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Biosynthesis of Glycosylphosphatidylinositol Is Essential to the Survival of the Protozoan Parasite *Toxoplasma gondii*

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The *PIGA* gene from *Toxoplasma gondii* has been cloned and characterized. Like mammalian PIGA, the transmembrane and C-terminal domains are sufficient to direct localization to the parasite endoplasmic reticulum. A functional copy of *PIGA* is required for tachyzoite viability, demonstrating that glycosylphosphatidylinositol biosynthesis is an essential process in *T. gondii*.

Glycosylphosphatidylinositol (GPI)-anchored proteins dominate the surface of the *Toxoplasma gondii* tachyzoite (3, 19) and have been implicated in both host cell attachment and modulation of the host immune response (7, 15, 19). To further our understanding of the synthesis, trafficking, and function(s) of GPIs and GPI-anchored proteins in *T. gondii*, we have begun to characterize the genes involved in the parasite's GPI biosynthetic pathway.

GPI biosynthesis is a conserved pathway among eukaryotes that occurs primarily in the endoplasmic reticulum (ER) and leads to the generation of both free GPIs and GPI-anchored proteins (reviewed in references 8, 9, 13, 17, and 21). In mammalian cells, the pathway is initiated on the cytosolic face of the ER with the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI) by the GPI-GlcNAc transferase complex (29–32). The GlcNAc transferase activity of the complex is thought to reside in the PI-glycan class A (PIGA) protein (18, 29). Loss-of-function mutations in *PIGA* result in a complete GPI deficiency, which consequently abolishes the surface expression of GPI-anchored proteins (reviewed in reference 2). The ability to tolerate a GPI deficiency is species specific and sometimes even life cycle stage specific (2, 10, 12, 14, 20, 22–25).

The complete *PIGA* cDNA of *T. gondii* strain RH(EP) (GenBank accession no. AY216495) was obtained and sequenced (using primers designed from an expressed sequence tag [EST1206547; http://ParaDB.cis.upenn.edu] that exhibited homology to the C-terminal region of multiple PIGA orthologues) from products of 5′ and 3′ rapid amplification of cDNA ends. Subsequent sequencing and characterization of the RH(EP) *PIGA* gene (GenBank accession no. AY216496) showed it to be a relatively large (~10 kb) single-copy gene harboring 11 introns. The predicted protein sequence of *T. gondii* PIGA (616 amino acids) exhibits significant similarity to sequences of other PIGA orthologues (Fig. 1), particularly between residues 22 and 383 (~50% identity and ~70% homology), a region which contains the putative GlcNAc transferase domain. As with other PIGA proteins, *T. gondii* PIGA harbors a potential transmembrane domain (residues 517 to 539) followed by a stretch of mostly hydrophilic residues (residues 541 to 616) extending to the C terminus (Fig. 1). *T. gondii* PIGA contains an insert of ~100 amino acids not found in other PIGA orthologues (residues 384 to 506; Fig. 1). This insert, which is also present in both genomic (http://toxodb.org/ToxoDB.shtml) and cDNA (data not shown) sequences of *PIGA* of *T. gondii* strain P(LK), exhibits no homology to any known proteins. Its functional significance, if any, is unknown.

Stably expressed recombinant versions of *T. gondii* PIGA, containing either an N-terminal c-Myc epitope tag or a C-terminal green fluorescent protein (GFP) fusion, localized predominantly to ER-like structures encircling the nucleus of the tachyzoite (Fig. 2A and B). When transiently expressed, an engineered ER marker (*Trypanosoma brucei* GPI-phospholipase C [GPI-PLC] containing a secretory signal sequence and ER retention signal [secGPI-PLC^{HDEL}]) localized to a perinuclear compartment (Fig. 2E) indistinguishable from that of PIGA.

Previous studies have shown that the transmembrane domain of mammalian PIGA, together with the 23 residues immediately C terminal to this domain, is sufficient for ER targeting and/or retention (31). Like its mammalian counterpart, *T. gondii* PIGA lacks an obvious N-terminal signal sequence but appears to localize to the ER. To determine whether the transmembrane and C-terminal domains of *T. gondii* PIGA direct localization, residues 517 to 616 were fused to the C terminus of GPI-PLC (GPI-PLC$^{PIGA517-616}$). In transient-expression studies, GPI-PLC$^{PIGA517-616}$ exhibited a localization pattern similar to that of PIGA (Fig. 2D) and strikingly different from the peripheral localization of GPI-PLC lacking this domain (Fig. 2C), indicating that the signals required for ER targeting of *T. gondii* PIGA are also located within the transmembrane and C-terminal domains. Heterologously expressed GPI-PLC can induce a GPI deficiency in other parasites through the cleavage of GPI intermediates (10, 22). Stable expression of wild-type GPI-PLC exhibited little effect on tachyzoites (data not shown); however, it was not possible to generate clones stably expressing GPI-PLC targeted to the ER, suggesting that GPI-PLC expression at the site of GPI biosynthesis was lethal.

When chemical mutagenesis (27), insertional mutagenesis (6), and targeted gene disruption of *PIGA*, each coupled with

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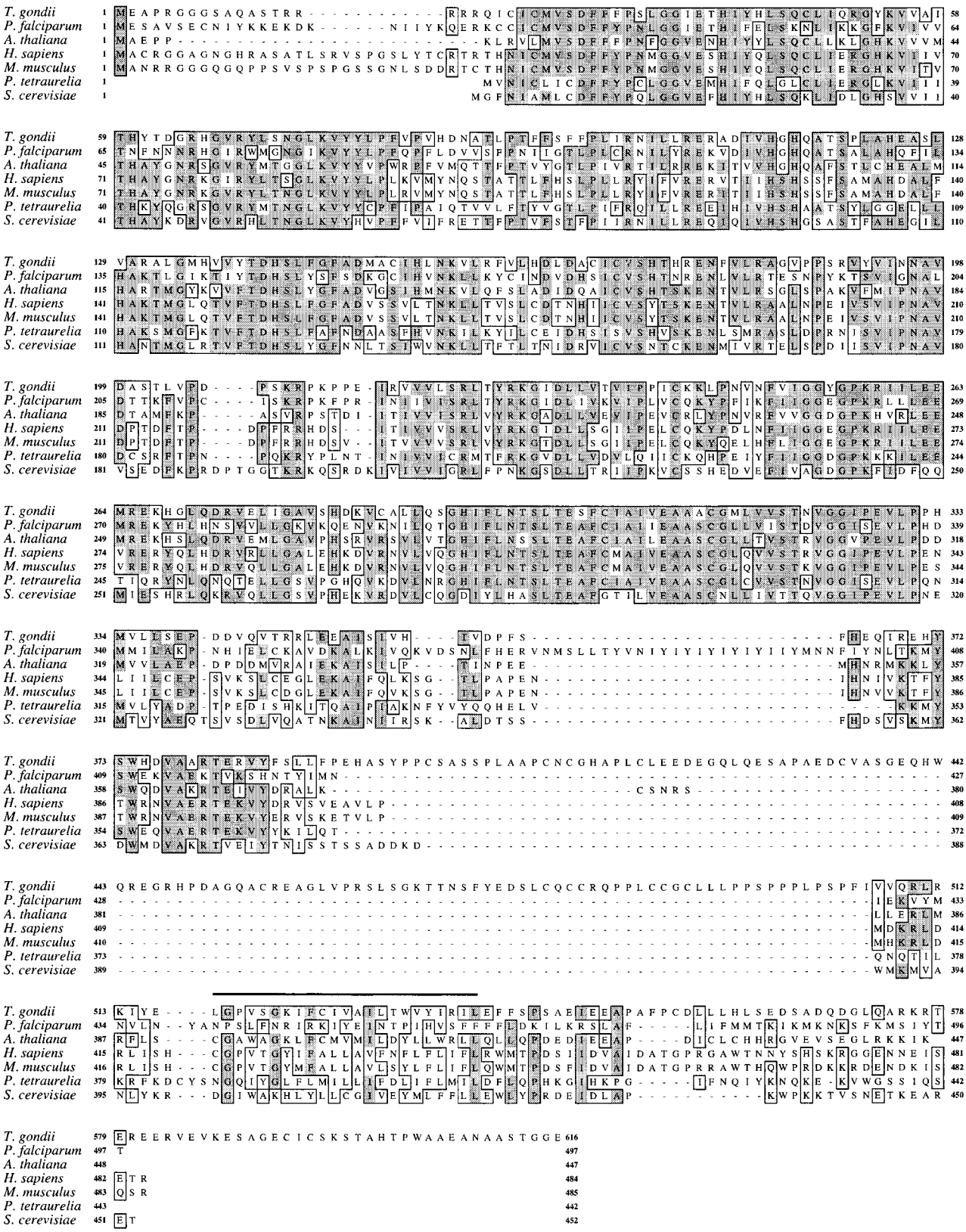

FIG. 1. ClustalW sequence alignment of PIGA orthologues from *T. gondii*, *Plasmodium falciparum* (gi23495183), *Arabidopsis thaliana* (gi18407913), *Homo sapiens* (gi219994), *Mus musculus* (gi1402592), *Paramecium tetraurelia* (gi8571458), and *Saccharomyces cerevisiae* (gi9755344). Regions of homology are boxed, with identity denoted by dark shading and conserved amino acid changes denoted by light shading. The predicted *T. gondii* PIGA transmembrane domain (amino acids 517 to 539) is denoted by a thick horizontal line.

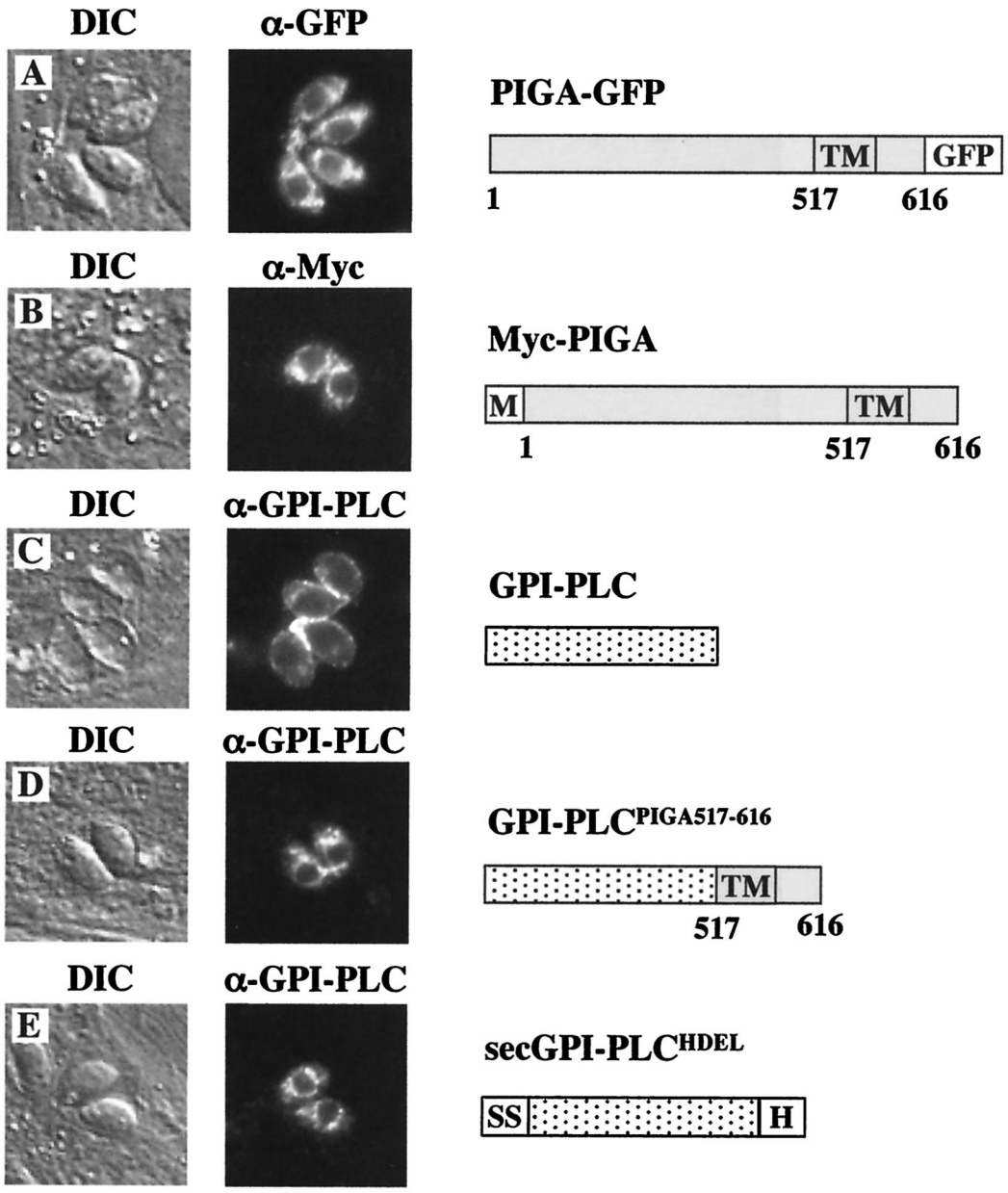

FIG. 2. Subcellular localization of *T. gondii* PIGA. PIGA with a C-terminal fusion to GFP (A) or an N-terminal c-Myc epitope tag (B) localized to ER-like structures encircling the nucleus of the parasite. As a marker for ER localization, a recombinant version of *T. brucei* GPI-PLC, which normally localized to the periphery of the tachyzoite (C), was engineered to localize to the ER (secGPI-PLC^{HDEL}) by the addition of an N-terminal secretory signal sequence and the C-terminal ER-retention motif, HDEL (E). When fused to the C terminus of GPI-PLC (GPI-PLC$^{PIGA517-616}$), the putative transmembrane and ER-lumenal domains (amino acids 517 to 616) of *T. gondii* PIGA were sufficient to alter the localization of GPI-PLC to an ER-like localization (D) indistinguishable from that of PIGA. Bar, 5 μm. M, c=Myc; SS, signal sequence; H, HDEL; TM, transmembrane; DIC, differential interference contrast.

selection (using *Clostridium septicum* alpha toxin) (11, 33) for a GPI-anchored protein deficiency, were used to attempt to generate GPI-deficient parasites, the results were uniformly unsuccessful, suggesting that GPI biosynthesis is required for tachyzoite viability. To show this conclusively, we attempted to disrupt *PIGA* in the presence of a second copy of the gene. A knockout vector (pHXGPRT/ΔPIGA^{5kb}) was designed such that integration within the region located upstream of the internal deletion (type I) would result in a pseudodiploid (5, 26, 28) harboring two nonfunctional *PIGA* alleles (Fig. 3A).

Parental [RH(EP)Δ*HXGPRT*) (26, 28)] and RH(EP)Δ*HXGPRT*/*PIGA-GFP* (referred to hereafter as RH*PIGA-GFP*) parasites were each transfected (16, 28) with undigested pHXGPRT/ΔPIGA^{5kb} in six independent experiments. Stable expression of PIGA-GFP was confirmed by both immunofluorescence (Fig. 2A) and Western blot (data not shown) experiments. A

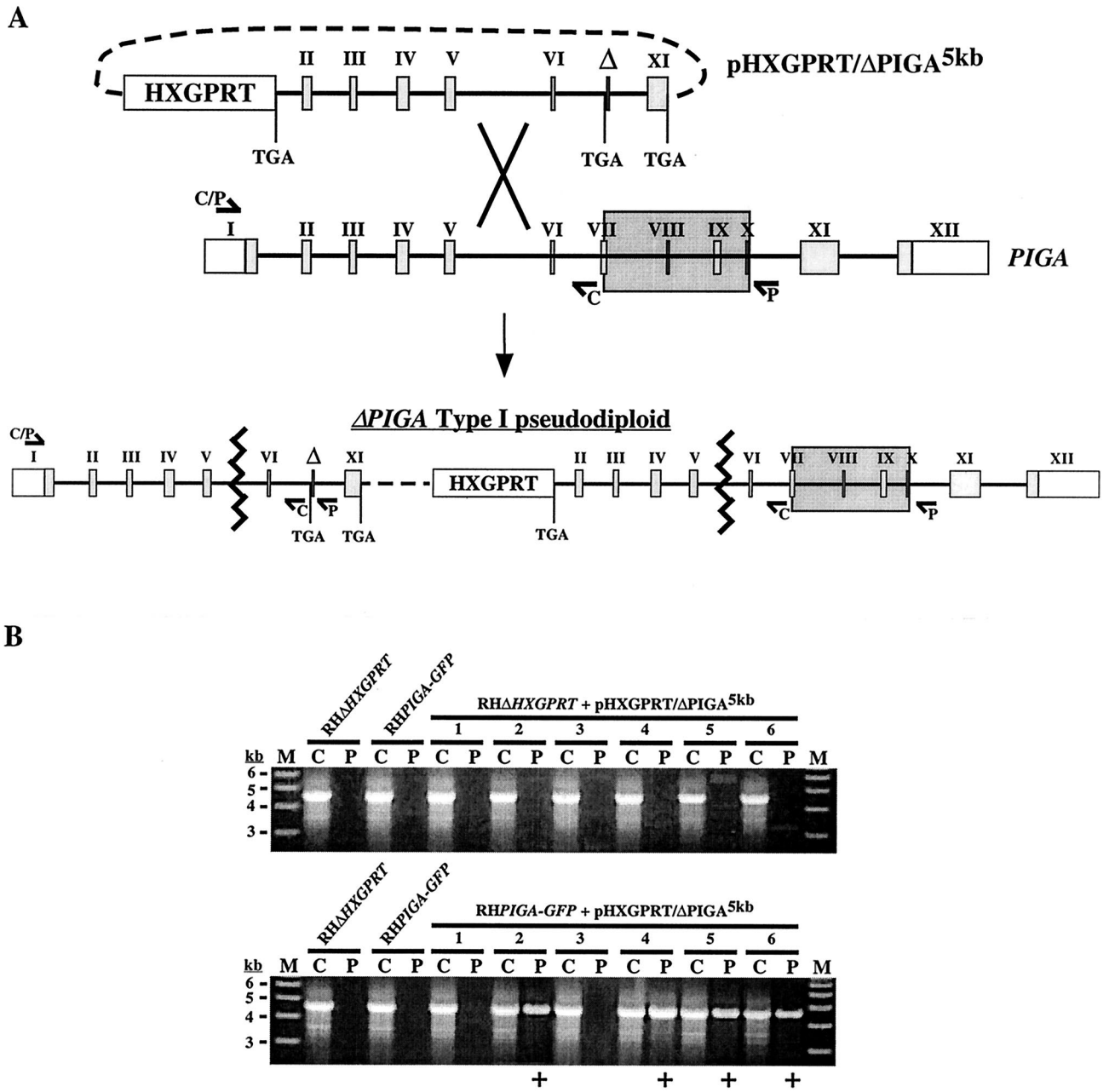

FIG. 3. Targeted disruption of *PIGA*. (A) Schematic of the gene disruption strategy. The pHXGPRT/ΔPIGA^{5kb} knockout vector was constructed such that single-crossover homologous recombination into the *PIGA* locus upstream of the deletion would generate a type I pseudodiploid with two nonfunctional *PIGA* alleles. To generate ΔPIGA^{5kb}, the 5′ end of *PIGA* was truncated within exon 1 (removing the 5′ untranslated region and coding sequence corresponding to residues 1 to 47) and the 3′ end was truncated into exon 11 (removing the coding sequence for the putative transmembrane and ER-lumenal domains [residues 519 to 616]). An internal sequence corresponding to a highly conserved region of PIGA (amino acids 303 to 368) was also deleted, and stop codons were engineered into all cloning junctions within the ΔPIGA^{5kb} allele. Type I integration resulted in the harboring by the pairs upstream allele of the ~1.8-kb deletion (marked by Δ; large shaded box indicates the corresponding sequence in the wild-type allele), which could be detected by PCR (using primer set P) as a product of ~4.6 kb. The P primer set was found to be incapable of amplifying the predicted product (~6.5 kb) from wild-type genomic DNA. (B) Six independent stable transgenic populations (lane pairs 1 to 6) derived from either parental parasites (RHΔ*HXGPRT*) or parasites harboring a stable second copy of *PIGA* (RH*PIGA-GFP*) were screened for integration of the plasmid into *PIGA* (see text and elsewhere in this legend). Δ*PIGA* type I pseudodiploids were not detected in any of the six populations derived from parental parasites, while four out of six populations from RH*PIGA-GFP* parasites yielded a positive PCR product result (+). Lanes C, control primers; lanes P, pseudodiploid-specific primers; lanes M, molecular mass markers.

forward primer directed against the 5′ untranslated region of *PIGA* (which was not present in ΔPIGA^{5kb}) and a reverse primer located directly downstream of the deleted region (primer set P) (Fig. 3A) were used for PCR to screen stable transgenic populations for the presence of type I Δ*PIGA* pseudodiploids. These primers yield a product of ~4.6 kb that is pseudodiploid specific, since amplification through the deleted region was not possible using genomic DNA as a tem-

plate (Fig. 3B). As a control, the same upstream primer and a reverse primer located immediately upstream of the deleted region in the ΔPIGA^{5kb} allele (primer set C) were used to amplify a product of ~4.6 kb from both the wild-type and pseudodiploid *PIGA* alleles (Fig. 3A). Type I Δ*PIGA* pseudodiploids were not detected in any of the six independent populations from the parental parasites. However, they were present in four of the six populations derived from RH*PIGA-GFP* parasites (Fig. 3B), indicating that a second copy of *PIGA* was sufficient to rescue the parasite upon disruption of the wild-type gene. Integration into the genomic locus was confirmed both by Southern blot analysis of independent clones and by the absence of a reverse transcription-PCR product corresponding to the *PIGA* transcript in these clones (data not shown).

These results demonstrate that GPI biosynthesis is essential for viability in *T. gondii*, and they identify the GPI biosynthetic pathway as a potential target for the development of new chemotherapeutics against this parasite. It is not clear whether the lethal consequences of *PIGA* disruption in *T. gondii* result from a deficiency in GPI-anchored proteins, free GPIs, or both. Future experiments aimed at disrupting components of the *T. gondii* GPI transamidase complex (1, 4, 12, 20, 34) should resolve this question and reveal the relative importance of GPI-anchored proteins in the *T. gondii* life cycle.

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