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# *Plasmodium* male development gene-1 (*mdv-1*) is important for female sexual development and identifies a polarised plasma membrane during zygote development

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ABSTRACT

Successful development of *Plasmodium* sexual stages is essential for parasite survival, but the genes involved are poorly understood. We 'knocked out' the male development gene-1 (*mdv-1*) locus in *Plasmodium berghei* and found it to be important in female gametocyte activation. Indirect immunofluoresence assays show MDV-1 has a punctate cytoplasmic distribution in gametocytes. After activation of both females and males, MDV-1 is more peripherally located but in males exclusively it becomes concentrated in a few large foci. In vitro ookinete conversion assays that test the ability of activated female gametocytes to develop into retort stage ookinetes, suggests a complicit role for MDV-1, with the knock-out parasite producing 86% reduction in ookinetes. The retort stage ookinete develops from the zygote by increasing growth of an apical protrusion and MDV-1 locates at the 'leading' extracellular apical pole of this protrusion. In the fully developed ookinete MDV-1 is localised to the posterior pole. In vivo, the knock-out parasites demonstrate a phenotype in which there is a 90% reduction of parasite transmission to oocysts in mosquitoes.



## 1. Introduction

*Plasmodium*, the causal agent of malaria, has a complex life cycle, alternating between the mammalian host and the mosquito vector. In the mammalian red blood cell (RBC), parasite replication produces schizonts containing daughter merozoites, which upon release, invade a new RBC. In every cycle of blood stage replication a small subset of merozoites are committed to form sexual gametocytes upon re-invasion. All merozoites from a single schizont go on to form either asexual parasites or gametocytes of the same sex (Bruce et al., 1990; Silvestrini et al., 2000). The development of gametocytes (gametocytogenesis) requires sex-specific genes such as Pfs16 (Kongkasuriyachai et al., 2004) and Pfg27 (Lobo et al., 1999), whose expression can be induced by environmental factors such as immunological stress from the host (Ade-Serrano et al., 1981). Female and male gametocytes differ morphologically and recent data has described the contributing sex-specific proteomes (Khan et al., 2005). A significant number of mRNA transcripts produced in the female gametocyte are translationally repressed, bound to a protein termed 'Development Of Zygote Inhibited' (DOZI) (Mair et al., 2006).

Gametocytes are ingested by the feeding female mosquito and, triggered by a fall in temperature and specific mosquito factors (Billker et al., 1998), rapidly activate to produce gametes (gametogenesis). During both female and male gametogenesis regulated secretory vesicles, termed osmophilic bodies, fuse with the plasma membrane and secrete their contents into the surrounding parasitophorous vacuole (PV), immediately prior to gamete emergence (Sinden et al., 1976). Osmophilic bodies are 4- to 5-fold greater in number in the female gametocyte than in the male (Sinden et al., 1978) and contain the protein Pfg377. Pfg377 knock-out gametocytes produce few osmophilic bodies and emergence of the female gametes is significantly impaired (de Koning-Ward et al., 2008). During male gametocyte activation DNA is rapidly replicated from ~1.5 to 8 N (Janse et al., 1986), possibly involving the highly up-regulated chromatin remodelling protein SET (Pace et al., 2006). Three mitotic divisions organise the DNA into the developing eight microgametes (Sinden et al., 1976). Within 15 s of male gametocyte activation, the amorphous cytoplasmic microtubule organising centre (MTOC) changes into highly organised kinetosomes which organise axonemes, which dramatically propel the newly formed microgametes from the cell, in a process termed

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exflagellation (Sinden and Croll, 1975; Sinden et al., 1976). After exflagellation, the beating male gametes draw RBCs into exflagellation centres, a process involving Pfs230 (Eksi et al., 2006) and the PfCCP family of proteins (Scholz et al., 2008).

The surface proteins Pfs48/45 and Hap2 are pivotal to gamete adherence (van Dijk et al., 2001) and fusion, respectively (Liu et al., 2008). The resulting zygote undergoes meiosis and ~6 h later develops into the retort form ookinete by the formation of a cylindrical extrusion from one side of the zygote surface, which ~22 h later results in the formation of the ookinete. Underlying the retort's plasma membrane in the developing protrusion is the apical complex, comprised of the inner membrane complex (IMC), subpellicular microtubules (SM) and micronemes. The IMC and SM both extend in the fully developed elongated ookinete to the posterior end and three quarters of the length of the ookinete, respectively (Canning and Sinden, 1973; Mehlhorn et al., 1980). During retort development, translationally repressed transcripts such as Pb28 are released from the DOZI complex, allowing protein translation (Mair et al., 2006).

The protein expressed by male development gene-1 (*Mdv-1*), with a signal peptide but no recognisable domains, was identified in the *Plasmodium berghei* (PB000248.02.0) (Hall et al., 2005) and *Plasmodium falciparum* (PFL0795c) (Florens et al., 2002) gametocyte proteomes. *Pfmdv-1* (also named Peg3) was identified as being up-regulated in developing gametocytes (Silvestrini et al., 2005) and in microarrays of *P. falciparum* strains with reduced male gametocytogenesis (Furuya et al., 2005). Immunogold localisation of PfMDV-1 shows a wide distribution pattern: in osmophilic bodies, at the parasite plasma membrane, the parasite vacuole membrane and at cleft-like 'Garnham bodies' (Sinden et al., 1978) in the erythrocyte (Furuya et al., 2005; Lanfrancotti et al., 2007). *Pfmdv-1* gene deletion disrupted male gametocyte development inducing extensive multi-membrane vesicles in deformed 'Garnham bodies' (Sinden et al., 1978; Furuya et al., 2005). Here we characterise *P. berghei* MDV-1 and demonstrate its importance to female gametocyte activation. We show that the localisation of MDV-1 dramatically shifts upon gametocyte activation in a sex-dependent manner. MDV-1 was recently identified in the *Plasmodium gallinaceum* zygote proteome (Patra et al., 2008) and we demonstrate it is concentrated in the apical pole tip of the leading apical protrusion during zygote to ookinete development, where it locates at the posterior pole.

## 2. Materials and methods

All studies involving laboratory animals were performed in accordance with the EU regulations 'EU Directive 86/609/EEC' and with the regulations of the United Kingdom Animals (Scientific Procedures) Act 1986.

### 2.1. Antibody production and Western blot analysis

The complete open reading frame of *Pbmdv-1* was amplified from wild-type genomic DNA and cloned in-frame into the pET-46 Ek/LIC expression vector (Novagen). The resulting recombinant His-tagged fusion protein (MDV-1-His) was expressed in *Escherichia coli* BL21 Star™ (DE3) cells (Invitrogen), extracted from cell pellets using Bugbuster lysis buffer (Novagen) and purified using Nickel agarose slurry (Qiagen). The purified recombinant protein was used to raise antiserum in rabbits at Eurogentec, Belgium. Pre-immune rabbit sera acted as a control for specificity of anti-Pbmdv-1 antibody in both Western blots and Immunofluorescence assays (IFAs). Anti-PbMDV-1 sera was affinity purified (APαMDV-1) using purified MDV-1-His coupled to cyanogen bromide activated Sepharose.

*Plasmodium berghei* ANKA wild-type strain 2.34 was maintained in Theiler's Original outbred mice as described previously (Sinden et al., 2002). Gametocytes and ookinetes were prepared for Western blot analysis. Gametocytes were purified from infected blood as described (Beetsma et al., 1998) with some modifications. Briefly, blood was resuspended in CLB buffer (20 mm Hepes, 20 mM glucose, 4 mM sodium bicarbonate, 1 mM EDTA, 0.1% BSA in 0.83 × PBS pH 7.25) and separated on a 48% nycodenz cushion (in CLB). For ookinetes, infected blood was harvested and placed into culture for 22–24 h at 19 °C (Sinden et al., 1985). Ookinetes were purified by ammonium chloride lysis of the RBCs and nycodenz density gradient separation (Dessens et al., 1999). Parasite lysates were compared by Western blot using affinity purified antibody APαMDV-1 at 1/1000 and detected by anti-rabbit ECL™ IgG horseradish peroxidase conjugate (HRP), which was further detected with the ECL-Plus Western blotting detection system (Amersham). To determine the relative cell numbers loaded in each lane, Western blots were stripped and re-probed for α-tubulin with TAT1 1/5000 (Billker et al., 2002) and detected by anti-mouse ECL™ IgG HRP.

### 2.2. Immunofluorescence assays

The intracellular localisations of MDV-1 in gametocytes, activated gametocytes, zygotes, retorts and ookinetes were determined by IFA. Unactivated gametocytes in mouse blood were smeared onto glass slides. Gametocytes were activated by extraction of infected mouse blood and addition of ookinete medium (Sinden et al., 1985). At the designated time point after gametocyte activation, pelleted cells were smeared onto glass slides in FBS.

Cells were fixed and permeablised with acetone/methanol (50:50) on dry ice for 10 min. Alternatively, cells were fixed with 4% formaldehyde in PBS pH 7.4 for 10 min at room temperature, washed once with Tris buffered saline (TBS), permeabilised with 0.2% Triton X-100 for 5 min, then washed three times in TBS for 5 min each. Fluorescence was quenched with 0.1% sodium borohydride in TBS for 5 min and slides were washed in TBS for 5 min. All slides were blocked with 3% BSA/5% goat sera for 15 min, then incubated with APαMDV-1 (1/1000) for 2 h and revealed with anti-rabbit Alexa 488 (Molecular Probes, 1/1500) for 1 h in the dark. Zygote development slides were subsequently incubated with Cy3-conjugated 13.1 (antibody to Pb28, 1/1000) for 1 h. Slides were washed and mounted with Mowiol, 25 mg/ml 1,4-Diazabicyclo(2,2,2)octane (DABCO) and 25 μg/ml DAPI.

For live cell IFAs, zygotes were cultured for 5 h after gametocyte activation, washed twice in incomplete ookinete media, blocked as above and incubated in APαmdv-1 (1/1000) for 1 h. After three washes, labelled cells were detected with anti-rabbit Alexa 488 for 1 h in the dark and subsequently washed three times. Cells were resuspended in ookinete medium with Hoechst 33258 (Molecular probes, 1/500) and mounted under Vaseline rimmed cover slips. All images were captured using a Leica DMR microscope with a Zeiss Axiocam camera with AxioVision AxioVs4.6.1.0 software and compiled in Adobe Photoshop 6.0.

### 2.3. Targeted disruption of P. berghei mdv-1

To replace all the protein-coding sequence of the *Pbmdv-1* gene (PB000248.02.0) with a *Toxoplasma gondii dhfr/ts* expression cassette conferring resistance to pyrimethamine, a targeting vector was constructed in plasmid pBS-DHFR (Fig. 4A). A 910 bp fragment comprising 5′-flanking sequence immediately upstream of the start codon was amplified from *P. berghei* genomic DNA using primers U1_up5pKpnI (5′-ATATGGTACCCACATCTTTGATTTTAATA