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Regulation of Sexual Development of *Plasmodium* by Translational Repression

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Translational repression of messenger RNAs (mRNAs) plays an important role in sexual differentiation and gametogenesis in multicellular eukaryotes. Translational repression and mRNA turnover were shown to influence stage-specific gene expression in the protozoan *Plasmodium*. The DDX6-class RNA helicase, DOZI (development of zygote inhibited), is found in a complex with mRNA species in cytoplasmic bodies of female, blood-stage gametocytes. These translationally repressed complexes are normally stored for translation after fertilization. Genetic disruption of *pbdoozi* inhibits the formation of the ribonucleoprotein complexes, and instead, at least 370 transcripts are diverted to a degradation pathway.

Translational repression (TR) of mRNAs in higher eukaryotes controls temporal expression of specific protein cascades or directs the location of translation within a cell, and is important after gamete fertilization (zygote formation) in the early embryo when de novo transcription of mRNA is restricted (1–5). The hallmark of repression is the assembly of certain mRNAs together with proteins into quiescent messenger ribonucleoprotein particles (mRNPs), where these transcripts are stored for translation at a later time. The DDX6 family of DEAD-box RNA helicases is tightly linked both to storage of mRNAs encoding proteins associated with progression through meiosis into translationally silent mRNPs and with the transport of mRNA to degradation centers in the cell (P-bodies). These helicases are found in organisms as diverse as yeast (e.g., Dhh1p) and humans (e.g., RCK/p54).

Earlier studies in *Saccharomyces cerevisiae* suggested that Dhh1p was localized to cytoplasmic P-bodies that contain both mRNA and enzymes central to the RNA degradation pathway (e.g., the decapping enzyme), implying that P-bodies harbor transcripts destined for degradation (6–8). More recently, it was proposed that mRNAs also exit P-bodies and re-engage polyosomes for translation in a Dhh1p-dependent mechanism (9). With the exception of human RCK/p54, homologs of DDX6 helicases in metazoans (e.g., RCK/p54). TR has been described in *Plasmodium* (10–16) in the female gametocyte, the stable, blood-stream precursor cell of the female gamete, where two abundant transcripts are present but not translated. These mRNAs, p25 and p28, en...
code proteins essential for zygote development and mosquito midgut invasion (17) and are kept in a translationally quiescent state. Translation is only initiated after these sexual forms have been activated during ingestion by a female mosquito, thus triggering gamete formation and subsequent fertilization. Later work showed that in gametocytes, TR most likely affected multiple transcripts and could be an important mechanism of gene regulation at this developmental time point involving mechanisms established in metazoans (16). In the gametocyte sex-specific proteomes (18), we identified an RNA helicase (DOZI) that is highly up-regulated in female gametocytes and showed high sequence homology to the DDX6 family of RNA helicases. Sequence alignments showed that DOZI formed a specific clade within all annotated Plasmodium falciparum RNA helicases, their P. berghei and apicomplexan homologs that cluster specifically with several DDX6 helicases known to be involved in mRNP formation (figs. S1 to S3). DOZI appeared to be the only DDX6-family homolog present in Plasmodium and contained the domains involved in RNA-binding and RNA-unwinding activity (19).

For cellular localization of DOZI, we generated a modified P. berghei line expressing a C-terminal green fluorescent protein (GFP) fusion of endogenous DOZI (DOZI::GFP) (Fig. 1, A to C; fig. S4). A punctate GFP-fluorescence pattern that appeared to be restricted to the cytoplasm of female gametocytes was observed in live and fixed cells after immunofluorescence assay (IFA) analysis with antibodies to GFP (anti-GFP) (Fig. 1A). These transgenic parasites showed wild-type fertilization rates and zygote/ookinete production (Fig. 1B). Steady-state levels and translational repression of p28 mRNA were the same as those in wild-type parasites (Fig. 1C). Fluorescence in situ hybridization (FISH) analysis of the localization of p28 and p25 transcripts, combined with IFA for DOZI, showed that both transcripts were highly abundant in female gametocytes (Fig. 1A) with a punctate localization pattern comparable to that of the helicase. This suggested that the repressed transcripts and DOZI are distributed similarly in the cytoplasm and prompted an analysis of transcripts associated with DOZI.

Immunoprecipitations of the DOZI::GFP fusion protein from gametocyte lysates were made with monoclonal anti-GFP (GFPMAB). Eluates were analyzed for DOZI protein and transcript content. Western analysis of total gametocyte lysate input, as well as specific and control precipitates, identified DOZI::GFP only in the specific immunoprecipitates, whereas other control proteins (P47 and eEF1A) were only found in the input material (Fig. 2A). Northern analysis showed the presence of a substantial amount of p25 and p28 transcripts and not control RNA species in the GFPMAB eluate (Fig. 2B), suggesting that these mRNAs and DOZI occurred together in an mRNP. Additionally, reverse transcription–polymerase chain reaction (RT-PCR) analysis of the eluates demonstrated that many other transcripts that had been predicted to be translationally repressed (16) coelute with DOZI::GFP (Fig. 2C). Reverse transcription–quantitative polymerase chain reaction (RT-qPCR) analysis indicated that these same mRNA species were significantly enriched in the DOZI::GFP coelute (fig. S5) and that they showed the punctate localization pattern in the cytoplasm (fig. S6).

P. berghei parasites were generated that lacked pbdozi (fig. S7). The pbdozi− null mutants showed normal development of the asexual blood stages and normal production of gametocytes and gametes, but there was a total lack of development of fertilized female gametes (zygotes) into mature ookinetes (Fig. 3A). Normal development into ookinetes requires meioic DNA replication in the zygote 2 to 3 hours after fertilization of female gametes (18). In pbdozi− null mutants, all zygotes aborted development before meiosis. DOZI is also produced in males (18), although in much lower abundance than in females. We crossed the male and female gametes of pbdozi− with gametes of mutant parasite lines that are defective in male sterility (20, 21) or in female gamete production (22) (Fig. 3A). Such cross-fertilization assays revealed that male pbdozi− gametes were able to fertilize wild-type DOZI-expressing female gametes, resulting in the development of ookinetes. By contrast, development of zygotes from pbdozi− females fertilized by wild-type males was incomplete and similar to development of zygotes from the pbdozi− line. These crosses demonstrate that the block in zygote/ookinete development is essentially due to a lack of DOZI of female gametocyte origin.

The phenotype of the pbdozi− parasites might be expected from the predicted function of DOZI in the storage of translationally repressed mRNAs of P28 and P25 in the female gamocyte, and the later, essential, developmentally regulated use of these and other transcripts in postfertilization (zygote and ookinete develop-
development) events. Northern analysis of mRNA showed not only a nearly complete loss of transcripts of p25 and p28 (Fig. 3B), explaining the absence of P25 and P25 (Fig. 3C), but also downregulation of an additional three transcripts—warp, which encodes an ookinete-specific protein, as well as pb000245.02.0 and pb000633.00.0 (Fig. 3B), which earlier studies had predicted to be translationally repressed (16)—indicating a wider role of DOZI in mRNA maintenance.

The full extent of the effect of DOZI depletion on steady-state mRNAs of blood-stage, unactivated gametocytes was analyzed with an oligonucleotide microarray that consisted of 5283 P. berghei gene models (16, 23). We observed 370 transcripts to be significantly reduced by more than a factor of 2 in pbdozi− as compared to wild-type gametocytes (table S1), including seven of nine genes previously shown to be translationally repressed (16). This subset also included groups of genes that ensure successful development of the parasite in the mosquito, e.g., genes linked to ookinete motility and invasion (table S2). Unexpectedly, transcripts of 92 genes were observed that concomitantly increased in abundance, which might reflect transcriptome responses to altered biological processes in the mutant or indicate a more complex role of DOZI in the regulation of mRNA abundance. These observations were confirmed by RT-qPCR analyses of selected transcripts (table S3, fig. S8).

Together, these data indicate that DOZI has a central role in the silencing and maintenance of steady-state levels of a population of gametocyte-specific transcripts. Furthermore, the loss of DOZI apparently severely affected the capacity of the parasite to store and stabilize a discrete subset of mRNAs in the female gametocyte, resulting in a failure to synthesize specific proteins and to complete normal zygote development.

Translational repression in Plasmodium may function to specifically regulate gene expression during meiosis in the zygote. Posttranscriptional regulatory mechanisms of gene expression in Plasmodium, such as translational repression and mRNA homeostasis, might play a central role in development, because the annotation of Plasmodium genomes indicates a relative scarcity of transcription factors (24). Indeed, the timing of the appearance of proteins from transcripts that undergo TR in gametocytes can be quite different. For example, P25 and P28 are first detected about 2 hours after female gamete activation and fertilization, whereas WARP may only be detected in developing ookinetes 8 hours after fertilization (25).

Until recently, DDX6 RNA helicases were implicated in mRNA storage in translationally silent MRNP complexes and in the transport of mRNA to P-bodies that serve as centers for mRNA degradation. More recently it has been shown that mRNA may be cycled to and from P-bodies in vegetative S. cerevisiae in a DDX6 helicase-dependent manner, at least in unicellular eukaryotes. DDX6 helicases are also expressed in germline cells and control the fate of mRNA species that are required for further development of the cell once it has been fertilized. Our data support a role for DOZI in the storage and silencing of mRNA species in Plasmodium gametocytes required after gamete fertilization (fig. S9). However, gametocytes in the blood circulation have a short half-life: The overwhelming majority fail to be transmitted to the mosquito and rapidly decay. Decaying gametocytes contain repressed transcripts that if translated, could produce proteins targeted by transmission-blocking antibodies (10). Consequently, in decaying blood-borne gametocytes, DOZI may be involved in rapid destruction of the stored mRNAs. By contrast, in the activated female gamete in the mosquito, TR is relieved, allowing the coordinated production of proteins essential for the further development of the parasite and establishment of the infection in the mosquito vector.

References and Notes
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Materials and Methods
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References
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