GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing

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Abstract

Malaria is caused by *Plasmodium* parasites that proliferate in the bloodstream. During each replication cycle some parasites differentiate into gametocytes, the only forms able to infect the mosquito vector and transmit malaria. Sexual commitment is triggered by activation of AP2-G, the master transcriptional regulator of gametocytogenesis. Heterochromatin protein 1 (HP1)-dependent silencing of *ap2-g* prevents sexual conversion in proliferating parasites. Here, we identified *Plasmodium falciparum* gametocyte development 1 (GDV1) as an upstream activator of sexual commitment. We found that GDV1 targeted heterochromatin and triggered HP1 eviction thus de-repressing *ap2-g*. Expression of GDV1 was responsive to environmental triggers of sexual conversion and controlled via a *gdv1* antisense RNA. Hence, GDV1 appears to act as an effector protein that induces sexual differentiation by antagonizing HP1-dependent gene silencing.

Heterochromatin protein 1 (HP1) is a conserved regulator of heterochromatin formation, heritable gene silencing and variegated gene expression (1). In *Plasmodium falciparum*, HP1-dependent clonally variant expression allows parasites to adapt rapidly to environmental challenges encountered during infection (2–4). For example, immune evasion via antigenic variation of *var*/PfEMP1 is the hallmark of *Plasmodium* survival. Other processes, such as expression of red blood cell (RBC) invasion ligands or nutrient transporters, are similarly regulated in this parasite (4). Most clonally variant genes cluster in subtelomeric domains but some also occur in chromosome-internal heterochromatic regions. In addition, HP1 forms microdomains at some euchromatic genes (2). One of these encodes the transcription factor AP2-G that is required for sexual conversion and differentiation (2, 5–7). HP1-dependent regulation of *ap2-g* controls the rate at which parasites commit to sexual differentiation (7).

To explore the mechanisms regulating HP1 occupancy in *P. falciparum* we identified HP1-interacting proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of native HP1 complexes that were purified by co-immunoprecipitation (co-IP)

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from parasites expressing GFP-tagged HP1 (7) (Fig. 1A and Table S3). Interestingly, we consistently observed GDV1 among the potential HP1 interaction partners (Table S1). GDV1 is a nuclear protein implicated in sexual commitment and early gametocytogenesis but its exact function remains unknown (8). We therefore created a parasite line for the conditional expression of fluorescently labelled ectopic GDV1 (GDV1-GFP-DD) (Fig. 1B). Proteins tagged with the immunophilin protein-folding chaperone FKBP destabilisation domain (DD) are proteolytically degraded unless cells are cultured in presence of Shield-1, a small molecule ligand stabiliser (9, 10). Thus, GDV1-GFP-DD is barely detectable in parasites cultured in absence of Shield-1 (3D7/GDV1-GFP-DD^{OFF}), but its expression is markedly induced in parasites grown in presence of Shield-1 (3D7/GDV1-GFP-DD^{ON}) (Fig. 1, B and C). In agreement with the co-IP results GDV1-GFP-DD co-localizes with HP1 at the nuclear periphery (Fig. 1C and Fig. S1). Furthermore, we found that recombinant HP1 and GDV1 formed a complex (Fig. S1) and that HP1 co-purified with GDV1-GFP-DD in reverse co-IPs (Fig. 1D, Tables S2 and S4). The chromodomain-helicase-DNA-binding protein 1 (CHD1) and a protein of unknown function (PF3D7_1451200) also consistently co-purified with both HP1 and GDV1-GFP-DD (Tables S1 and S2). Given that CHD1 plays important roles in cell fate decision and heterochromatin remodelling in other organisms (11, 12) and that GDV1 is implicated in gametocytogenesis (8) it appears that this putative regulatory complex may function in activating sexual commitment.

Malaria parasites proliferate by iterative rounds of intra-erythrocytic replication through schizogony, merozoite release and RBC re-invasion. The decision to enter gametocytogenesis is made in the cell cycle prior to sexual differentiation; sexually committed schizonts release merozoites that invade RBCs and differentiate all into either female or male gametocytes (13, 14) (Fig. 2A). To test if GDV1 triggers sexual commitment, 3D7/GDV1-GFP-DD^{OFF} parasites were split and cultured in the absence or presence of Shield-1. After re-invasion, stage I gametocytes were quantified by immuno-fluorescence assays (IFA) using antibodies against the gametocyte marker Pfs16 (15). Strikingly, the 3D7/GDV1-GFP-DD^{ON} population displayed a sexual conversion rate of 57.2% (+/- 10.0 SD) compared to 11.0% (+/- 2.4 SD) in 3D7/GDV1-GFP-DD^{OFF} parasites, and these gametocytes differentiated normally into both male and female gametocytes and showed a typical female-biased sex ratio (Fig. 2B and Fig. S2). Moreover, Shield-1 titration revealed a positive correlation between ectopic GDV1-GFP-DD expression levels and sexual conversion rates (Fig. 2C). To test if endogenous GDV1 levels similarly correlate with gametocyte conversion we used CRISPR/Cas9-based gene editing to append a triple hemagglutinin (HA) tag to the N-terminus of GDV1 (3D7/3xHA-GDV1) (Fig. S3). Endogenous 3xHA-GDV1 co-localised with HP1 as expected (Fig. 2D and Fig. S3) but was only expressed in some parasites. We next quantified 3xHA-GDV1 expression under conditions that either suppress or favour sexual conversion. To this end, we made use of the recent discovery of choline as an inhibitor of sexual commitment (16). 3D7/3xHA-GDV1 parasites cultured in the presence or absence of 2 mM choline displayed sexual commitment rates of 1.8% (+/- 0.3 SD) or 30.9% (+/- 3.8 SD), respectively (Fig. 2E). Strikingly, parasites cultured in the absence of choline showed markedly increased 3xHA-GDV1 expression levels (Fig. 2F). This was accounted for by a higher proportion of 3xHA-GDV1-positive cells (48.6% (+/- 3.4 SD) in absence compared to 16.4% (+/- 1.8 SD) in presence of choline)

(Fig. 2F) and comparatively higher 3xHA-GDV1 expression levels in individual 3xHA-GDV1-positive parasites (Fig. S3). Together, these results show that GDV1 activates sexual conversion in a dose-dependent manner and that endogenous GDV1 expression can be induced by environmental signals triggering sexual commitment.

We next performed comparative transcriptome analyses using two-colour microarrays. 3D7/GDV1-GFP-DD^{OFF} ring stage parasites were split, cultured separately in absence or presence of Shield-1 and total RNA was harvested at seven paired time points spanning the remaining 24 hours of generation 1 (24-32 hours post-invasion (hpi); 32-40 hpi, 40-48 hpi) and the first 40 hours after re-invasion in generation 2 (8-16 hpi, 16-24 hpi, 24-32 hpi, 32-40 hpi) (Fig. 2A). As expected, GDV1-GFP-DD expression triggered a transcriptional response characteristic of sexual commitment and early differentiation. This was evident from the induction of *ap2-g* in generation 1, followed by activation of early gametocyte markers (5, 7, 8, 17) after re-invasion (Fig. 2G, Fig. S4 and Table S5). In F12 parasites, a 3D7-derived gametocyte-deficient clone carrying a loss-of-function mutation in *ap2-g* (5, 18), GDV1-GFP-DD expression still activated *ap2-g* but failed to launch a sexual differentiation response (Fig. 2H and Table S6). Next to *ap2-g* only eight other genes were significantly induced in F12/GDV1-GFP-DD^{ON} parasites, all of which are marked by HP1. This set included *dblmsp2*, which was also induced in 3D7/GDV1-GFP-DD^{ON} parasites (Fig. 2, G and H). Given that DBLMSP2 is a merozoite surface antigen expressed only in a small subpopulation of schizonts (19, 20) the GDV1-dependent activation of the *dblmsp2* locus suggests it may be expressed specifically in sexually committed schizonts. In summary, these findings show that GDV1 is an upstream activator of sexual commitment and likely triggers this process by antagonising HP1-dependent silencing of *ap2-g*.

To test if GDV1 associates with heterochromatin *in vivo* we conducted comparative ChIP-seq experiments. 3D7/GDV1-GFP-DD^{OFF} parasites were split at 28-34 hpi, cultured in parallel in the absence or presence of Shield-1 and paired chromatin samples were harvested two (30-36 hpi), six (34-40 hpi) and ten (38-44 hpi) hours after Shield-1 addition. We found that (1) GDV1-GFP-DD associates specifically with heterochromatin throughout the genome (Fig. 3A, Fig. S5, Table S7); (2) GDV1-GFP-DD occupancy was markedly higher in 3D7/GDV1-GFP-DD^{ON} compared to 3D7/GDV1-GFP-DD^{OFF} parasites (Fig. S5, Table S7); and (3) GDV1-GFP-DD occupancy is highly correlated with that of HP1 (Fig. 3B). Moreover, GDV1-GFP-DD occupancy peaked six hours post-induction and decreased substantially thereafter (Fig. 3A, Fig. S5, Table S7). This drop in GDV1-GFP-DD signal coincided with a reduced HP1 occupancy over heterochromatic genes in 3D7/GDV1-GFP-DD^{ON} compared to 3D7/GDV1-GFP-DD^{OFF} parasites (Fig. 3, A and C, Table S7). While the vast majority of heterochromatic loci, in particular those displaying high HP1 occupancy such as *var* genes, displayed only slightly decreased HP1 levels, some genes exhibited as much as 40% reduced HP1 occupancy (Fig. 3C and Table S7). This group of genes includes *ap2-g* and most known HP1-associated early gametocyte markers including *geco* (21), *pfgexp17* (22) and *pfg14_748* (8, 17) (Fig. 3, C and D, Table S7). These data are consistent with the microarray results, where GDV1-GFP-DD expression activated *ap2-g* and early gametocyte genes but had no effect on the expression of the bulk of heterochromatic loci including *var* genes (Fig. 2, G and H). Of note, given the 50-60% sexual conversion rate observed for 3D7/GDV1-GFP-DD^{ON} parasites (see above), a 30-40% reduction in HP1 occupancy indicates that HP1

may be depleted at these loci specifically in sexually committed parasites but single cell approaches are required to confirm this hypothesis. Overall, we suggest that GDV1 destabilises heterochromatin and thus allows specific transcription factors to activate expression of *ap2-g* and other gametocyte-specific heterochromatic genes, and this may play an important role in the positive auto-regulatory feedback loop proposed to reinforce AP2-G expression in committed parasites (5, 6, 23). How GDV1 achieves specificity in unlocking specific HP1-associated genes despite binding heterochromatin genome-wide is a challenging question to be addressed in the future.

Since GDV1 activates sexual commitment, the question arises of how parasites limit GDV1 expression to prevent sexual conversion in asexual schizonts. A recent study identified a multi-exon long non-coding *gdv1* antisense RNA (asRNA) that initiates downstream of the *gdv1* locus and overlaps with the ATG start codon of *gdv1* (24), which is a hallmark feature of regulatory asRNAs (25). To investigate if the *gdv1* asRNA participates in regulating sexual commitment we created a *gdv1* asRNA loss-of-function mutant in F12 parasites (F12/*gdv1*-asKO) (Fig. 4A and Fig. S6). Strand-specific RNA-seq analysis identified a small set of genes that were consistently differentially expressed between F12/*gdv1*-asKO and F12 wild-type parasites (17 up- and 23 down-regulated genes) (Fig. 4B, Table S8). Strikingly, and similar to F12 parasites expressing ectopic GDV1-GFP-DD (Fig. 2H), *ap2-g*, *dblmsp2* and two early gametocyte genes (*pfg14_748,* PF3D7_1477400) (8, 17) were markedly induced in F12/*gdv1*-asKO parasites, and all except one up-regulated gene are HP1-associated genes (Fig. 4B, Fig. S6, Table S8). *gdv1* sense transcripts were slightly increased in the F12/*gdv1*-asKO population, while *gdv1* antisense transcripts were undetectable as expected (Fig. 4, B and C, Fig. S6, Table S8). These results indicated that the *gdv1* asRNA acts as a negative regulator of GDV1 expression. To confirm this hypothesis, we tagged endogenous GDV1 in these parasites (F12/3xHA-GDV1/*gdv1*-asKO) and observed that indeed almost all parasites expressed 3xHA-GDV1 (96.7% +/- 2.5 SD) (Fig. S7). Lastly, we show that deletion of the *gdv1* asRNA locus in a conditional AP2-G mutant resulted in a markedly increased production of gametocytes (Fig. S8 and Supplementary text). Together, these findings demonstrate a central role for the *gdv1*-asRNA in regulating GDV1-dependent activation of sexual commitment. We anticipate this mechanism likely involves inhibiting GDV1 expression by interference with *gdv1* mRNA transcription, stability or translation, similar to asRNA-mediated gene regulation in other organisms (26).

We identified GDV1-mediated heterochromatin destabilisation as an epigenetic control strategy regulating sexual cell fate decision in *P. falciparum*. Our discovery of the *gdv1*-asRNA as a negative regulator of sexual commitment is reminiscent of lncRNA-mediated control of gametogenesis in yeasts (27, 28). In *S. cerevisiae*, nutritional stress triggers gametogenesis by activating the transcriptional regulator Inducer of Meiosis 1 (IME1) (28). A lncRNA in the *ime1* promoter and antisense transcription of *ime4* are key factors in preventing IME1 expression under non-inducing conditions (29, 30). These parallels raise the exciting possibility that evolutionary divergent unicellular eukaryotes may employ a conceptually similar regulatory logic to control entry into the sexual phases of their life cycles. Interestingly, all *Plasmodium* species infecting humans possess a GDV1 ortholog suggesting the GDV1-based regulation of sexual commitment is conserved in all human-infective malaria parasites. In conclusion, our study contributes to understanding the

molecular pathway underlying the formation of malaria transmission stages and provides opportunities for the development of intervention strategies targeting transmission of human malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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One sentence summary

The nuclear factor GDV1 induces gametocyte differentiation by activating expression of the master transcription factor AP2-G
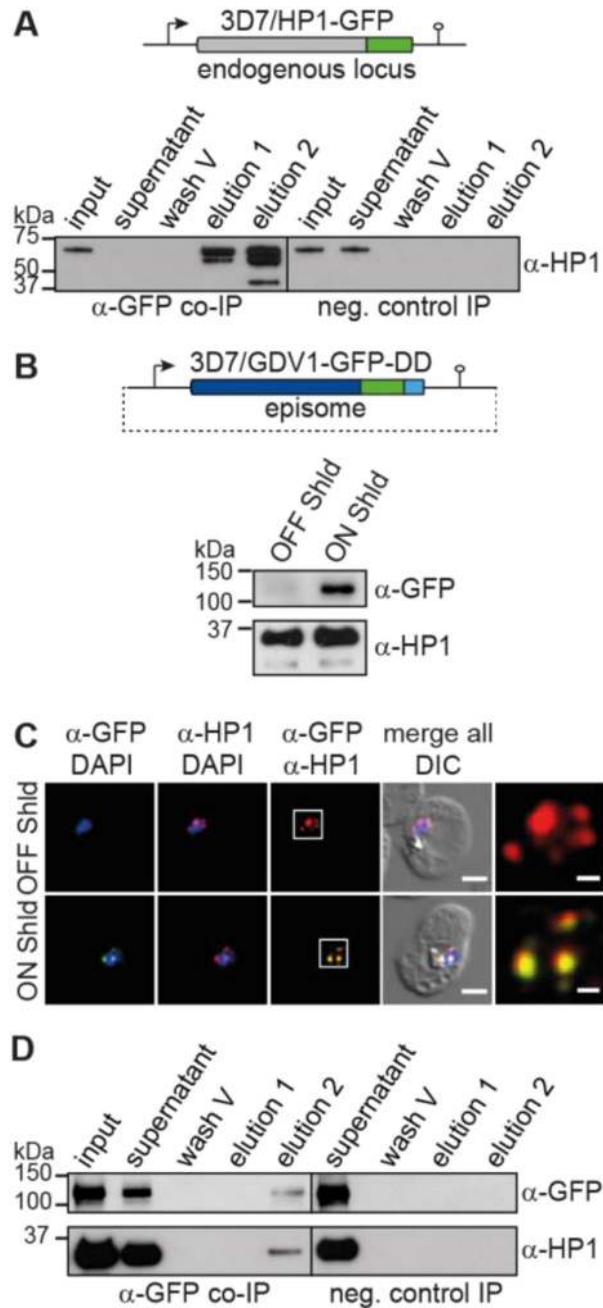
Fig. 1. GDV1 interacts with HP1.
(**A**) Endogenous *hp1* locus in 3D7/HP1-GFP parasites and α-HP1 Western blots of the α-HP1-GFP co-IP and negative control samples. Results are representative of three biological replicates. (**B**) *gdv1-gfp-dd* expression plasmid and α-GFP Western blots of 3D7/GDV1-GFP-DD^OFF and 3D7/GDV1-GFP-DD^ON parasites. α-HP1 antibodies served as loading control. (**C**) GDV1-GFP-DD/HP1 co-localisation IFAs in 3D7/GDV1-GFP-DD^OFF and 3D7/GDV1-GFP-DD^ON trophozoites (24-32 hpi). DIC, differential interference contrast. Scale bar, 2.5 μm (0.5 μm for the magnified views in the rightmost images). Results are

representative of three biological replicates. **(D)** α-GFP and α-HP1 Western blots of the α-GDV1-GFP-DD co-IP and negative control samples. Results are representative of three biological replicates.
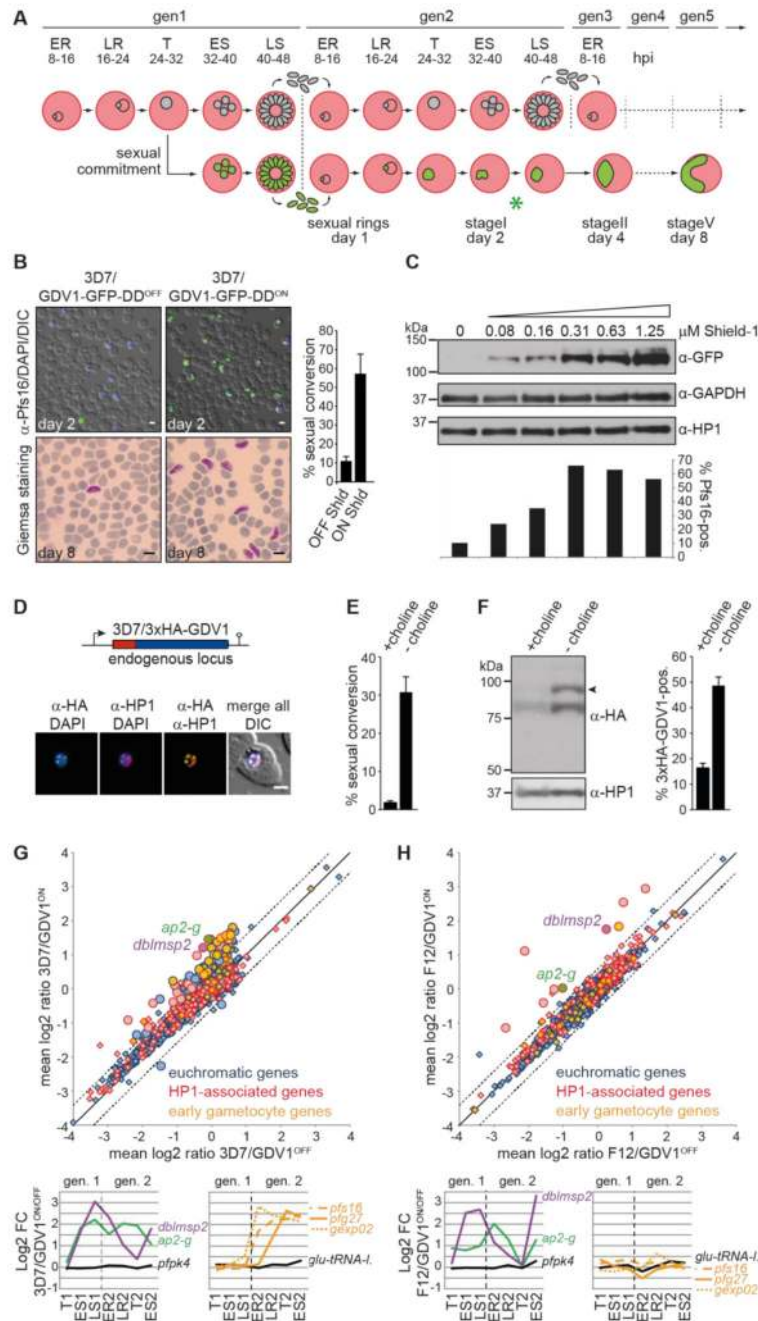
**Fig. 2. GDV1 induces sexual commitment and differentiation.**
(**A**) Schematic illustrating the iterative cycles of schizogony and RBC re-invasion (top) or sexual commitment, RBC re-invasion and gametocyte differentiation (bottom). ER/LR, early/late ring stages; T, trophozoites; ES/LS, early/late schizonts; gen, generation; hpi, hours post-invasion; asterisk, time point of α-Pfs16 IFAs. (**B**) Top panel: α-Pfs16 IFAs identifying stage I gametocytes. Quantification of Pfs16-positive parasites is shown at the right (results are the mean of three biological replicates (200 infected RBCs counted per sample); error bars indicate SD). Bottom panel: Giemsa-stained blood smears showing stage

V gametocytes. Scale bars, 5 μm. **(C)** Western blot showing GDV1-GFP-DD expression in presence of increasing Shield-1 concentrations. α-GAPDH and α-HP1 antibodies served as loading controls. Percentages of Pfs16-positive parasites are shown at the bottom (400 infected RBCs counted per sample). **(D)** Endogenous *gdv1* locus in 3D7/3xHA-GDV1 parasites and 3xHA-GDV1/HP1 co-localisation IFAs in trophozoites (24-32 hpi). Scale bar, 2.5 μm. **(E)** Sexual conversion rates in 3D7/3xHA-GDV1 parasites cultured in presence or absence of choline (results are the mean of three biological replicates (>190 infected RBCs counted per sample); error bars indicate SD). **(F)** Left panel: Western blot showing 3xHA-GDV1 expression levels in 3D7/3xHA-GDV1 parasites cultured in presence or absence of choline. α-HP1 antibodies served as loading control. Right panel: Percentages of 3xHA-GDV1-positive parasites in presence or absence of choline (results are the mean of three biological replicates (>100 infected RBCs counted per sample); error bars indicate SD). **(G,H)** Comparison of mean expression levels of all genes in 3D7/GDV1-GFP-DD^{ON} versus 3D7/GDV1-GFP-DD^{OFF} (G) and F12/GDV1-GFP-DD^{ON} versus F12/GDV1-GFP-DD^{OFF} parasites (H). Significantly de-regulated genes are indicated by circles (mean fold change cut-off >1.5; q-value (fdr) cut-off <0.15). Known early gametocyte markers (7, 8, 17) are labelled orange. Line graphs show fold changes in expression across seven consecutive TPs. *pfs16*/*pfg27*/*gexp02*, early gametocyte markers (15, 22, 32); *pk4 (*PF3D7_0628200*)*/*glu-tRNA-l.* (PF3D7_1331700), control genes (7).
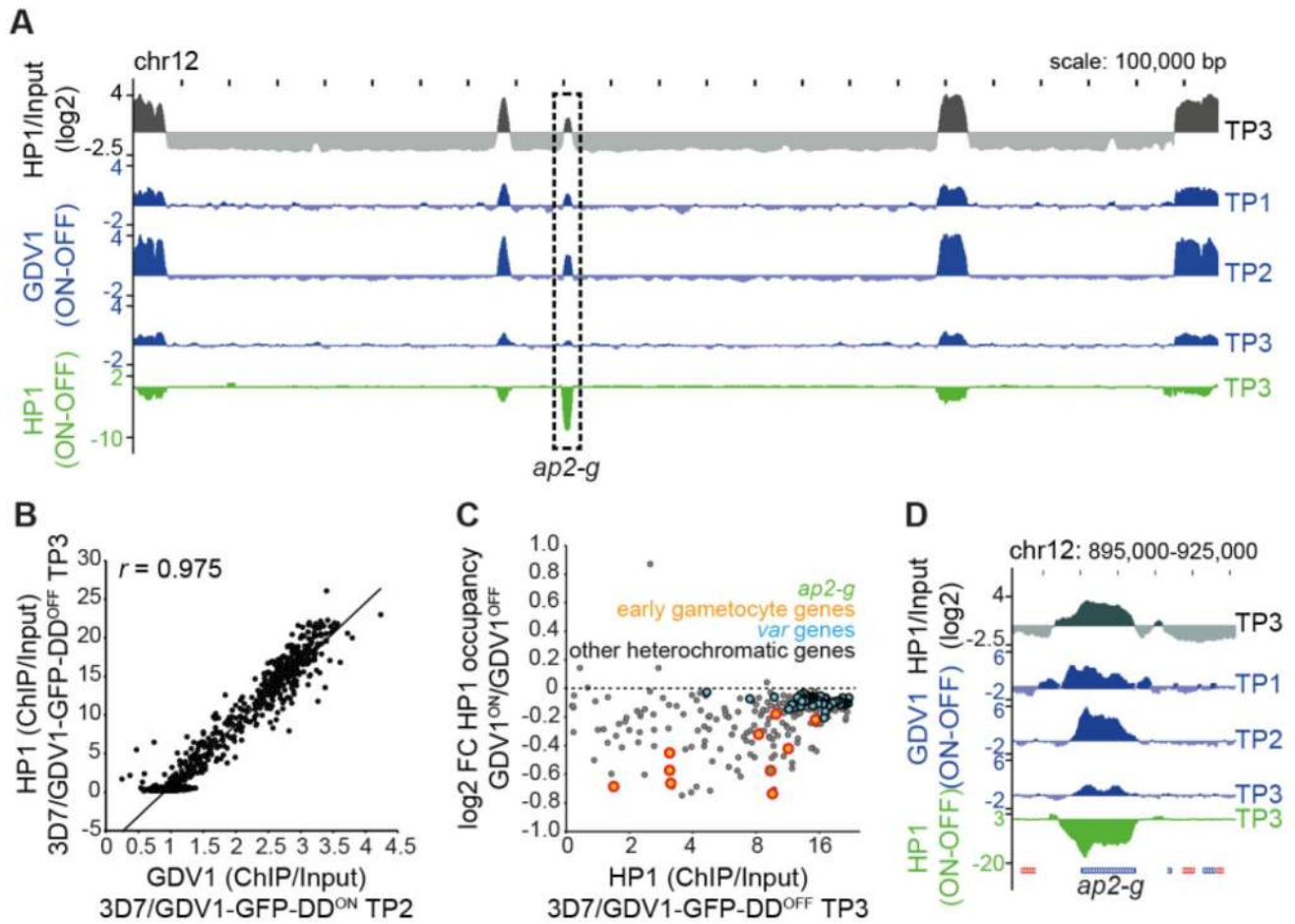
Fig. 3. GDV1 associates with heterochromatin throughout the genome and triggers HP1 removal at *ap2-g*.

(**A**) HP1 over input ratio track from 3D7/GDV1-GFP-DD^{OFF} schizonts (38-44 hpi, TP3) (grey). ChIP-seq subtraction tracks display relative enrichment of GDV1-GFP-DD in 3D7/ GDV1-GFP-DD^{ON} schizonts two (30-36 hpi, TP1), six (34-40 hpi, TP2) and ten (38-44 hpi, TP3) hours after Shield-1 addition (blue), and relative depletion of HP1 in 3D7/GDV1-GFP-DD^{ON} parasites at TP3 (green). (**B**) Correlation between GDV1-DD-GFP enrichment in 3D7/GDV1-GFP-DD^{ON} (34-40 hpi, TP2) and HP1 enrichment in 3D7/GDV1-GFP-DD^{OFF} schizonts at each coding region. *r*, Pearson correlation coefficient. (**C**) Fold change in HP1 enrichment upon GDV1-GFP-DD overexpression in relation to HP1 enrichment in 3D7/ GDV1-GFP-DD^{OFF} schizonts for each heterochromatic gene. (**D**) Zoom-in view of the enrichment/subtraction tracks at the *ap2-g* locus.
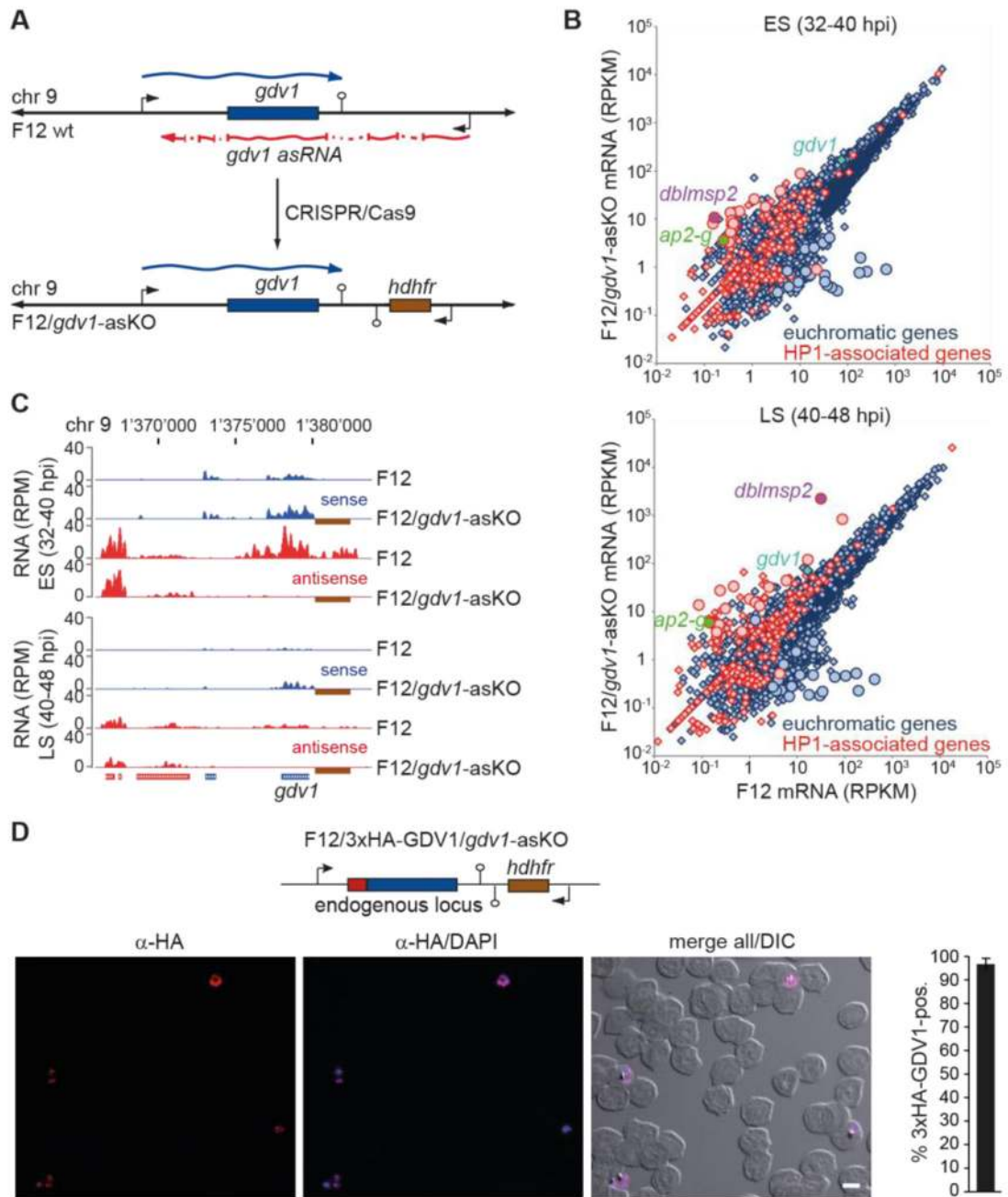
Fig. 4. A *gdv1* antisense RNA antagonises GDV1-dependent sexual commitment.
(A) *gdv1* locus in F12 wild-type and F12/*gdv1*-asKO parasites. The *gdv1* sense transcript
(blue), five-exon *gdv1*-asRNA (24) (red) and h*dhfr* resistance marker (brown) are
highlighted. **(B)** Comparison of gene expression levels in F12 wild-type and F12/*gdv1*-asKO
early (ES) and late (LS) schizonts. Genes de-regulated > 5-fold in both TPs are indicated by
circles. **(C)** UCSC genome browser screenshots of RNA-seq coverage plots over the *gdv1*
locus in F12 wild-type and F12/*gdv1*-asKO early (ES) and late (LS) schizonts. The h*dhfr*
resistance cassette downstream of the *gdv1* locus in F12/*gdv1*-asKO parasites and absent in

the 3D7 reference genome is indicated by a brown box. **(D)** Endogenous *gdv1* locus in F12/3xHA-GDV1/*gdv1*-asKO parasites and α-HA overview IFA in early schizonts (ES, 32-40 hpi). DIC, differential interference contrast. Scale bar, 5 μm. Percentage of 3xHA-GDV1-positive parasites is shown at the right (results are the mean of three biological replicates (100 infected RBCs counted per sample); error bars indicate SD).