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Translational Repression is essential for *Plasmodium* sexual development and mediated by a DDX6-type RNA helicase

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Abstract

Translational repression (TR) of mRNAs plays an important role in sexual differentiation and gametogenesis in multicellular eukaryotes. We show here that TR and mRNA turnover are key influences on stage specific gene expression in the protozoan *Plasmodium*. The DDX6 class RNA helicase, DOZI (development of zygote inhibited), is found in cytoplasmic bodies of female, blood stage gametocytes in a complex with mRNA species known to be translationally repressed. Genetic disruption of *pbdozi* inhibits the formation of translationally quiescent mRNPs and targets these and other (>350 different) transcripts to the degradation pathway rather than directing them to translating polysomes, preventing zygote development prior to meiosis in the mosquito. Thus TR is essential in malaria parasite development. A full catalogue of the proteins and processes associated with DOZI (the first described malaria parasite TR-effector protein) might lead to novel approaches to prevent parasite development.

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 prominent after gamete fertilisation (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

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Post-fertilisation development of *Plasmodium* zygotes in the mosquito relies on the formation of translationally quiescent mRNPs in blood-stage, female sexual precursor cells

Supporting Online Material

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Materials and Methods

Figs. S1 to S4

Tables S1 to S4

References

transport of mRNA to degradation centers in the cell (P-bodies). These helicases are found in organisms as diverse as yeast (DHH1p) and man (rck/p54).

In *S. cerevisiae* DHH1p has historically been localised to cytoplasmic P-bodies (6,7), which contain both mRNA and two enzymes central to the RNA degradation pathway; the de-capping enzyme Dcp1p and the de-adenylase Pop2p implying that P-bodies harbor transcripts destined for degradation (7,8). However, it has been proposed recently that mRNAs may also exit P-bodies and re-engage polysomes for translation in a DHH1p dependent mechanism (9). To date, homologs of DDX6 helicases in higher eukaryotes and metazoans on the other hand are exclusively localised to mRNPs involved in TR (2–4).

TR has been described in *Plasmodium* (10–16) in the female gametocyte, the precursor cell of the female gamete, where two abundant transcripts are present but not translated. These mRNAs, *p25* and *p28*, encode 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. It is clear now that repression of translation uses mechanisms established in metazoans (18) and is mediated by cis-acting RNA motifs that are located in the untranslated regions of *p25* and *p28* transcripts (19).

In a search for proteins involved in TR in *Plasmodium* we identified in the gametocyte sex-specific proteomes (20) an RNA helicase (DOZI) that is highly upregulated in female gametocytes and shows remarkable sequence homology to the DDX6-family of RNA helicases. Sequence alignments with all annotated *P. falciparum* RNA helicases, their *P. berghei* and apicomplexan homologs and a number of DDX6 helicases known to be involved in mRNP formation, clearly showed a clustering of DOZI with the latter (see Supplementary Figures S1–S3). Also, DOZI appears to be the only DDX6-family homolog that is present in *Plasmodium* and contains all characterised domains involved in RNA binding and RNA unwinding activity (21).

To determine the cellular localisation of DOZI we generated a modified *P. berghei* line expressing a C-terminal GFP-fusion of endogenous DOZI (DOZI::GFP, Fig 1A-E). A strong, speckled and punctate GFP-fluorescence pattern that appeared restricted to the cytoplasm of female gametocytes was observed in live and fixed cells after IFA analysis with anti-GFP antibodies (Fig. 1F). These transgenic parasites showed normal asexual blood stage development, production of gametocytes (results not shown) and wild type fertilization rates and zygote/ookinete production (Fig. 1G), strongly indicating that DOZI-function is not affected by the C-terminal GFP-fusion. Fluorescent In Situ Hybridisation (FISH) analysis of the localisation of *p28* and *p25* transcripts combined with IFA for DOZI showed that both transcripts are highly abundant in female gametocytes (Fig. 1F) and show a punctate localisation pattern comparable to that of the helicase. This suggested that the repressed transcripts and DOZI are distributed similarly and prompted an analysis of transcripts associated with DOZI.

Therefore, we performed immunoprecipitations (IP) of the DOZI::GFP fusion protein from gametocyte lysates with monoclonal anti-GFP antibodies and eluates were analysed for their protein and RNA contents. Western analysis of input, specific and control IPs identified DOZI::GFP only in the specific IP, whereas other control proteins (P47 and eEF1A) were only found in the input material (Fig. 2A). Northern analysis showed the presence of a significant amount of *p25* and *p28* transcripts in the specific IP-eluate (Fig. 2B), strongly suggesting the presence of these mRNAs and DOZI in a stable mRNP.

To further investigate the role of DOZI, *P. berghei* parasites were generated that lack *pbdozi* by standard targeted disruption (Figure 3A-C). The *pbdozi* null mutants showed normal

development of the asexual blood stages and normal production of gametocytes and gametes (results not shown). However, a clear, 100% developmental block exists manifested as a total lack of development of fertilized female gametes (zygotes) into mature ookinetes (Fig. 3D). The development into ookinetes involves meiotic DNA replication in the zygote, 2–3 hours after fertilization of female gamete (20). In *pbdozi* mutants only zygotes are observed that abort development before meiosis and fail to produce the ookinete surface proteins P28 (Fig. 3E) and P25 (not shown).

Since the proteomes of male and female gametocytes indicated that DOZI is also produced in males (20) (although of considerably lower abundance than in females) it was investigated whether the inhibition of zygote/ookinete development was the result of the lack of DOZI protein in males or females, or both. Therefore, we crossed the males and female gametes of *pbdozi* with gametes of mutant parasites lines that are either defective in male (22,23) or in female gamete production (24) (Fig. 3D). In these standard cross-fertilization assays it was observed that male *pbdozi* gametes were able to fertilize wild type, DOZI-expressing female gametes resulting in the development of ookinetes. On the other hand development of zygotes from *pbdozi* females fertilized by wild type males was incomplete and similar to 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 is in agreement with a predicted function of DOZI in the storage of translationally repressed mRNAs in the female gametocyte and the later, essential, developmentally regulated use of these transcripts in early, post-fertilisation (zygote development) events. Therefore the status of *p25* and *p28* transcripts was assessed in *pbdozi* gametocytes. Northern analysis of mRNA not only showed a nearly complete loss of these transcripts, explaining the absence of P28 (Fig. 3E) and P25 (data not shown), but also strong down-regulation for an additional three transcripts; *warp*, as well as *pb000245.02.0* and *pb000633.00.0* (Fig. 3F) that in an earlier study were predicted to be translationally repressed (16).

The full extent of the effect of DOZI depletion on steady state mRNAs of blood-stage, unactivated gametocytes was analysed using a oligonucleotide microarray that comprises 5283 *P. berghei* gene models (16) (www.sanger.ac.uk). The steady state level of 370 transcripts was observed to be significantly reduced by greater than 2-fold in *pbdozi* gametocytes compared to wild type (Supplementary Table S1) including 7 out of 9 genes previously shown to be translationally repressed based on their transcription and translation profiles (16). This subset also includes groups of genes that ensure successful development of the parasite in the mosquito, e.g. genes linked to ookinete motility and invasion (Supplementary Table S2). Unexpectedly, the abundance of 92 transcripts was concomitantly increased 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 quantitative RTPCR on selected transcripts (Supplementary Table S3). Taken together, these data strongly indicate that DOZI has a central role in the silencing and maintenance of steady state levels of a population of gametocyte-specific transcripts and that the loss of DOZI apparently severely affects the capacity of the parasite to store and stabilise a discrete subset of affected mRNAs specifically in the female gametocyte resulting in failure to synthesise specific proteins leading to arrested zygote development. Therefore, TR in *Plasmodium* may function to specifically regulate gene expression during meiosis in the zygote when active transcription might be limited as is the case during meiosis in many eukaryotes (5). On the other hand, post-transcriptional regulatory mechanisms of gene expression in *Plasmodium*, such as TR and mRNA homeostasis, might play a more central role in development, since the

annotation of *Plasmodium* genomes indicates a relative scarcity of transcription factors (25). Indeed, the timing of the appearance of proteins from transcripts that undergo TR in gametocytes can be quite different. For example, P25 and P28 can be first detected about 2 hours after female gamete activation and fertilisation whereas WARP may only be detected in developing ookinetes 8 hour after fertilization (26).

Until recently DDX6 RNA helicases have been implicated in either mRNA storage in translationally silent mRNP complexes or in transport of mRNA to P-bodies that serve as centers for mRNA degradation. The recent demonstration that mRNA may be cycled to and from P-bodies in *S. cerevisiae* in a DDX6 helicase- dependent manner indicates that within a single organism (at least in unicellular eukaryotes) such helicases may fulfil a dual role dependent upon the cellular context. Typically, these helicases are expressed in germ-line cells and control the fate of mRNA species that are required for the further development of the cell once fertilised. Our data show a clear role of DOZI in the stable storage of mRNA species in gametocytes that are needed after fertilization. However, gametocytes in the blood circulation have a short half live and the overwhelming majority fail to be transmitted to the mosquito and rapidly decay. Decaying gametocytes contain a high level of repressed transcripts that if translated could produce proteins amongst which are those known to be the target of transmission blocking antibodies⁷. Therefore, DOZI might also play a dual role in *Plasmodium* mRNA homeostasis that is consistent with the alternative fates of the gametocyte (Figure 4). In the decaying blood-borne gametocyte DOZI might be involved in rapid destruction of the stored mRNAs whereas in the activated (female) gamete in the mosquito TR is relieved allowing the co-ordinated production of proteins essential for the further development and establishment of the infection in the mosquito vector.

This study demonstrates for the first time the essential role of TR in malaria parasite development and description of a TR-effector protein. Further studies on TR in *Plasmodium* to produce a full catalogue of the function of proteins encoded by mRNA species involved in these processes should lead to a greater understanding of the control of *Plasmodium* development. Moreover, identification of the constituent proteins in TR complexes may also yield novel approaches of inhibition of parasite development and transmission through interference with proteins like DOZI and therefore lead to novel therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Generation of parasite, PbDOZI::GFP, expressing GFP-tagged DOZI which shows a punctuate localisation in the cytoplasm of female gametocytes. They show normal TR and zygote development

A Schematic representation of GFP-tagging of the *pbdozi*-locus using a construct that integrates through single cross-over homologous recombination and contains the *tgdhfr/ts* selectable marker. **B** Diagnostic PCRs of mutant population. PCRs show 5' and 3' integration (lanes int), lack of WT *pbdozi*, presence of *tgdhfr/ts* and the *p28* locus as a control. **C** RT-PCR confirming correct use of splice sites in the *pbdozi::gfp* pre-mRNA. **D** Northern analysis of transcripts of PbDOZI::GFP gametocytes showing the presence of *pbdozi::gfp* transcripts, absence of WT *pbdozi* mRNA and 'storage' of *p28* transcripts like in WT. * indicates the *pbdozi* transcript specific to gametocytes. **E** Western blot analyses of gametocyte proteins of PbDOZI::GFP shows the correct size (~75kDa) for the DOZI::GFP protein and absence of P28 protein in WT and PbDOZI::GFP. P28 is present in ookinetes. Polyclonal serum against eEF1A is used as a control. **F** FISH and IFA analysis of female gametocytes show a similar, punctuate localisation in the cytoplasm of DOZI::GFP and *p25* and *p28*. Live imaging also shows punctuate localisation. **G** PbDOZI::GFP gametocytes show WT development of zygotes into ookinetes. Data show mean $\pm$ s.d. WT = wild type, DG = PbDOZI::GFP. BF = Bright Field

Figure 2. Immunoprecipitation (IP) experiments show localisation of the transcripts of *p25* and *p28* in complexes containing the DOZI::GFP fusion protein

A Western blot analyses of gametocyte lysates from IPs, using anti-GFP-antibodies, anti-c-myc-antibodies or beads only, show the presence of DOZI::GFP only in the specific (i.e. IP-GFP) fraction. No contamination with the control proteins P47 or eEF1A is observed (only present in the input-lysate). * c-myc antibody, ♦ protein G. **B** Northern blot analysis of RNA recovered from the IPs as shown in A) identify *p25* and *p28* transcripts in the IP-GFP fraction and in the washes. The transcripts of the controls *p47*, *eef1a*, and *rrna* were not detected (only present in the washes).

Figure 3. *Pbdozi* null mutants show complete inhibition of zygote development, and gametocytes fail to store *p25* and *p28* transcripts

A. *Pbdozi* parasites were generated by double crossover homologous recombination replacing *pbdozi* with the *tgdhfr/ts* selectable marker. **B.** Diagnostic PCRs of mutant population. PCRs show 5' and 3' integration (lanes int), lack of WT *pbdozi*, presence of *tgdhfr/ts* and *p28* as a control. **C.** Northern blot analysis of RNA from *pbdozi* null parasites shows the lack of *pbdozi* transcripts (*rrna* is included as input control). **D.** Analysis of zygote development in standard *in vitro* assays shows the lack of development of *pbdozi* null zygotes into ookinetes. *Pbdozi* null female gametes (KO) cross-fertilized with 'wild type' males (270) do not develop into ookinetes, whereas 'wild type' females (137) cross-fertilized with *pbdozi* null males (KO) show normal ookinete development. Data show mean $\pm$ s.d. **E.** Western blot analyses of gametocyte lysates show the absence of P28 in both WT and *pbdozi* null gametocytes. P28 is present only in ookinetes. **F.** Northern blot analyses of gametocyte transcripts show that *p25* and *p28* steady state levels are heavily reduced in *pbdozi* null parasites. In addition, we show transcripts that are also affected (left panel) or unaffected (right panel) by the lack of DOZI. WT = wild type, KO = *pbdozi* null mutant, ook = ookinete

Figure 4. Proposed role of DOZI in TR in sexual cells (gametocytes) of *Plasmodium*

In the presence of the RNA helicase DOZI specific transcripts are assembled into translationally quiescent mRNPs in the female gametocytes that are the precursors of the female gametes. These mRNAs are stored for later translation after gamete formation and fertilisation, which occurs in the mosquito. The stable assembly of such translational repressor complexes for the transcripts *p25* and *p28*, encoding surface proteins of the zygote, is absolutely dependent on the activity of DOZI; in the absence of DOZI these transcripts are neither stored in silent mRNPs nor transported to translating polysomes but instead are specifically destroyed.