *et al*. 1999; Taron et al. 2000). Because loss of GPI-Etn-P function leads to accumulation of a Man_4-GPI with Etn-Ps on Man-1 and Man-3, GPI-Etn-P-II may normally add Etn-P to Man-2 after GPI-Etn-P-T-III has modified Man-3. However, because Man_3- and Man_4-GPIs with a single Etn-P on Man-2 accumulate in the *smp3* mutants and in temperature-sensitive *gpi11Δ* strains complemented by the human Gpi11 orthologue (Taron *et al*. 2000; Grimme *et al*. 2001), GPI-Etn-P-II has the capacity to act on Etn-P-free GPIs.

Diverse phenotypes of *gpi7Δ* cells indicate that the Etn-P moiety on Man-2 is important for a number of reasons. First, the combination of *gpi7Δ* with the GPI transamidase mutation *gpi8* leads to a synthetic growth defect, indicating that an Etn-P on Man-2 enhances transfer of GPIs to protein (Benachour *et al*. 1999). Second, *gpi7Δ* cells have defects in ER to Golgi transport of GPI-proteins and GPI lipid remodeling to ceramide (Benachour *et al*. 1999). Third, *GPI7* deletion leads to cell wall defects and

shedding of GPI-proteins, indicating defective transfer of such proteins into the wall (Toh-e and Oguchi, 1999; Richard *et al*., 2002). Lastly, *gpi7Δ* cells show a cell separation defect that results from mistargeting of Egt2, a GPI protein expressed in daughter cells and implicated in degradation of the septum (Fujita *et al*. 2004). These phenotypes suggest that the Etn-P group on Man-2 is recognized by GPI transamidase, the intracellular transport machinery, GPI lipid remodeling enzymes, and cell wall crosslinkers. An inability to remove Etn-P from Man-2 also leads to phenotypes (see *Remodeling of protein bound GPIs*).

Phosphoethanolamine addition to Man-3 by Gpi13 and the role of Gpi11. Gpi13 is the catalytic subunit of GPI-Etn-P-T-III, and, as expected from the fact that it adds the Etn-P that participates in the GPI transamidase reaction, *GPI13* is essential. The major GPI accumulated by yeast strains depleted of Gpi13 is a Man_4-GPI with a single Etn-P on Man-1 (Flury *et al*. 2000; Taron *et al*. 2000). Gpi11 is likely involved in the GPI-Etn-P-T-III reaction as well, because a recently isolated *gpi11*-Ts mutant also accumulates a Man_4-GPI with its Etn-P on Man-1 (K. Willis and P. Orlean, unpublished results), and human Gpi11 interacts with and stabilizes human Gpi13 (Hong *et al*. 2000). Human Gpi11 (Pig-F) also interacts with human Gpi7 (Shishioh *et al*. 2005). The lipid accumulation phenotypes observed in various types of *gpi11* mutants may prove to be explainable in terms of differential abilities of wild type Gpi11, mutant Gpi11, and human Gpi11 to interact with Gpi7, Gpi13, and possibly even Mcd4, and permit varying extents of Etn-P modification. Because GPIs with the same chromatographic mobilities may be isoforms modified with Etn-P at different positions, and because accumulating GPIs may be mixtures of isoforms, detailed structural analyses should give a clearer picture of the role of Gpi11 in Etn-P modification.

GPI transfer to protein:

Depletion of Gab1 and Gpi8 leads to actin bar formation. Additional functions for Gab and Gpi18 are suggested by the finding that depletion of Gab1 or Gpi8 from yeast, but not of Gaa1, Gpi16, or Gpi17, leads to accumulation of bar-like structures of actin that associate with the perinuclear ER and are decorated with cofilin (Grimme *et al*. 2004). This phenotype, which is not a general result of defective GPI anchoring, might reflect disruption of some functional interaction between resident ER membrane proteins and the actin cytoskeleton and consequent collapse of the ER around the nucleus (Grimme *et al*. 2004).

Remodeling of protein-bound GPIs:

Roles of Bst1, Per1, and Gup1 in ER exit and transport of GPI proteins. Modifications of the GPI lipid by Bst1, Per1, and Gup1 are necessary for efficient transport of GPI proteins from the ER to the Golgi. Loss of Bst1 function leads to retarded transport of GPI-proteins from the ER to the Golgi (Vashist *et al*. 2001), and delayed ER degradation of misfolded GPI proteins, suggesting that inositol deacylation generates sorting signals for ER exit of GPI proteins and for recognition by a quality control

mechanism for GPI-proteins (Fujita *et al*. 2006; Fujita and Jigami, 2008). *per1Δ* and *gup1Δ* cells also show significantly delayed ER to Golgi transport of GPI-proteins (Bosson *et al*. 2006; Fujita *et al*. 2006b). Lipid remodeling events generate a GPI able to associate with and be concentrated in membrane microdomains at ER exit sites prior to their export from the ER (Castillon *et al*. 2009). At these sites, the p24 complex of membrane proteins then serves as an adapter between GPI-proteins and the COP II machinery to promote incorporation of GPI proteins into COP II vesicles specialized for transport of GPI-proteins from the ER. Remodeled GPIs may bind p24 with higher affinity, therefore promoting export of the proteins bearing them (Castillon *et al*. 2011). In the Golgi, GPI-proteins with remodeled anchors are released and proceed onwards along the secretory pathway. However, p24 complexes, which cycle between the ER and Golgi, again monitor the remodeling status of GPIs and exert a quality control function in the Golgi by sensing and retrieving proteins with unmodified GPIs to the ER, where they may encounter the resident ER remodeling enzymes (Castillon *et al*. 2011).

Remodeling of the GPI lipid moiety to ceramide by Cwh43. Cwh43, which replaces the diacylglycerol moiety of GPIs with ceramide, is a large protein with 19 predicted transmembrane domains (Martin-Yken *et al*. 2001; Ghugtyal *et al*. 2007; Umemura *et al*. 2007). *cwh43Δ* cells accumulate GPI-proteins whose lipids are diacylglycerols with a very long acyl chain similar to the lipid generated after action of Bst1, Per1, and Gup1. Because ceramide remodeling requires prior action of Bst1, and *per1Δ* and *gup1Δ* strains show severe defects in remodeling, the exchange reaction seems to take place after the first three lipid modification steps. The mechanism is so far unknown, but could involve a phospholipase-like reaction that replaces diphosphatidic acid with ceramide phosphate or diacylglycerol with ceramide (Ghugtyal *et al*. 2007; Fujita and Kinoshita, 2010). However, alternatives to such a linear remodeling pathway, in which Cwh43 acts instead on the Bst1 or Per1 products, have been discussed (Umemura *et al*. 2007). The C-terminal domain of Cwh43 contains a motif that may be involved in recognition of inositol phosphate (Umemura *et al*. 2007). Because *mcd4* and *gpi7*, mutants defective in addition of Etn-P to Man-1 and Man-2, are affected in ceramide remodeling, Cwh43 may also recognize Etn-P side-branches. Cwh43 appears to act in the ER, where it remodels GPIs with a ceramide consisting of phytosphingosine bearing a C_{26} acyl chain, as well as in the Golgi, where the ceramide it introduces contains phytosphingosine with a hydroxy-C_{26} acyl group (Reggiori *et al*. 1997).

Removal of Etn-P moieties from Man-1 and Man-2. The ER-localized Ted1 and Cdc1 proteins are homologous to mammalian PGAP5, which removes EtN-P moieties from Man-2 (Fujita *et al*. 2009), and genetic interactions connect these two proteins processing and export of GPI-proteins. Export of Gas1 is retarded in *ted1Δ* cells, and *ted1Δ*'s buffering genetic interactions with *emp24Δ* and *erv5Δ*, mutants deficient in two components of the p24 complex involved in maturation and trafficking of GPI proteins, indicate a functional relationship between the three proteins (Haass *et al*. 2007). Further, *cdc1*

mutations are suppressed by *per1*/*cos16* and *gup1* mutations (Paidhungat and Garrett, 1998; Losev *et al*. 2008). Ted1 and Cdc1 contain a lumenal metallophosphoesterase domain (Haass *et al*. 2007; Losev *et al*. 2008), and, consistent with this, *cdc1*'s temperature-sensitivity is suppressed by Mn^{2+}, the cation required by PGAP5 (Fujita *et al*. 2009). These findings are in turn consistent with Ted1 and Cdc1 being GPI-Etn-P phosphodiesterases, but this possibility awaits biochemical confirmation.

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