

## Genome-wide identification of genes upregulated at the onset of gametocytogenesis in *Plasmodium falciparum*

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### Abstract

A genome-wide expression analysis was undertaken to identify novel genes specifically activated from early stages of gametocytogenesis in *Plasmodium falciparum*. A comparative analysis was conducted on sexually induced cultures of reference parasite clone 3D7 and its gametocyteless derivative clone F12. Competitive hybridisations on long-oligomer microarrays representing 4488 *P. falciparum* genes identified a remarkably small number of transcripts differentially produced in the two clones. Upregulation of the mRNAs for the early gametocyte markers Pfs16 and Pfg27 was however readily detected in 3D7, and such genes were used as reference transcripts in a comparative time course analysis of 3D7 and F12 parasites between 30 and 40 h post-invasion in cultures induced to enter gametocytogenesis. One hundred and seventeen genes had expression profiles which correlated to those of *pfs16* and *pfg27*, and Northern blot analysis and published proteomic data identified those whose expression was gametocyte-specific. Immunofluorescence analysis with antibodies against two of these gene products identified two novel parasite membrane associated, sexual stage-specific proteins. One was produced from stage I gametocytes and the second showed peak production in stage II gametocytes. The two proteins were named Pfpeg-3 and Pfpeg-4, for *P. falciparum* proteins of early gametocytes.

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### 1. Introduction

The spread of malaria in human populations is caused by the alternate life cycle of its aetiological agent, the protozoan parasites of the genus *Plasmodium*, between human hosts and *Anopheles* mosquitoes. The global efficiency of this process is well summarised by the figures of 300–500 million clinical cases of malaria and 1.1–2.7 million deaths per year, largely caused by *Plasmodium falciparum* in African children [1]. Parasite gametocytes play a central role in this process as they ensure transmission of *Plasmodium* from an

infected individual to the insect. Gametocytes are formed in the human host, where some blood stage parasites cease to multiply asexually and enter the pathway of sexual differentiation. Gametocytogenesis in *P. falciparum* lasts about 10 days, and it is traditionally divided into five morphological stages of maturation [2].

Onset and early events of *P. falciparum* sexual differentiation are still poorly understood events in the parasite life cycle, and are the objects of the present study. Available experimental evidence indicate that during parasite multiplication in the bloodstream some asexual schizonts commit their progeny to develop as gametocytes upon reinvasion [3]. Such sexually committed merozoites invade new red blood cells, and in the first 30–40 h post-invasion distinct molecular

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changes occur in the parasite, which together identify a gametocyte of stage I. While no obvious morphological difference in light or electron microscopy distinguishes this cell from a small asexual trophozoite [4] the early sexual cell actively produces two specific proteins: Pfg27, an abundant dimeric cytosolic phosphoprotein essential for gametocyte formation [5] and Pfs16, a membrane protein localised in the gametocyte parasitophorous vacuole [6,7]. These events are preliminary to the appearance of a network of subpellicular microtubules and the morphological changes leading to the crescent-shaped stage II gametocyte detectable since 48 h p.i. [4]. Besides these events, early sexual stages are otherwise poorly characterised biochemically and physiologically.

Transcriptome analysis, to date carried out on various developmental stages of *P. falciparum* with different microarray platforms [8–11], has been conducted also on gametocytes, resulting in at least a 10-fold increase in the number of identified genes expressed in sexual stages [8,11]. These analyses however invariably recurred to Percoll purified gametocytes, mainly at stages III and IV of maturation, and they were never conducted on the young sexual stages. The present work aimed instead to analyse genome-wide the transcriptional changes associated to induction and formation of early gametocytes in *P. falciparum*. To this aim we utilised the reference parasite clone 3D7 [12], and microarrays containing 7462 long oligonucleotides specific for 4488 of the 5409 ORFs annotated by the *P. falciparum* genome sequencing consortium [10]. This approach had to face two technical difficulties. Unlike described developmental studies in protozoa or fungi [13–16], induction of *P. falciparum* sexual differentiation in vitro is poorly controlled, and it routinely achieves gametocyte conversion rates of 10–20% of the parasite culture. In addition sexually committed schizonts cannot be physically purified, and enrichment of stage I gametocytes requires exposure to drug treatment that we preferred to avoid in our experimental design. For these reasons our experiments, in which gametocytogenesis was induced by growing parasite cultures to high density [17,3], recurred to a control parasite clone unable to produce gametocytes as a source of background asexual mRNA. Gametocyteless clone F12 used here was obtained from 3D7 after long term asexual propagation, and it is unable to produce either morphologically recognisable gametocytes or the early sexual stages expressing the Pfg27 marker [18]. Gene expression was recently compared between asexual forms of the two clones on a collection of 153 parasite genes involved in signalling, cell cycle and transcription. Only eight genes showed minor differences in transcript levels between the two clones, confirming on one hand the isogenic background of these parasites, but failing on the other hand to suggest the nature of the molecular defect of the gametocyteless mutant [19]. The first step of the present investigation was thus to preliminary explore transcriptome differences between 3D7 and F12 in parasite cultures grown at high density, a condition inducing sexual differentiation.

## 2. Materials and methods

### 2.1. Parasite lines and cultivation

*P. falciparum* clones 3D7A [12] and F12 [18] were grown in 0<sup>+</sup> red blood cells in RPMI 1640 plus hypoxanthine 50 µg/ml, supplemented with 10% heat-inactivated 0<sup>+</sup> human naturally-clotted serum, at 37 °C, in a 2% O<sub>2</sub> and 5% CO<sub>2</sub> atmosphere. For parasite synchronisation, cultures at 8–10% parasitaemia at 10% haematocrit were centrifuged at 3000 rpm for 10 min through a 60% Percoll cushion and slow sedimenting schizonts used to invade fresh red blood cells. Morphological analysis of Giemsa stained slides indicated that resulting cultures typically contained >95% ring stages within 90 min of incubation. Cultivation and Percoll gradient purification of stage III–IV gametocytes is described in [20]. Method used to measure gametocyte conversion rates was described in [3]. In brief it consisted in staining parasites 40 h post-invasion with antibodies specific for Msp1 and Pfg27 and measuring numbers of specifically stained asexual schizonts and young gametocytes.

### 2.2. Microarray fabrication and hybridisation

Array fabrication and slide hybridisation were performed as described in [10]. In brief, the DNA microarray contained 7462 70-mer oligonucleotides representing 4488 of the 5409 ORFs annotated by the malaria genome sequencing consortium, and slides were printed in the laboratory of Dr. DeRisi (UCSF, USA). Information on array design is available at MIAME express (<http://www.ebi.ac.uk/MIAMEexpress/>) with accession code A-MEXP-130. For RNA preparations, parasitised red blood cells were harvested in prewarmed incomplete medium, lysed in 0.05% saponin solution in 1xPBS, pelleted, and flash frozen in liquid nitrogen (total time 15 min). Frozen pellets were extracted according to [21], and 10–15 µg of total RNA and oligo-dT<sub>18</sub> primers were used to produce aminoallyl-dUTP (Sigma)-labelled cDNA for chemical coupling to monofunctional Cy3 and Cy5 (Amersham Pharmacia Biotech). Purified cyanine-labelled cDNAs were hybridised on microarray chips in 20 microliters of 3xSSC, 0.5% SDS, 1 µg/µl of polyA DNA (Pharmacia Biotech) as described in [22] in custom made hybridisation chamber incubated for 16–24 h at 68 °C. After washing, fluorescent microarray images were acquired with a GenePix 4000B and analysed by GenePix Pro 3.01 (Axon Instruments, Union City, California, USA). Microarray spots were inspected for size and shape, and passed quality control if their mean pixel intensity was greater than the local background plus two standard deviations for both channels. Array data were median-scaled normalised and only quality-filtered features, with values present in more than 80% of experiments were subjected to the cluster analysis using the Acuity 3.1 software (Axon Instruments, Union City, California, USA). As a further measure of data set quality, correlation coefficients

were calculated for hybridisation values of multiple oligonucleotides for the same genes. Average of correlation coefficients was 0.83 (S.D. = 0.24).

### 2.3. Protein expression and purification

Production of a recombinant fragment of Pfpeg-3 (PFL0795c). Primers n.1 and n.2 in Table I (supplementary data) were used to PCR amplify 378 bp (126 aminoacids) of the *pfpeg-3* coding region which was digested with *Bam*HI–*Not*I, and inserted in frame at the C-terminal portion of the glutathione S-transferase protein encoded in vector pGEX-1 (Amersham Pharmacia Biotech). Recombinant fusion protein, produced in *E. coli* BL21 strain, was attached to a Glutathione-Sepharose column and digested with PreScission™ Protease (Amersham Pharmacia Biotech) in order to cut the Pfpeg-3-specific portion of the fusion protein and release the Pfpeg-3 recombinant polypeptide of approximately 16 kDa from the column. Production of a GST fusion protein with a recombinant portion of Pfpeg-4 (PF10.0164). Primers n.3 and n.4 in Table I (supplementary data) were used to PCR amplify 237 bp (79 aminoacids) of the *pfpeg-4* coding region which was digested with *Eco*RI–*Not*I, and inserted as described above in vector pGEX-1 (Amersham Pharmacia Biotech). Recombinant fusion protein was produced in *E. coli* BL21 strain and was extracted from *E. coli* inclusion bodies by the addition of lysozyme (1 mg/ml), Triton (1%) and Sarkosyl (1.5%) prior to sonication.

### 2.4. Antibodies and immunofluorescence analysis

*Rat immunisation.* Purified 16 kDa Pfpeg-3 fragment, the GST-Pfpeg-4 described above, and GST alone were used to immunise male Sprague–Dowley rats. Each animal received subcutaneous injection of 200 µg of polypeptide in complete Freund adjuvant, after which a second injection of peptide with incomplete Freund adjuvant and two intramuscular injections of the peptide alone followed at 2-week intervals. Blood was eventually collected by cardiac puncture from the anaesthetised animal, and serum was obtained after clotting for 3 h at 37 °C.

Parasites for immunofluorescence analysis were fixed on microscope slides either in acetone or in 4% paraformaldehyde (30 min at room temperature) followed by 10 min incubation in 0.1% Triton X100. Antibodies were then incubated at the indicated dilutions with 1% BSA 0.1% Tween 20, washed in 1xPBS, and incubated with 1:200 dilution of rhodamine- or fluorescein-conjugated affinity purified anti-rat IgG antibodies (Cappell) and 0.1 µg/ml DAPI. After final washes slides were examined with Leitz DMR fluorescent microscope equipped with filters BP 340–380 (DAPI), BP 470–490 (fluorescein), and BP 515–560 (rhodamine), and images merged with Photoshop 5.0 software (Adobe).

### 2.5. Probes and northern analysis

*Polymerase chain reaction amplification (PCR).* PCR amplification reactions were obtained with AmpliTaq Gold polymerase (Perkin-Elmer) in 50 µl reactions with 2 mM Mg, 0.2 mM dNTP and 20 pmol of each primer after target DNA denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 68 °C for 2 min, and a final elongation at 68 °C for 7 min. Table I (supplementary data) contains the list of primers used to amplify various fragments used as probes for Northern blot analysis. Total RNA extracted as described above or with the RNeasy kit (QIAGEN) was separated through 1% agarose and formaldehyde gel and blotted by standard methods onto Extra-C nitrocellulose (Amersham). <sup>32</sup>P-labelled DNA probes were hybridised overnight in 0.5 M phosphate buffer 7% SDS at 58 °C and washed in 1xSSC 0.1% SDS at the same temperature before autoradiography. Sizes of PCR fragments obtained from genomic DNA for the various genes were consistent with the sizes expected from the genome sequence information, and they were as follows: PF14\_0108 669 bp; PF10\_0164 313 bp; PFL0795c 660 bp; PF11\_0040 (pfsep11.1) 278 bp; PF11\_0477 462 bp; PF13\_0161 795 bp; PFI0915w 290 bp, PFB0685c 616 bp.

## 3. Results

### 3.1. Pairwise comparison of 3D7 and F12 transcriptomes.

Three parallel pairs of cultures (biological replicates) were obtained for 3D7 and F12, and grown to high parasitaemia. 3D7 cultures contained asexual parasites and variable fractions of young (mainly stage I) gametocytes, with conversion rates ranging between 10 and 20%. Parallel cultures of clone F12 failed instead to produce Pfg27-positive young sexual stages, and parasites exhibited only asexual morphology. RNA samples from each pair of cultures were used in microarray competitive hybridisation experiments in order to explore transcriptional differences between the parasite clones, and to identify genes specifically expressed in the 3D7 parasite subpopulation entering sexual differentiation. cDNAs from one replicate (R1) were used in a dye swap experiment, while in the other replicates (R2, R3) 3D7 cDNA samples were labelled with Cy5 and F12 samples with Cy3. At least two microarray hybridisations per replicate were produced and analysed as described in the experimental procedures (average correlation coefficient for the technical replicates = 0.71 ± 0.08), and expression ratios were obtained for 832 genes in replicate R1, 2365 in R2 and 2885 in R3.

Established an arbitrary threshold of two-fold difference in transcript abundance, a total of 78 genes were upregulated in 3D7 in all experiments, of which however only eight resulted reproducibly upregulated in at least two out of three replicates (Table II, supplementary data). Despite such high

biological variation between replicates, the genes encoding the early gametocyte markers *Pfg27* (PF13\_0011) and *Pfs16* (PFD0310w) were readily detected as upregulated, and were present amongst the eight genes. The remaining six coded for two heat shock proteins (PF10875, PF08\_0054), three non-annotated hypothetical proteins (PF10\_0164, PF14\_0744, PF13\_0338), and one member of the PfSep protein family [23] (PF11\_0040). The entire data set was also inspected for expression levels of genes encoding known sexual stage-specific proteins which appear at later stages of gametocyte maturation, such as *Pfs45/48* and *Pfs230*, produced since stage II gametocytes [24], *Pftubulin- $\alpha$ II* and *Pfg377*, since stage III [25,26], *Pfs230II*, specific for stage V male gametocytes [27], and *Pfs25*, appearing in gametes [28]. This analysis showed that none of these mRNAs was upregulated in 3D7 parasites (Table III, supplementary information), which confirmed morphological observations that sexual differentiation was still at an early phase in the 3D7 cultures analysed and, importantly, that mRNAs from mature gametocytes were not significantly contributing to the RNA pool obtained from 3D7 parasites.

In summary these experiments indicated that 3D7 and F12 parasites have remarkably similar transcriptomes in the gametocyte inducing conditions used here, and that 3D7 does not produce a large set of reproducibly upregulated genes compared to F12. On the other hand this analysis readily detected mRNA specific upregulation of both the early gametocyte markers *pfs16* and *pfg27* in the fraction of parasites entering sexual differentiation, probably given the abundance of these transcripts in young gametocytes [29,30]. This result thus validated the use of *pfg27* and *pfs16* transcripts as reliable references in microarray analysis of early sexual stages, but suggested to recur to a more sensitive experimental design to detect gene expression specific for early sexual development.

### 3.2. Comparative time course analysis of high parasitaemia cultures of 3D7 and F12

A time course experiment was thus designed to analyse parasite cultures developing in the first 30–40 h after red blood cell invasion, a moment in which differentiation of asexual schizonts and of stage I gametocytes occurs. Three parallel time courses were analysed. Percoll purified schizonts of 3D7 and F12 were used to start two synchronous cultures, which were sampled from 30 to 40 h p.i. (cultures 3D7-1 and F12-1 in Fig. 1A and B). As however such highly synchronous cultures routinely show lower gametocyte conversion rates compared to asynchronous parasites (conversion rate for the above 3D7-1 culture was 4%), a second 3D7 culture with higher gametocyte production was included in the experiment. Culture 3D7-2 was started as described above, but parasites underwent two subsequent asexual cycles, and RNA samples were obtained between approximately 30–40 h after the second erythrocyte invasion (Fig. 1, panel C). At these time points higher numbers of stage

I gametocytes were present, and stage II gametocytes from the previous cycle were also detectable (data not shown).

cDNAs from all time points were Cy5-labelled and hybridised against aliquots of a reference cDNA pool obtained mixing the above points, yielding a total of 13 microarray hybridisations. Data of these microarray experiments were submitted to MIAMEexpress database (<http://www.ebi.ac.uk/MIAMEexpress/>). Log<sub>2</sub> transformed expression ratios were calculated for 2217 array elements, representative of 1810 predicted genes, and expression profiles of these genes across the three time courses were obtained. A hierarchical clustering algorithm, based on an uncentered Pearson correlation matrix, was used to organise correlated expression profiles in a tree structure [31], and the minimal correlation node which contained expression profiles from the array elements specific for *pfg27* and *pfs16* was thus identified (Fig. 1D, asterisk). In this node 143 array elements were present, representative of 119 genes whose expression profiles showed an average correlation of 0.91 (Table IV, supplementary data). In culture 3D7-1 relative abundances of these mRNA were initially low but showed a marked increase around 40 h p.i. (Fig. 2A, time points a to d in 3D7-1). In culture F12-1 levels of these transcripts remained constantly low at all time points (Fig. 2A, e to h in F12-1). Finally, in gametocyte enriched culture 3D7-2 levels of these mRNAs were comparatively higher over the background reference, and tended to increase with time (Fig. 2A, i to m in 3D7-2). Representative hybridisation signals for some of the 143 array elements are shown in Fig. 2B.

The same data set was analysed with the independent approach of non-hierarchical *k*-medians clustering [32], applying the same criterium of finding the minimum correlation set including all array elements for *pfs16* and *pfg27*. Gap statistics analysis [33] estimated an optimal cluster number of 14, and indicated that one of them contained *pfg27* and *pfs16* with additional 149 array elements, representing 122 genes. 94 of these were clustered together also in the above hierarchical approach (Table IV, supplementary data), which indicated a robust correlation between expression profiles for most of the genes identified in this analysis.

In conclusion the comparative time course analysis presented here detected a group of parasite transcripts specifically upregulated between 30 and 40 h after merozoite invasion only in 3D7 parasites and not in F12 gametocyteless parasites. The fact that their expression profiles clustered with those of genes *pfg27* and *pfs16*, and that they were upregulated in a culture enriched for young gametocytes suggested that their expression was associated to early sexual differentiation.

### 3.3. Analysis of the gene cluster upregulated in 3D7 parasites

Besides *pfg27* and *pfs16*, only 32% of the 117 genes identified above were functionally annotated according to Gene Ontology classification, as reported in the PlasmoDB



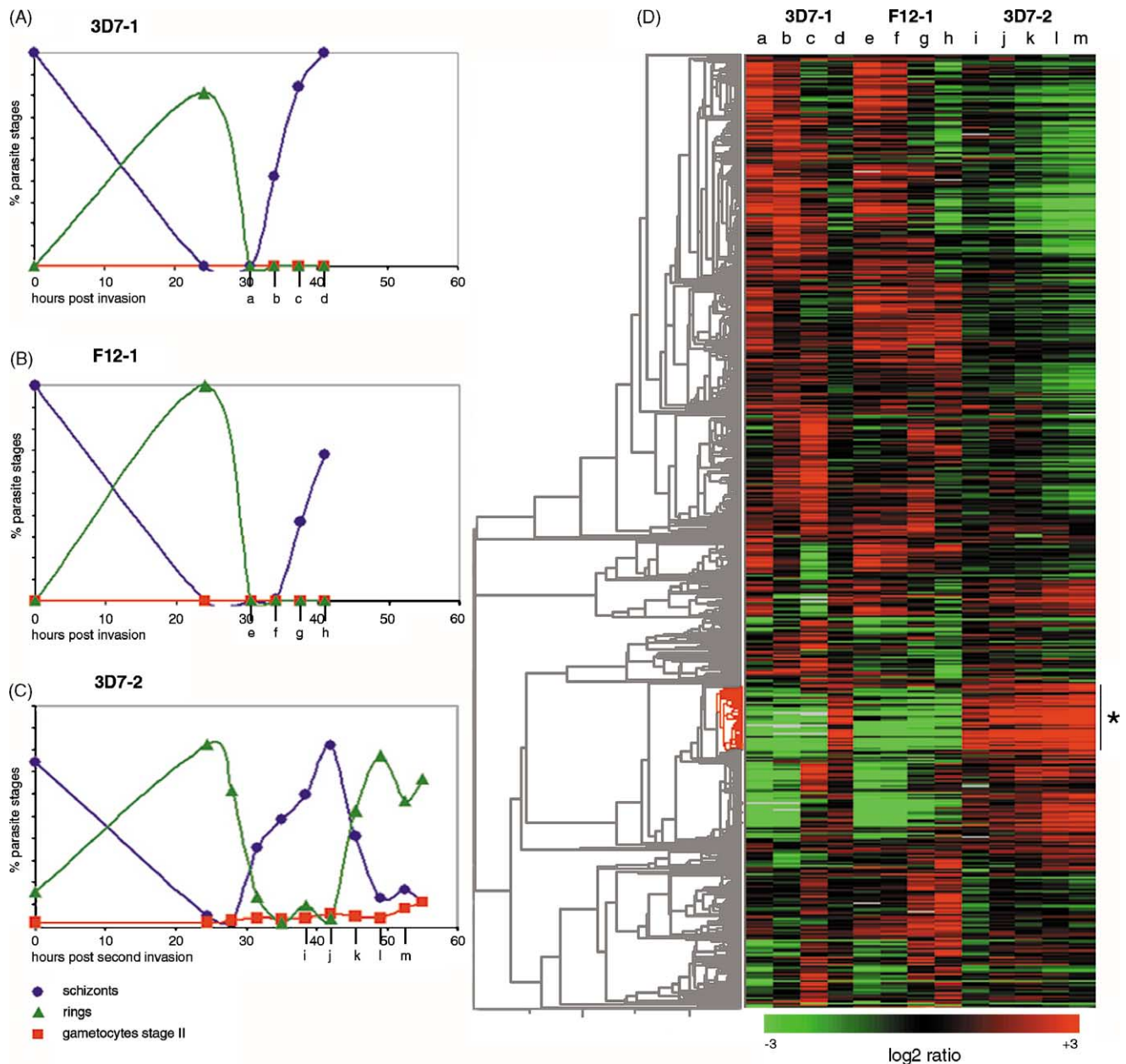


Fig. 1. Comparative time course experiment. (A–C) Description of cultures 3D7-1, F12-1 and 3D7-2 from synchronisation to times points of RNA sampling (indicated by letters under the *x*-axis). Parasitaemias of cultures 3D7-1, F12-1 and 3D7-2 were 7.5, 13, and 11%, respectively. Symbols for parasite stages are red squares: morphologically recognisable stage II gametocytes; blue circles: schizonts; green triangles: ring forms. (D) Tree diagram showing result of cluster analysis on 2217 expression profiles from the 13 time points, indicated above the diagram. The tree node highlighted in red and marked by an asterisk contains the expression profiles of the long-oligomers specific for *pfg27* and *pfs16* plus 141 additional array elements.

database (release 4.3, November 2004), or in the KEGG Genes Database (release 32.0+/12-03, December 2004), while the vast majority were putative hypothetical proteins lacking homologous counterparts in protein sequence databases (Table IV, supplementary data). No distinct functional class of genes was overrepresented amongst the annotated sequences to suggest that a specific cellular or metabolic process was upregulated in 3D7 or defective in F12 in the time course analysed. The presence in this cluster of individual annotated genes however suggested possible involvement in

early sexual differentiation of specific molecular processes. Presence of a putative diacylglycerol kinase (PFI1485c) and a putative cAMP-specific 3'/5'-cyclic phosphodiesterase (MAL13P1.119) was for instance noticeable as the former class of molecules is implicated in differentiation processes [34], while the cyclic AMP-dependent pathway was specifically proposed to be involved in *Plasmodium* sexual differentiation [35]. Presence of three putative transcription factors (PF11\_0477, MAL7P1.86 and PFB0290c) was also noticeable because, despite their annotation as general tran-

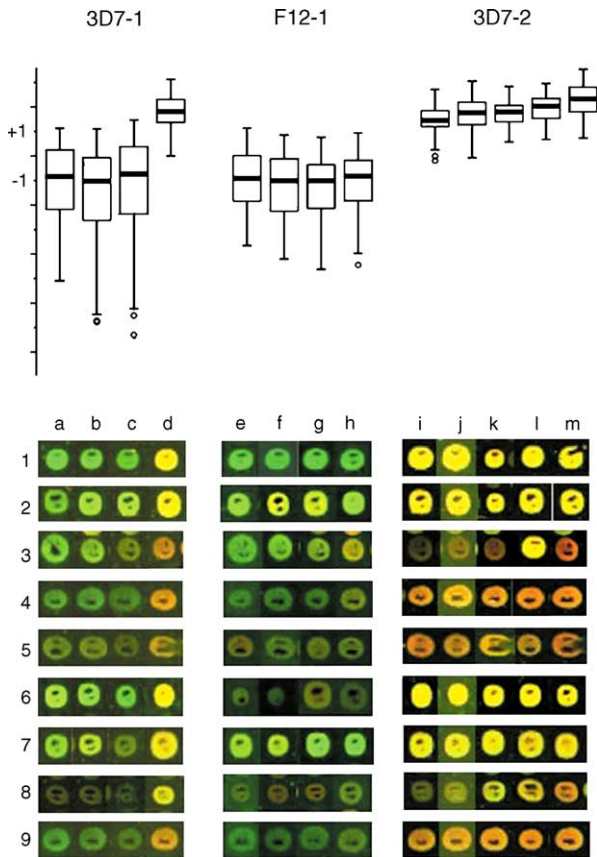


Fig. 2. Expression profiles and representative microarray hybridisation signals of genes clustered with *pfg27* and *pfs16*. (A) Box plot of the average expression profiles for the 143 array elements clustered with *pfg27* and *pfs16* in the time course experiment of Fig. 1. The y-axis reports the log<sub>2</sub> values of the Cy5/Cy3 ratios. (B) Hybridisation signals for nine representative oligonucleotide spots from the above cluster, aligned under the corresponding time points. Spots were randomly located within the microarray chip. Identity of the array elements and corresponding genes are 1: M32775.1 (PF13.0011); 2: D49176.46 (PFD0310w); 3: N150.33 (PF14.0108); 4: F28964.1 (PF13.0161); 5: Ks66.2 (PF11.0477); 6: Ks75.15 (PF11.0040); 7: J425.3 (PF10.0164); 8: F36721.5 (MAL7P1.86); 9: L2.280 (PFL0795c).

scription factors, their presence in the cluster might suggest that these molecules play instead specific, possibly regulatory, roles in governing gene expression in sexual differentiation. Genes were also noticed whose presence could be related to the major cellular transformation observed in early gametocytes [4], such as proteins catalysing phosphorus metabolism (five kinases, two transferases and three phosphatases), two myosins, one myosin interacting protein and one actin-binding protein. This group of gene products might suggest a role for signalling and post-translational modifications in the major cytoskeletal rearrangements accompanying transition from stage I to stage II gametocytes. Finally, presence of three of the seven members of the *P. falciparum* *sep* gene family, encoding conserved proteins transported to the erythrocyte cytoplasm both in the rodent and the human parasites [23] might suggest involvement of protein trafficking in such rearrangements. Other annotated genes, however, encoded molecules, which do not play obvious roles in sex-

ual differentiation, such as three merozoite surface proteins and one erythrocyte and one reticulocyte binding antigens. Although it might be tempting to speculate that presence of the latter is related to the observed increased efficiency of gametocyte production in young red blood cells [36], differential expression of such transcripts are more likely due to parasite clone-specific gene expression or uncontrolled environmental differences between the asexual parasites in the 3D7 and F12 cultures.

Although some functional clues could be derived from the annotation of the genes identified in the above experiment, this analysis indicated that deeper insights into physiology and specific molecular mechanisms governing parasite early sexual differentiation will require functional characterisation of the hypothetical, non-annotated gene products identified here.

#### 3.4. Characterisation of novel gene products specifically upregulated at the onset of gametocytogenesis

Since the main objective of this study was to identify novel sexual-stage specific genes whose expression started in early gametocytes, an assumption was made that such genes were likely to express their sexual stage-specific products also at later stages of gametocyte maturation. *P. falciparum* stage-specific proteome data (available for 80 out of 119 genes) were thus inspected revealing that 18 genes specifically produced polypeptides in mid-stage gametocytes [37,38]. A survey on eight of such genes was then conducted to analyse their stage-specific pattern of mRNA production in asexual parasites of 3D7, and in stage III–IV gametocytes, equivalent to those used for the above proteomic analysis. Northern blot results showed that six out of eight genes (PFL0795c; PF10.0164; PF13.0161; PFI0915w; PF14.0108, PFB0685c) were transcribed specifically or predominantly in sexual stages, while two (PF11.0040; PF11.0477) produced mRNA also in asexual parasites, indicating that stage-specificity of expression of this group of genes was in most cases controlled at the level of mRNA abundance (Fig. 3). Observation that genes first identified for their coexpression with *pfg27* and *pfs16*, were specifically transcribed and translated in maturing gametocytes, strongly suggested that their expression started since the early phase of gametocytogenesis.

This hypothesis was tested for two of them, PFL0795c and PF10.0164, by using antibodies raised against the respective recombinant proteins on asexual parasites and gametocyte at different stages of maturation. The above genes were selected also because both putative gene products contained a signal sequence, and one of them – PF10.0164 – a transmembrane region, suggesting that they coded for novel secreted or membrane associated proteins specific of the early gametocytes.

Immunofluorescence analysis indicated that the anti-serum specific for PFL0795c reacted only with gametocytes, and not with asexual blood forms (Fig. 4, panels 1a and c). Importantly, anti-PFL0795c antibodies specifically reacted

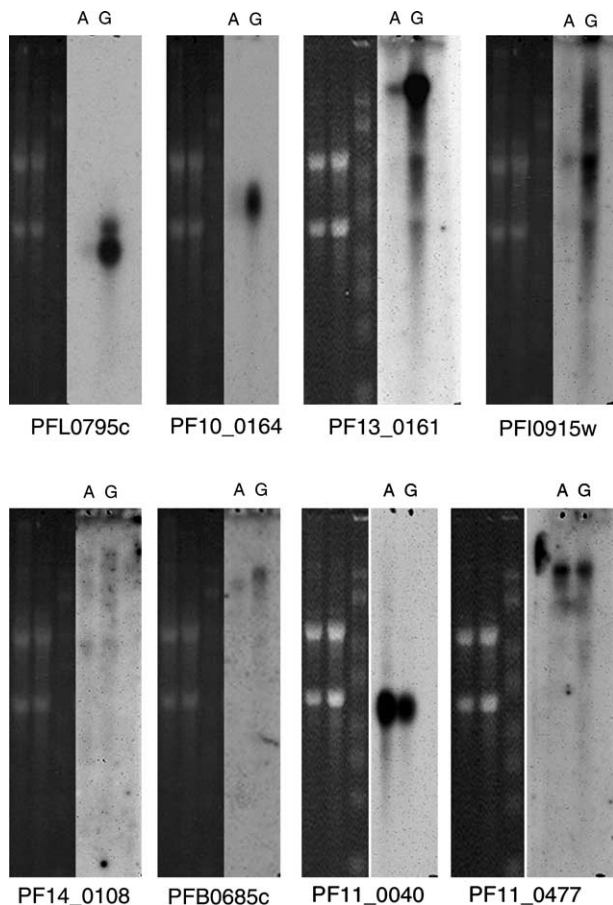


Fig. 3. mRNA production in 3D7 asexual parasites and mid-stage gametocytes for eight genes from the *pfs16/pfg27* cluster, which showed gametocyte-specific product in proteomic analysis. Panels show ethidium bromide stained agarose gels with identical samples of total RNA from 3D7 asynchronous asexual parasites (lanes A), and from Percoll-purified stage III–IV gametocytes (lanes G), and autoradiographies of the respective nitrocellulose filters hybridised with PCR fragments specific for the genes indicated under each panel. RNA molecular marker is the 0.1–1 kb Perfect RNA<sup>TM</sup> Markers (Novagen).

with the small, round shaped stage I gametocytes, whose identification was confirmed with anti-Pfg27 antibodies in double immunofluorescence experiments (Fig. 4, panels 1a, b and c). Fluorescent reaction on the more mature stage III and IV gametocytes indicated that the PFL0795c gene product persisted throughout gametocyte maturation. The pattern of fluorescence on the sexual cells indicated that the protein was associated to membranous structures surrounding the parasite, possibly representing the gametocyte surface or the parasitophorous vacuole membranes (Fig. 4, panels 2, 3 and 4). This analysis in summary showed that the PFL0795c gene product is sexual-stage specific, starts to be produced in stage I gametocytes, and it is present throughout gametocyte maturation.

Immunofluorescence analysis with antibodies specific for the PF10.0164 gene product showed that also this protein was produced only in gametocytes and not in asexual stages (Fig. 4, panel 5a, b and c). Unlike the above case, stage I game-

toocytes were almost negative to the anti-PF10.0164 specific serum. The brightest fluorescence was instead specifically observed on stage II gametocytes (Fig. 4, panels 6a and b), and it was comparatively weaker on more mature sexual stages (Fig. 4, panel 5a, b and c). Staining of the anti-PF10.0164 serum exhibited a distinctive granular pattern on and around the gametocytes, indicating that the protein is compartmentalised in distinct subcellular structures of the gametocytes. This analysis showed that the PF10.0164 gene product is a novel sexual-stage specific protein, exhibits a peak of production in stage II gametocytes, and it is localised in subcellular structures precedently undescribed in this stage of gametocyte formation. Based on the above results we propose here to name the genes PFL0795c and PF10.0164 and their products as *P. falciparum* protein of early gametocytes, *Pfpeg-3* and *Pfpeg-4*, respectively, to indicate that they represent the third and fourth protein, beyond Pfs16 and Pfg27, described to be produced from early gametocytogenesis.

#### 4. Discussion

The work presented here applied for the first time a genome-wide approach to specifically identify *P. falciparum* transcripts upregulated at the onset of gametocytogenesis, and resulted in the discovery of two novel gametocyte-specific gene products expressed since stages I and II of gametocyte maturation.

Focussing microarray analysis on this specific window of parasite development required to adjust microarray experimental design to compensate for the low gametocyte conversion rates achievable in *P. falciparum* cultures. Significant gametocyte production in culture can be obtained exposing parasites to drug treatment [39], or clearing residual asexual parasites by a 48 h exposure to *N*-acetyl-glucosamine [40]. In the present analysis however we decided to avoid pharmacological interference with parasite metabolism, nor could we utilise the *N*-acetyl-glucosamine protocol, as its time scale was incompatible with analysis of early events in gametocytogenesis. The present experimental design thus analysed cultures of the gametocyte producer parasite clone 3D7 in which a fraction of parasites was undergoing sexual differentiation, and recurred to a gametocyteless 3D7 derivative clone as a background reference for gene expression associated to asexual parasites. Unlike other described parasite lines or clones defective in sexual differentiation, either affected in late stages of gametocyte maturation [41] or allowing for minimal residual production of gametocytes [18], the defective clone F12 used here does not produce any morphologically recognisable gametocytes nor the early sexual stages detectable with anti-Pfg27 antibodies [18].

The first general observation from the present study is that remarkably few differences in gene expression are observed between these parasite clones grown under conditions triggering sexual differentiation. This result might suggest that commitment and early events in gametocytogenesis recur



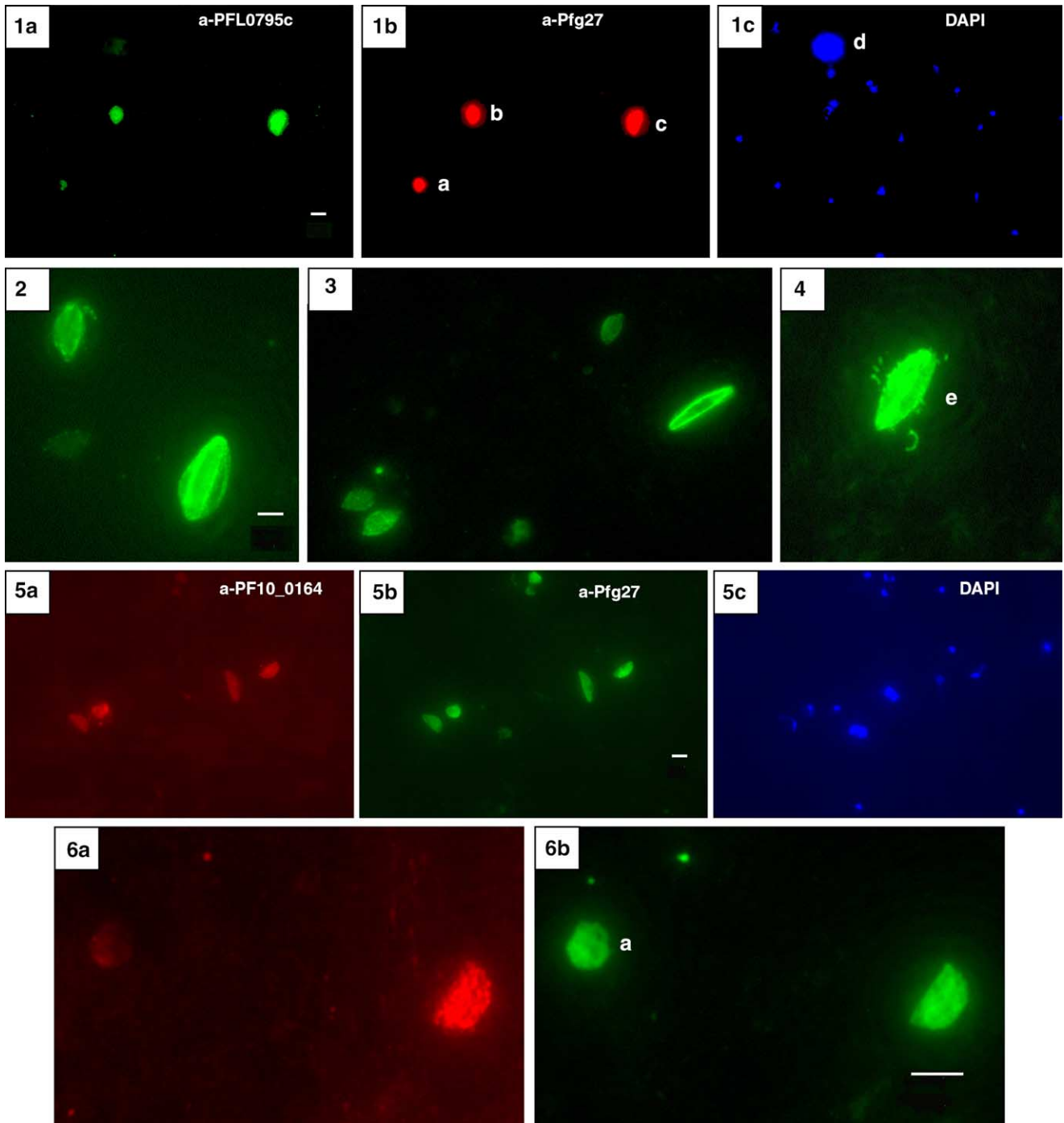


Fig. 4. Immunofluorescence analysis on 3D7 parasites with antibodies specific for PFL0795c and PF10.0164 recombinant proteins. Panels 1a to c show the same field of an acetone-fixed parasite smear reacted with: (1a) rat polyclonal antibodies against PFL0795c (green fluorescence), (1b) rabbit polyclonal anti-serum against Pfg27 (red fluorescence) and (3) DAPI nuclear staining. In these panels, a and b mark a young and an old stage I gametocyte, respectively, c a stage II gametocyte, and d indicates a schizont stained only by DAPI. Panels 2–4 show paraformaldehyde-fixed stage III gametocytes reacted with the anti-PFL0795c serum. Panels 5a to c shows the same field of an acetone-fixed parasite smear reacted with: (5a) rat polyclonal antibodies against PF10.0164 (red fluorescence), (5b) rabbit polyclonal antibodies against Pfg27 (green fluorescence) and (5c) DAPI. Anti-PF10.0164 serum specifically reacts on sexual stages and not with the multinucleated schizonts detectable only by DAPI in panel 5c. Panel (6a): stage I and II gametocyte reacted with anti-PF10.0164 serum. Stage II gametocyte shows the granular pattern of PF10.0164-specific fluorescence. Both gametocytes are detected by anti-Pfg27 antibodies in panel 6b. All bars are 4  $\mu$ M.



predominantly to post-transcriptional control of gene expression. In an alternative interpretation, however, this could propose that F12 parasites might not be totally defective in sexual differentiation, but could be affected at a very early developmental step, resulting in limited transcriptome differences with the parental clone. No molecular marker for such an early event in gametocytogenesis is however available to distinguish between the two hypotheses.

The reproducible detection of both *pfg27* and *pfs16* mRNAs between the genes upregulated in 3D7 indicate that these rank amongst, or possibly are, the most abundant gametocyte-specific genes activated in stage I gametocytes. The described repertoire of specific genes specifically activated since such an early stage of differentiation is otherwise very limited, and it includes, to our knowledge, only the genes for RNA binding proteins Pfpu1 and Pfpu2 [42]. In our microarray experiments signals from such genes were too close to background levels to be detected as upregulated, probably because young gametocytes were invariably only a fraction of the 3D7 cultures in our experiments, and only most abundant transcripts from this subpopulation were likely to be detected. Indeed *pfpu* mRNAs, readily detectable by Northern blots in mid and late gametocytes, were comparatively less abundant in stage I and II gametocytes [42].

The novel gametocyte-specific genes described here – *pfpeg-3* and *pfpeg-4* – expand the number of developmental markers for this relatively uncharacterised part of the parasite life cycle, and are likely to provide novel molecular information on early events in gametocytogenesis. Protein Pfpeg3 is expressed since stage I gametocytes and persists throughout sexual development, associated in mid stage gametocytes to a membrane compartment surrounding the parasite. Protein Pfpeg4 is maximally produced in stage II gametocytes, which contains high numbers of Pfpeg4-positive granules, although a few granular structure per cell are detectable also in late phase of stage I gametocyte formation (data not shown). Since similar structures were never molecularly or morphologically described in gametocytes at such young stages of maturation, further investigation on Pfpeg4 localisation is expected to give insights into the extensive cellular modifications occurring at this moment of gametocytogenesis.

The comparative time course on 3D7 and F12 parasites induced to sexual differentiation, identified a cluster of 117 genes upregulated in 3D7, whose expression profiles strongly correlated with those of *pfg27* and *pfs16*. In this experiment young gametocytes were a minor subpopulation of 3D7 parasites, and consequently such genes could in principle represent either gametocyte-specific genes truly co-regulated with *pfg27* and *pfs16* or alternatively genes upregulated also, or possibly only, in the asexual forms of clone 3D7. In the present work we focussed attention only on gametocyte-specific genes, even though we are aware that some of the genes upregulated in 3D7 asexual parasites might be of potential interest, as they could be involved in predisposition and commitment of such stages to sexual differentia-

tion. Inspection of available proteomic data for 80 of the 117 genes indicated that 18 produced the respective proteins only in gametocytes, and a Northern blot survey on eight of them indicated that gametocyte-specific expression was controlled at the level of transcript abundance in six cases out of eight. The remaining two genes were instead transcribed also in asexual parasites of clone 3D7, suggesting that their upregulation in the comparative time course was parasite clone-specific, rather than young gametocyte-specific. Although proteomic data were absent for a third of the analysed genes, thus possibly underestimating the actual number of gametocyte-specific genes in this cluster, this analysis nevertheless indicated that upregulation of *pfg27* and *pfs16* at the onset of sexual differentiation is accompanied by the induction of comparable magnitude of a limited group of gametocyte-specific transcripts. This suggests that the set of sexual-stage specific genes active in early gametocytes is more limited, and possibly different, from that specifically activated in more mature gametocytes. This hypothesis is supported by comparison of our data to those derived from analysis of the specific transcriptome of stage III–IV gametocytes reported in Le Roche et al., 2003 [11], which finds an overlap of only five genes between the two gene sets (PFD0310w; PF13\_0269; MAL13P1.119; PFI0915w; PFL2205w). Technically, this observation confirms that upregulated transcripts detected in this work did not derive from subpatent late gametocytes in the 3D7 cultures analysed, while it suggests that they were induced in young developing gametocytes. More importantly the above comparison suggests that gene expression in early sexual differentiation is substantially different from that of gametocytes in the mid-late phases of maturation. This is consistent with several observations, such as that metabolic and cytoadhesive properties of early sexual stages are distinct from those of late gametocytes [44–46], that proteins contributing to gametocyte sex dimorphism appear only from stage III of maturation [26], and that specific isoforms of constitutive cellular components like the sexual stage-specific  $\alpha$ -tubulin, actin, and large ribosomal RNA molecules appear in mid-stage gametocytes [25,47,48]. Transcriptome analysis presented here thus suggests on one hand that *P. falciparum* sexual stages largely utilise constitutive molecules in the very early phases of differentiation. On the other hand the present work and previous evidence indicate that young gametocytes express specific proteins either in significant abundance, such as Pfg27 and Pfs16, or with possible regulatory functions, such as Pfpu1, Pfpu2 [42] or the upregulated protein modifying enzymes identified here. This suggests that constitutive molecular components of the young gametocyte might be therefore regulated and modified in the earliest phases of sexual development by a set of specific molecules in order to properly drive the subsequent specialisation of the mature gametocyte. Investigation on these specific molecules will elucidate molecular mechanisms active in this still poorly described phase of malaria parasite differentiation, with the objective to design novel practicable strategies to interfere with parasite transmission.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2005.04.015.

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