Supplemental Figure Legends

Supplemental Figure S1: Alignment of full-length ROP5 isoforms from type I and II strains ($ROP5B_{II}$ has a frame-shift resulting in a premature stop codon and type III isoforms are nearly identical to those found in type I (1); Genbank IDs for ROP5 isoforms are AEA41142-AEA41154, inclusive). Secondary structure is shown above the alignment. The predicted helices of the Arginine-rich Amphipathic Helix (RAH) domain are shown in light gray. Polymorphic residues for which we have no structural information are shaded gray, buried residues are noted in black, residues near the active site are yellow, and other surface exposed polymorphic residues are green. Note that $ROP5A_{III}$ is identical to $ROP5A_I$ except for substitution of F460V. Polymorphic residues that lie on the opposite face from the pseudoactive site are indicated with blue circles. Note that these are enriched in "isoform-specific" rather than "strain-specific" substitutions, as are the polymorphic residues in the activation segment. Motifs important for catalysis in active kinases are indicated below the alignment in red. The activation segment is indicated with a green line. Cys458 and Cys492, which are disulfide bonded, are indicated with orange stars.

Supplemental Figure S2: Localization of $ROP5A_{III}$-HA R389D/K263S in parasites that are (A) in the process of dividing (note the twin "pre-rhoptry" staining of ROP2/4) or (B) in parasites with mature rhoptries. Rhoptries are stained with an antibody recognizing the rhoptry markers ROP2/4. (C) Mice (n=10) were inoculated with 10^6 Δ*rop5* parasites that had been complemented either with wild-type $ROP5A_{III}$ or with R389D/K263S $ROP5A_{III}$ and monitored for 30 days.

Supplemental Figure S3: Structural alignment of $ROP5B_I$ with four pseudokinases of known structure (ROP8; 3BYV, VRK; 2JII, ILK; 3KMW, and STRADα; 2WTK), the atypical Mg-independent kinase CASK (3C0I), and two canonical kinases, p38 (1CM8) and PKA (1ATP). The alignment was created by structural superposition with TM-align (2) and manual inspection and realignment of areas of interest. The N-terminal extension of ROP5 and C-terminal linker of PKA are boxed in orange. Canonical motifs are highlighted in red, with substitutions predicted to be deleterious to kinase function shaded gray. The activation segment is noted with a green line and the activating phosphorylation site of PKA and p38 noted with an orange hexagon below the alignment (note: for full activation, p38 also requires phosphorylation of the Tyr 2 residues C-terminal to this site). Residues thought to be directly involved in catalysis based on simulation are noted with orange arrows below the alignment. Residues coordinating Mg^{2+} are noted with a yellow circle. Basic residues lining the posterior of the ROP5 surface and conserved in ROP8 are noted with blue arrows.

Supplemental Figure S4: The ATP-bound (gray) and -unbound (blue) $ROP5B_I$ structures are overlaid. ATP is shown as sticks.

Supplemental Figure S5: (a) Organization of the $ROP5B_I$ pseudoactive site. Coordination of Mg^{2+} is noted with black dashes, hydrogen bonds with gray dashes. Residues that directly coordinate Mg^{2+} are colored black. Residues that provide

supporting hydrogen-bonding to the Mg^{2+} coordination are colored green. The conserved HRD motif His387 is yellow. ATP is cyan. (b) A homology model of $ROP5A_I$ was generated with Modeller (3), using the ATP-bound $ROP5B_I$ structure as a template. This model shows a likely conformation of the Arg389 substitution in the ROP5 active site, which places its guanidino group 2.8Å from the γ-phosphate of the ATP.

Supplemental Figure S6: Comparison of the casein kinase 2 (CK2) and $ROP5B_I$ active sites. (a) Structural alignment of CK2 (white) and PKA (grey). The CK2-bound Mg^{2+} is shown in green, the PKA-bound Mn^{2+} in purple. CK2 Ser51 and the bound ATP (or the non-hydrolysable ATP-analog ANP, in the case of CK2) are shown as sticks. (b) The $ROP5B_I$ pseudoactive site is shown in the same orientation as the CK2 active site, and colored as in (a). ROP5 Ser246, which is equivalent to CK2 Ser51, is shown as sticks.

Supplemental Figure S7: The ROP5 pseudokinase domain is in a degenerate active conformation. The ROP5 pseudokinase domain (gray) aligns more closely with (a) the active conformation of PKA (blue; 1ATP; 3.1Å RMSD for 217 out of 351 Cα atoms); than with the (b) inactive conformation (pink; 1SYK; 3.9Å RMSD for 224 out of 351 Cα atoms). Note, in particular, the conformation of the P-loops in each structure. The ROP5 ATP is shown as sticks. (c) The ROP5 helix C is swung "out" from the conformation in active PKA (d). Note the malonate in the ROP5 structure whose carboxylate contacts Lys263 in an analogous manner to interaction between PKA Lys 77 and the helix C Glu91.

Supplemental Figure S8: ROP5 has a degenerate, but well-ordered activation segment. The activation segments of (a) PKA, (b) $ROP5B_I$ are shown. The activation loops are colored blue and the P+1 loops are colored green. The catalytic Asp (or His389, in $ROP5B_I$), the HRD Arg (PKA only; ROP5 has a Gly at this position), and the PKA GT-motif Thr201 are indicated as sticks, as is ATP. The PKA phosphorylation site (pT197) and the equivalent structural position in $ROP5B_I$ (Pro419) are indicated as purple sticks. $ROP5B_I$ Pro424, which structurally aligns with the GT-motif Thr201 in PKA, is shown as well. (c-d) Surface representation of the same region, with the P+1 loops shown in green.

Supplemental Figure S9: The ROP5 N-terminal extension spans the N- and C-terminal lobes of the pseudokinase domain. (a) The structure of $ROP5B_I$ (tan with NTE in red) is overlaid with the structure of ROP8 (gray with NTE in blue). The ROP8 loop that protrudes into the active site is circled. (b) A homology model of the structure of ROP18 was created with Modeller (3) using the structure of ROP8 as a template. The ROP18 model (gray with NTE in blue) is shown overlaid on the $ROP5B_I$ structure (tan with NTE in red). Residues highlighted in (c) are shown as sticks, with $ROP5B_I$ Thr211 and ROP18 Thr214 indicated in cyan. (c) Structural alignment of rhoptry kinases was generated by first structurally aligning ROP8 with $ROP5B_I$, and using the resulting alignment as a profile to align the sequences for four additional rhoptry kinases, each of which possesses a RAH domain N-terminal to their pseudokinase domain. The conserved hydrophobic buried hydrophobic residue (ROP5 Trp215) that caps the NTE is highlighted in yellow. Boxed in gray are the residues that occlude the ATP binding site in the structure of ROP8

but are not conserved in the family. Residues proposed as regulatory phosphorylation sites for ROP18 are boxed and indicated with arrows; the only one conserved to any degree across the alignment is highlighted in blue ($ROP5B_I$ Thr211). Note that this residue is polymorphic in the ROP5 family.

References for Supplemental Materials

1. Reese, M. L., Zeiner, G. M., Saeij, J. P., Boothroyd, J. C. & Boyle, J. P. (2011) *Proc Natl Acad Sci U S A* **in press**.
2. Zhang, Y. & Skolnick, J. (2005) *Nucleic Acids Res* **33,** 2302-9.
3. Sali, A. & Blundell, T. L. (1993) *J Mol Biol* **234,** 779-815.