SUPPLEMENTAL MATERIALS AND METHODS:

Verification of transgene expression by immunofluorescence microscopy. FCU-GFP expression in transgenic parasite lines was verified by immunofluorescence imaging analysis. Mixed stage transgenic *P. falciparum* infected erythrocytes were fixed with 4% formaldehyde/0.0075% gluteraldehyde (Polysciences, Inc.) using the method of Tonkin *et al*. (Tonkin et al. 2004). Fixed cells were permeabilized with 0.01% Triton-x 100 (Sigma-Aldrich) and blocked with 10% normal goat serum and 3% bovine serum albumin (BSA). To detect FCU-GFP localization, the suspension was incubated with a 1:1000 dilution of a mouse anti-GFP antibody (Thermo Fisher Scientific) followed by secondary antibody Alexaflour-488 rat anti-mouse (Molecular Probes) diluted to 1:500. Cells were resuspended in Slowfade Antifade reagent with DAPI to stain parasite nuclei (Thermo Fisher Scientific) and mounted on slides using Flouromount-GTM (Southern Biotech). Fluorescent microscopic images were obtained with an Olympus BX61 system and deconvoluted using SlideBook 5.0 software (Intelligent Imaging Innovations).

Gametocyte generation. To determine the ability of each strain to produce mature gametocytes, a standard protocol for gametocyte induction was utilized (Eksi et al. 2005). In brief, 1% synchronous ring-stage parasites in 5% hematocrit were induced to generate gametocytes by nutrient deprivation. More specifically, 50% of 24 hour spent medium was removed and replaced with fresh medium to initiate gametocyte development. Medium was refreshed 48 hours later and subsequently maintained under standard conditions. Five days post-induction, Giemsa-stained thin-blood smears were assessed for percentage gametocytemia (numbers of gametocytes/numbers of uninfected red blood cells). Gametocyte percentages are plotted for each strain and error bars represent the standard deviation of two independent experiments.

Assessment of FCU-GFP transgene ability to salvage 4-thioluracil via Northern blot. Incorporation of 40µM 4-TU throughout 3D7^{cam} intraerythrocytic development was carried out by incubating highly synchronized ring-, trophozoite- and schizont-stage parasites for one, two and four hours. Incorporation of 4-TU into the total RNA pool was verified by northern blot as described above.

After generation of transgenic FCU-GFP expressing lines F12^{cam} and F12^{pfs16}; ability to incorporate 4-TU was verified by adding 40µM 4-TU to mixed-stage parasite culture and incubating for 12 hours. Incorporation of 4-TU into the total RNA was performed as described above. The length of film exposure time was varied based on the visualization of biotinylation.

Effect of 4-TU on parasite growth. Highly synchronized parasite cultures of 3D7^{cam} (2.5% parasitemia, 5% hematocrit) in the presence of increasing concentrations of 4-TU (0, 20, 40, 80 and 160µM). Growth was monitored by assessing the change in percent parasitemia every 12 hours for 1.5 life cycles by Geimsa-stained thin blood smears. The optimal concentration of 4-TU was decided by the highest concentration that had the least effect on parasite growth as compared to growth in culture medium containing no 4-TU.

LC-MS detection of pyrimidine salvage driven thiol-incorporation. The ability of transgenic parasites to synthesize or salvage pyrimidine was assessed by whole metabolite LC-MS/MS analysis. Parasite cultures (10% trophozoites, 5% hematocrit) were grown in complete medium supplemented with 40µM ^{15}N-uracil for 10 and 30 minutes. Alternatively, FCU-mediated salvage into the pyrimidine pool was determined by incubating wild-type 3D7 and 3D7^{cam} with 40µM ^{15}N-uracil and 24mM ^{13}C-bicarbonate modified complete medium (lacking unlabeled bicarbonate) for 10 min and 30 min in the presence or absence of 10nM atovaquone, an indirect inhibitor or *de novo* pyrimidine synthesis. Whole parasite metabolite extraction was carried out as previously described with minor modifications (Olszewski and Llinas 2013). Briefly, parasites were saponin (0.01%) lysed, collected by centrifugation and washed in ice-cold 1xPBS. Whole metabolites were extracted and stabilized by the addition of 1mL ice-cold 90% methanol to the parasite pellet. The methanol/parasite suspension was centrifuged for 10min at 4°C and supernatant transferred to a clean tube which can be stored at -80°C. Just prior to analysis, metabolite extracts were dried under nitrogen gas flow at room temperature. Following evaporation, samples were reconstituted in 200µl HPLC-grade water and analyzed. Metabolites detected by liquid chromatography-mass spectrometry (LC-MS) were quantified using the MAVEN software program (Clasquin et al. 2012) (version 2011.6.17) which aided in the determination of total UMP pool and proportions that were heavy labeled via *de novo* biosynthesis (^{13}C-UMP) versus pyrimidine salvage (^{15}N-UMP). ^{13}C-UMP and ^{15}N-UMP intensity values were corrected for natural abundance and converted to percentages of total UMP. Unlabeled UMP percentages were normalized to 100%, representing the total pyrimidine pool and the labeled UMP intensity values are represented as a percentage thereof. Labeling was performed in triplicate and the error bars represent the standard deviation of two independent biological replicates.

Growth Inhibition Assay. Genetic supplementation of a *de novo* pyrimidine synthesis metabolic bypass was assessed by growth in the presence of various concentrations of atovaquone. All parasite growth inhibition assays were performed in 96-well plates as described by Smilkstein *et al*. (Smilkstein et al. 2004). 3D7 wild-type and 3D7^{cam} *P. falciparum*-infected erythrocytes at 1.0% initial parasitemia and 4% hematocrit were exposed to various concentrations of atovaquone in the

presence and absence of 40μM uracil for 48 hours. Following drug treatment, plates were placed at −80° overnight and thawed at room temperature to promote cell lysis. Once lysed, parasite cultures were incubated with SYBR green I dye (Sigma) (0.4 μL/mL) in 100 μL of buffer (20mM Tris-HCl, pH 7.5; 5mM EDTA; 0.08% Triton X-100; 0.008% saponin). Fluorescence was quantified using a BioTek Synergy MX plate reader equipped with Gen5 software. Fluorescent quantification using SYBR Green detection of nucleic acids served as a measure of cell proliferation and was plotted as a percentage growth compared to an untreated control using GraphPad Prism 6.

Reverse Transcription and Microarray Analysis. Total RNA, unbound, and bound fractions of mRNA are precipitated by traditional methods (0.1vol NaCl, 0.1vol linear acrylamide and 3vol 100% EtOH) followed by resuspension in 20μl DEPC-treated water. The concentration of each sample is determined by NanoDrop ND-1000. Starting with 1.0-2.5μg of RNA, single-strand aminoallyl-containing cDNA synthesis and Amersham CyDye-coupling (GE Healthcare) was carried out as previously described (Bozdech et al. 2003) with the addition of control RNAs to aid in the normalization between samples and arrays (two color RNA spike-in kit, Agilent Technologies). To prevent Cy5 degradation by ozone (Branham et al. 2007), all steps starting with dye resuspension were carried out in an ozone-free environment. Final cDNA concentration and dye-incorporation was assessed on a NanoDrop ND-1000 spectrophotometer. To reduce sample and time-point bias, cDNA from each time-point was labeled with Cy5 and combined with an equal amount of Cy3-labelled cDNA reference pool generated from equal amounts of ring, trophozoite, and schizont stage mRNA and hybridized to *P. falciparum* custom arrays (Agilent Technologies 60mer SurePrint platform, AMADID #037237 (Kafsack et al. 2012)). Hybridized arrays were incubated for 16h in a rotating hybridization oven (10rpm) at 65 °C. Prior to scanning, arrays were washed in 6X and 0.06X SSPE (both containing 0.005 % N-lauryl-sarcosine (Sigma-Aldrich, St. Louis, MO, USA), followed by an acetonitrile rinse.

Array scanning, data acquisition, and analysis. Arrays were scanned on an Agilent G2505B Microarray Scanner (Agilent Technologies) with 5μm resolution at wavelengths of 532nm (Cy3) and 633nm (Cy5) using the extended dynamic range (10–100%) setting. Normalized intensities were extracted using Agilent Feature Extractor Software Version 9.5 employing the GE2_1100_Jul11_no_spikein extraction protocol and the resultant text files were analyzed using the Rnits (v1.2.0) Bioconductor package in R (Sangurdekar 2014). All array intensity values were background subtracted and normalized within each timecourse and sample (ex: 4-TU labeled, unlabeled mRNA) and converted to Log_2(Cy5/Cy3) ratios. The same data are available on Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under study accession number

GSE72695. Probes excluded in this analysis were those representing the following: Agilent Controls, Cy3 or Cy5 signal below background (<200), *P. falciparum* surface variants (*rifin, stevor, var, pfmc-2tm*), and exogenous controls (Kafsack et al. 2012). All gene values (log_2 ratios) were within-strain median centered and clustered based upon the K10 means of $3D7^{cam}$ and $3D7^{pfs16}$. In total, 5168 genes are represented in the thiol-labeled mRNA pool (transcription) and 5175 genes are represented in the non-thiol-mRNA pool (stabilization) out of 5509.

Intra-strain correlation analyses were carried out by determining the Pearson correlation coefficient of the log_2 ratios of all genes over time between either *cam-fcu-gfp* or *pfs16-fcu-gfp* expressing lines. The Pearson correlation coefficients for both Transcription and Stabilization arrays were plotted over time for each strain. Similarly, inter-strain median Pearson's *r* correlations were calculated between $3D7^{cam}$ and $F12^{cam}$ using mean centered log_2 ratios across the timecourse (0-36h) for each strain.

The full dataset was K10 means clusters within $3D7^{cam}$ and $3D7^{pfs16}$ using Cluster 3.0. Subsequently, all genes in $F12^{cam}$ and $F12^{pfs16}$ were ordered based on the K10 means clustering from 3D7 and arranged in order of peak abundance from 0-36 hpi (Table S1). To determine fold changes of genes specific to parasites with an active *pfs16*-promoter (committed gametocytes), the log_2 ratio from *cam-fcu-gfp* expressing lines were subtracted from the partner *pfs16-fcu-gfp* expressing lines (Fold change = $3D7^{pfs16}(Log_2(Cy5/Cy3))/3D7^{cam}(Log_2(Cy5/Cy3))$). Significant fold changes in were determined based upon a log_2 fold change ≥ 1.0 and percentile ranking of ≥ 95 within 3D7 (Table S2).

Comparison of total transcript abundance throughout the IDC. Data from cDNA microarrays that were hybridized with total RNA extracted from wild-type 3D7, $3D7^{cam}$ and $3D7^{pfs16}$ throughout each timecourse was extracted as described above. Next, the Pearson correlation was determined for each gene using the $Log_2(Cy5/Cy3)$ ratios intensities over time between a previously published 3D7 mRNA abundance DNA microarray time course (Kafsack et al. 2012) and either $3D7^{cam}$ or $3D7^{pfs16}$.

Motif and GO-term enrichment analysis. Identification of motifs enriched in genes identified as stabilized or transcribed (log_2 ratio fold change ≥ 0.05 and p-value ≤ 0.01) in gametocytes was carried out using a regulatory element discovery algorithm (FIRE) (Elemento et al. 2007).

GO-term enrichment of select gene groups or clusters was carried out using the Analysis Tool at

http://www.PlasmoDB.org (Aurrecoechea et al. 2009).

References:

Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, Gao X, Gingle A, Grant G, Harb OS et al. 2009. PlasmoDB: a functional genomic database for malaria parasites. *Nucleic acids research* **37**: D539-543.

Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. 2003. The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*. *PLoS biology* **1**: E5.

Branham WS, Melvin CD, Han T, Desai VG, Moland CL, Scully AT, Fuscoe JC. 2007. Elimination of laboratory ozone leads to a dramatic improvement in the reproducibility of microarray gene expression measurements. *BMC Biotechnol* **7**: 8.

Clasquin MF, Melamud E, Rabinowitz JD. 2012. LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. *Current protocols in bioinformatics / editoral board, Andreas D Baxevanis [et al]* **Chapter 14**: Unit14 11.

Eksi S, Haile Y, Furuya T, Ma L, Su X, Williamson KC. 2005. Identification of a subtelomeric gene family expressed during the asexual-sexual stage transition in *Plasmodium falciparum*. *Molec Biochem Para* **143**: 90-99.

Elemento O, Slonim N, Tavazoie S. 2007. A universal framework for regulatory element discovery across all genomes and data types. *Mol Cell* **28**: 337-350.

Kafsack BF, Painter HJ, Llinas M. 2012. New Agilent platform DNA microarrays for transcriptome analysis of *Plasmodium falciparum* and *Plasmodium berghei* for the malaria research community. *Malaria Journal* **11**: 187.

Olszewski KL, Llinas M. 2013. Extraction of hydrophilic metabolites from *Plasmodium falciparum*-infected erythrocytes for metabolomic analysis. *Methods Mol Biol* **923**: 259-266.

Sangurdekar DP. 2014. Rnits: R Normalization and Inference of Time Series data. *R package version 120*.

Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrobial agents and chemotherapy* **48**: 1803-1806.

Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF, McFadden GI. 2004. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol* **137**: 13-21.