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REVIEW

Plasmodium falciparum R2TP complex: driver of parasite Hsp90 function

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Abstract

Heat shock protein 90 (Hsp90) is essential for the development of the main malaria agent, *Plasmodium falciparum*. Inhibitors that target Hsp90 function are known to not only kill the parasite, but also reverse resistance of the parasite to traditional antimalarials such as chloroquine. For this reason, Hsp90 has been tagged as a promising antimalarial drug target. As a molecular chaperone, Hsp90 facilitates folding of proteins such as steroid hormone receptors and kinases implicated in cell cycle and development. Central to Hsp90 function is its regulation by several co-chaperones. Various co-chaperones interact with Hsp90 to modulate its co-operation with other molecular chaperones such as Hsp70 and to regulate its interaction with substrates. The role of Hsp90 in the development of malaria parasites continues to receive research attention, and several Hsp90 co-chaperones have been mapped out. Recently, focus has shifted to *P. falciparum* R2TP proteins, which are thought to couple Hsp90 to a diverse set of client proteins. R2TP proteins are generally known to form a complex with Hsp90, and this complex drives multiple cellular processes central to signal transduction and cell division. Given the central role that the R2TP complex may play, the current review highlights the structure-function features of Hsp90 relative to R2TPs of *P. falciparum*.

Keywords *Plasmodium falciparum* · Heat shock protein 90 · R2TP proteins

Plasmodium falciparum Hsp90

Plasmodium falciparum constitutes the main agent of malaria. *P. falciparum* develops in cold-blooded mosquito vectors and is transmitted to warm-blooded human hosts when mosquitoes take up a human blood meal (Fig. 1). Thus, one of the main requirements for the parasite's survival is the need to adapt to physiological changes. The rapid replication of the parasites within the human host is driven by the synthesis of biomolecules such as lipids and proteins (Santiago et al. 2004; Absalon et al. 2016). As such, proteostasis is central to the development of the parasite within the host. Heat shock proteins, among them Hsp90, serve as protein folding facilitators in the parasite (Banumathy et al. 2003; Shonhai 2010; Daniyan et al. 2019). Apart from their role in protein folding, parasite heat shock proteins are also implicated in host red blood cell remodeling, which is crucial for clinical malaria development. About 500 parasite proteins (approximately 10% of the parasite proteome) are exported to the host red blood cell at the erythrocyte stages of the disease and, hence, are implicated in remodeling of the host cell to make it rigid (Maier et al. 2008).

Although Hsp90 is not part of the parasite exportome, its house-keeping role is essential for parasite survival (Banumathy et al. 2003). In addition, Hsp90, along with its functional partner Hsp70, is implicated in parasite resistance against the current first-line treatment of artemisinin-based combination therapies (ACTs) (Corey et al. 2016). Interestingly, both Hsp90 and Hsp70 along with Hop (Hsp90-Hsp70 organizing protein) are deemed to constitute part of several target proteins to which ACTs bind (Ismail et al. 2016). Altogether, this suggests that Hsp90 plays a central role in the survival of the parasite and may further augment parasite drug resistance. Indeed, inhibition of PfHsp90

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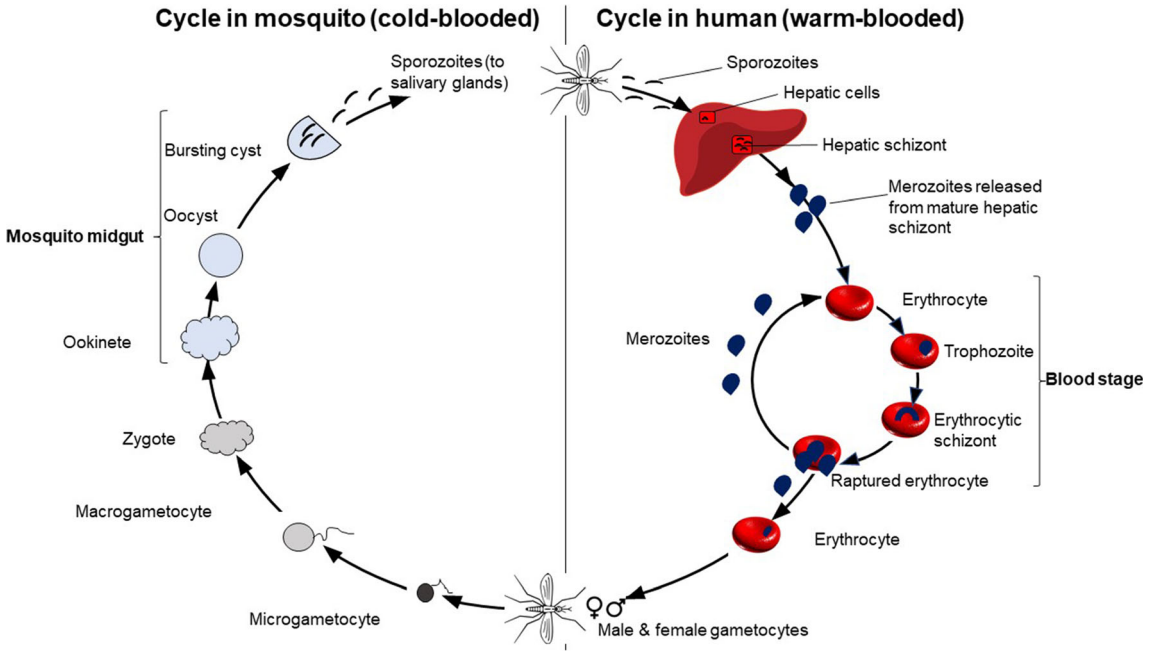

Fig. 1 Life cycle of *Plasmodium falciparum.* The parasites are introduced into the warm-blooded human host by the *Anopheles* mosquito vector during its blood meal. Initially, the mosquito injects *P. falciparum* sporozoites into the bloodstream, which subsequently migrate to the liver. In the hepatocytes, the sporozoites develop and multiply via schizogony to form merozoites, which then infect red blood cells. Once inside these cells, merozoites undergo multiplication via schizogony and red blood cells burst, releasing more merozoites. Some of the merozoites differentiate into gametocytes, which are then taken up by the mosquito when it ingests a blood meal from the human. Gametocytes then fuse together and differentiate in the mosquito midgut to form new sporozoites that can be further injected into the human host

arrests parasite growth both at the blood stages and liver stages of development (Banumathy et al. 2003; Shahinas et al. 2013).

Hsp90 is a highly ubiquitous molecular chaperone, and normally has four different isoforms in *P. falciparum*: two cytosolic (inducible PfHsp90α PF3D7_0708400 and constitutive Hsp90β PF3D7_1443900), and two other paralogs resident in the endoplasmic reticulum (PF3D7_1222300), and mitochondrion and apicoplast (PF3D7_1118200) (Liu et al. 2014; Seraphim et al. 2014). Most biochemical studies have focused on the inducible, cytosol-localized isoform of *P. falciparum* Hsp90 (PfHsp90α; PF3D7_0708400; Banumathy et al. 2003; Wang et al. 2016).

As part of its unique functional features, Hsp90 is capable of recognizing target proteins that are in a near-native state, thus facilitating the final stages of protein folding (Young et al. 2001). For this reason, it cooperates with Hsp70 which first refolds misfolded proteins before handing them over to Hsp90 for complete folding and functional maturation (Kravats et al. 2018; Daniyan et al. 2019). The formation of the Hsp70-Hsp90 complex is coordinated by the tetratricopeptide repeat (TPR)–rich, Hsp70-Hsp90 organizing protein (Hop) of which the *P. falciparum* Hop (PfHop) protein has previously been characterized (Gitau et al. 2012; Zininga et al. 2015; Silva et al. 2019).

One of the distinctly conserved functions of Hsp90 is its ability to promote protein complex assembly (Makhnevych and Houry 2012). To this end, the clientome of Hsp90 is quite large, spanning over several hundreds of proteins and continues to grow in numbers (Picard 2002; Zhao et al. 2005; Karagoz and Rudiger 2015; Li et al. 2018). Despite this large set of interactors, it was established that the sets of Hsp90 co-chaperones present in eukaryotic organisms are unique as no individual co-chaperones were present in 19 disparate species (Johnson and Brown 2009). Furthermore, it has been established that the type of co-chaperones and their respective ratios when bound to Hsp90 dramatically influence the nature of client proteins that Hsp90 interacts with (Riggs et al. 2003, 2004). Therefore, the plasticity of Hsp90 co-chaperones regulates its functional specificity across species and within the cell.

It has been established through structural and biochemical studies that PfHsp90 is a nucleotide-dependent chaperone that functionally operates as a homodimer *in vivo* (Pallavi et al. 2010; Wang et al. 2016). PfHsp90 monomers characteristically possess an N-terminal domain (NTD) which mediates ATP binding, a middle domain (MD) which interacts with client proteins, co-chaperones and plays a role in ATPase activity, and a C-terminal domain (CTD) which is responsible for Hsp90 dimerization (Hoter et al. 2018). The CTD also possesses a C-terminal Met-Glu-Glu-Val-Asp (MEEVD) motif that is crucial for interaction with TPR domain containing co-chaperones such as PfHop (Gitau et al. 2012; Krysztofinska et al. 2017). PfHsp90 NTD and MD are connected by a long, flexible, charged linker that modulates NTD/MD contacts affecting the Hsp90 function. Notably, cytosolic Hsp90 possesses a longer charged linker and this extended linker interacts with

several co-chaperones containing TPR motifs (Fig. 2; Scheufler et al. 2000; Hoter et al. 2018).

Notably, compared with the linkers of human and yeast Hsp90 homologs, the linker of PfHsp90 is much longer (it is 32 residues longer than that of hHsp90) and further possesses 16 glutamate and aspartate residues, which gives it a highly charged character (Fig. 2). The unique features of the linker of PfHsp90 may thus confer it with the capability to bind a

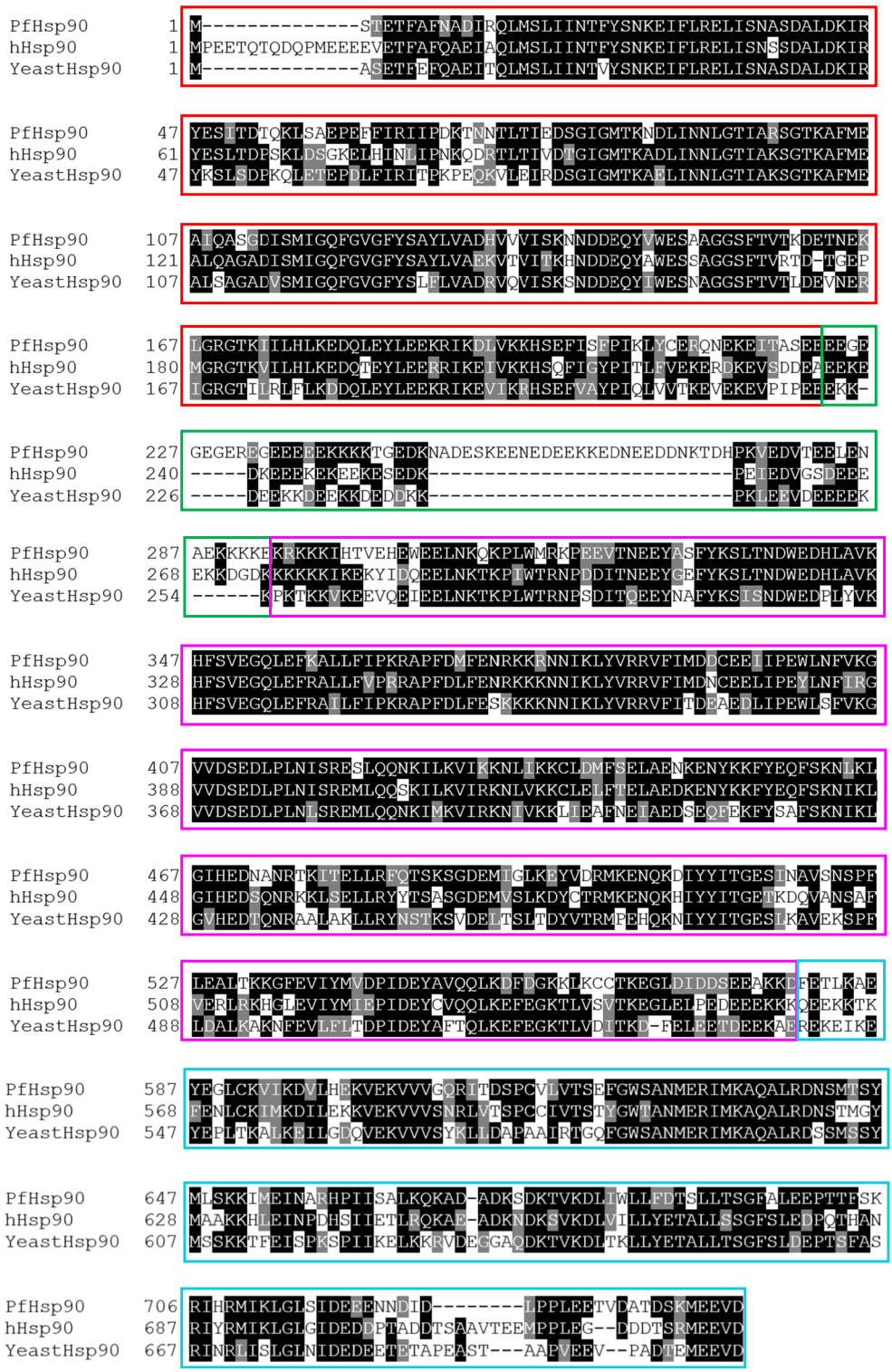

Fig. 2 Multiple sequence alignment of PfHsp90 and human and yeast homologs hHsp90. The NTD is denoted by a red box, linker in green, MD in magenta, and the CTD in blue

unique set of co-chaperones compared with the yeast and human Hsp90s and may also possess a unique mechanism of interacting with co-chaperones.

PfHsp90 activity and conformational dynamics are largely modulated by nucleotides. In the *apo*/ADP states, Hsp90 mainly adopts a V-shaped open conformation (Pallavi et al. 2010). However, upon ATP binding, Hsp90 undergoes drastic conformational rearrangements that lead to a closed conformation at the N-terminal thus allowing client proteins to become engulfed (Halpin et al. 2016). In order for ATP hydrolysis to occur, the ATP-binding site in the NTD must physically interact with the MD (Elnatan et al. 2017). Co-chaperone recruitment facilitates ATP hydrolysis and stabilizes Hsp90 allowing for the maturation and subsequent release of the client protein (Li et al. 2013). Hsp90s generally possess inherently low ATPase activity (hydrolysis rate of 0.1 μM ATP min^{-1} in humans) and ATP affinity (K_d of 400 μM) (Panaretou et al. 1998; Rowlands et al. 2010). Since Hsp90 conformation is tightly coupled to its nucleotide state, co-chaperones direct Hsp90 structural transitions allowing for client protein interaction and its subsequent functional maturation (Chua et al. 2012).

In light of PfHsp90's essential function as a regulator of parasite proteostasis, it remains important to establish its functional partners. It was recently proposed that Hsp90 interacts with multi-protein complexes such as the R2TP complex, which is a key regulator of cell growth and proliferation (Zhao et al. 2005; Rivera-Calzada et al. 2017). The R2TP complex is a specialized Hsp90 co-chaperone required for the assembly and maturation of a range of multi-protein complexes involved in cell growth and proliferation (Kakihara and Houry 2012). Below we highlight the structure-function features of one of the most recently described co-chaperones of *P. falciparum* Hsp90, i.e., the R2TP complex (Ahmad et al. 2013).

R2TP in Plasmodium falciparum

In a systematic genome-wide screen for Hsp90-interacting proteins in budding yeast *Saccharomyces cerevisiae*, the Houry group identified 627 putative Hsp90-interacting proteins. Among them, two unknown proteins were named and further characterized: Pih1 (protein interacting with HSP90-1) and Tah1 (TPR-containing protein associated with HSP90-1) (Zhao et al. 2005). Additionally, it was found that Pih1 and Tah1 tightly bound Rvb1 and Rvb2 to form a four-protein complex that was named R2TP (Rvb1-Rvb2-Tah1-Pih1) (Zhao et al. 2005). Rvb1 and Rvb2, also known in higher eukaryotes as Pontin/RUVBL1 and Reptin/RUVBL2, respectively, are essential and highly conserved helicases ubiquitously found in eukaryotes. The versatility of these proteins and their central role in cellular physiology is remarkable due to their involvement in several critical complexes, such as chromatin remodeling complexes INO80 and SRW-C, and the histone acetyl transferase complex TIP60, among many others (Shen et al. 2000; Jonsson et al. 2001; Cai et al. 2003; Jin et al. 2005; Zhao et al. 2005; Boulon et al. 2008; Venteicher et al. 2008; Zhao et al. 2008; Boulon et al. 2010). Subsequently, R2TP was immunopurified from mammalian cell lysates (Te et al. 2007; Boulon et al. 2008) and identified in *Drosophila* (Benbahouche Nel et al. 2014), establishing R2TP as a widely conserved complex in eukaryotes.

Functionally, the R2TP complex has been shown to work as an assembly factor in the biogenesis of box C/D snoRNPs (Gonzales et al. 2005; Boulon et al. 2008; Zhao et al. 2008; McKeegan et al. 2009; Prieto et al. 2015). R2TP physically interacted with core box C/D snoRNP proteins, and depletion or deletion of R2TP proteins resulted in instability of the snoRNPs (Gonzales et al. 2005; McKeegan et al. 2007; Boulon et al. 2008; Zhao et al. 2008; McKeegan et al. 2009; Prieto et al. 2015). R2TP has also been found as an assembly factor for phosphatidylinositol-3 kinase-related protein kinase (PIKK) signaling complexes (Horejsi et al. 2010; Izumi et al. 2010; Takai et al. 2010; Horejsi et al. 2014), telomerase reverse transcriptase (TERT) core complex (Venteicher et al. 2008), RNA polymerase II (Boulon et al. 2010) and, more recently, ciliary dynein motors (Zur Lage et al. 2018), among other complexes (McKeegan et al. 2007; Bizarro et al. 2015; Cloutier et al. 2017; Malinova et al. 2017). It has been proposed that R2TP acts as an assembly platform for macromolecular complexes, bridging interactions among many proteins (Rivera-Calzada et al. 2017; Martino et al. 2018). However, its exact mechanism of function remains unknown.

The domain organization of R2TP subunits is shown in Fig. 3a. Rvb1/RUVBL1 and Rvb2/RUVBL2 are AAA+ proteins (ATPases associated with diverse cellular activities) containing the canonical αβα subdomain, known as domain I, and the all-α subdomain, or domain III, that come together to form the AAA+ domain (Iyer et al. 2004; Ammelburg et al. 2006. Domain I forms part of the ATPase pocket, constituted by the Walker A and Walker B motifs, along with a highly conserved arginine residue (R-finger). In-between these two domains is the insertion domain (domain II), initially proposed to bind DNA/RNA (Matias et al. 2006). Structural studies have shown that Rvb1 and Rvb2 form single heterohexameric rings that can associate into double heterohexamers (Puri et al. 2007; Torreira et al. 2008; Gorynia et al. 2011; Lopez-Perrote et al. 2012). Pih1, or PIH1D1 (PIH1 domain–containing protein 1) in humans, was found as a phosphopeptide-binding protein interacting with phosphorylated DSDD/E motifs (Horejsi et al. 2014). This protein has an N-terminal PIH1 domain and a C-terminal CS (CHORD-containing proteins and SGT) domain (Pal et al. 2014). Tah1 is a minimal TPR domain–containing protein of 111 amino acid residues that binds to the C-terminus MEEVD

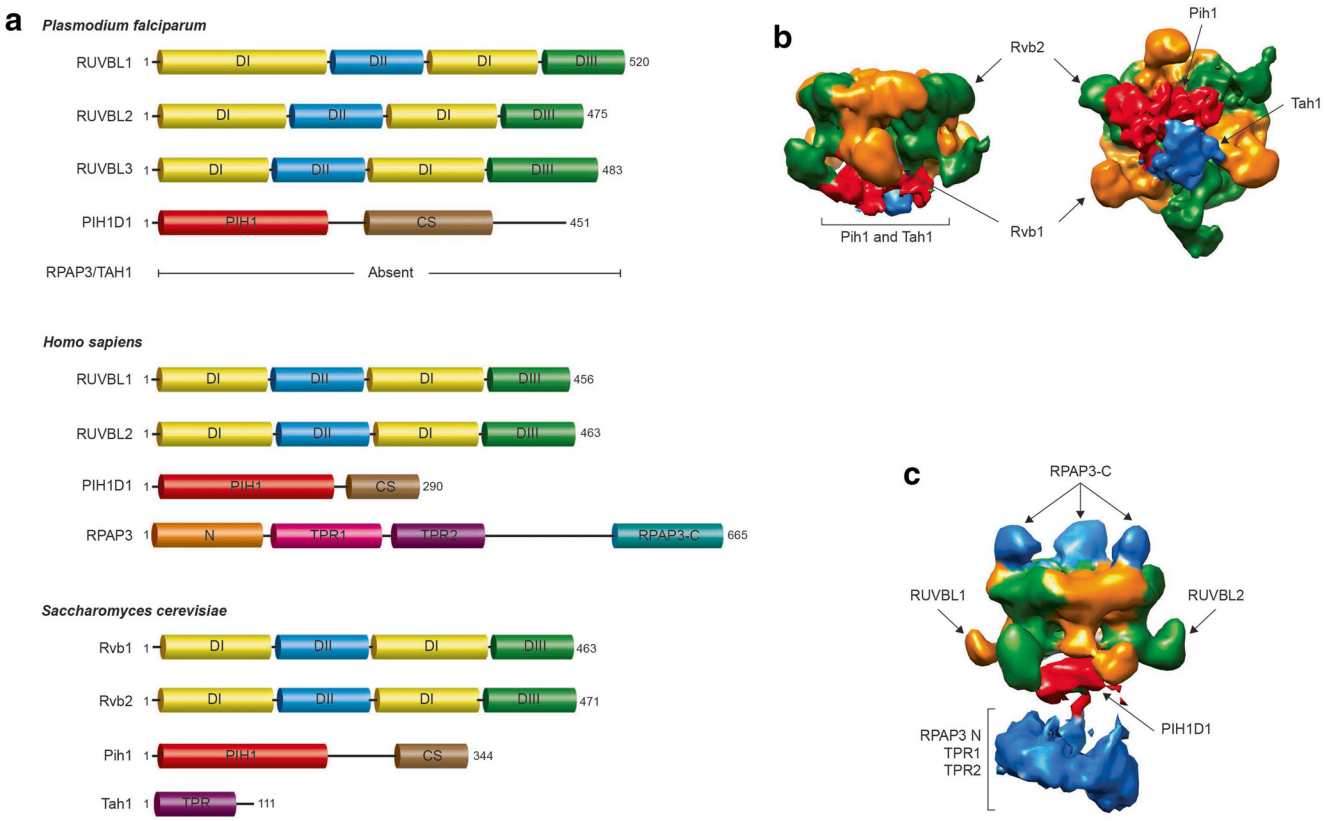

Fig. 3 Domain organization and structure of the R2TP complex. **a** Shown is the domain organization of *Plasmodium falciparum* R2TP proteins (*top*) compared with human (*middle*) and yeast (*bottom*) R2TP subunits. DI, domain I; DII, domain II; DIII, domain III; PIH1, protein interacting with Hsp90-1 domain; CS, CHORD-containing proteins and SGT domain, N, N-terminal domain; TPR, tetratricopeptide repeats-containing domain; RPAP3-C, RPAP3 specific C-terminal domain. Regions with no domains assigned or predicted to be disordered are shown in gray. **b** Cryo-electron microscopy structure of the yeast R2TP complex (EMDB ID: EMD-3678). Pih1 is in red and Tah1 is colored in blue; Rvb1 and Rvb2 are colored in orange and green, respectively. **c** Cryo-electron microscopy structure of the human R2TP complex (EMDB ID: EMD-4290). RPAP3 domains are shown in blue; RUVBL1 and RUVBL2 are colored in orange and green, respectively; PIH1D1 is shown in red

sequence of the Hsp90 molecular chaperone (Back et al. 2013; Morgan et al. 2015). The N-terminal region of Tah1 is formed by two TPR, followed by an unstructured C-terminal region that binds to the CS domain of Pih1 (Jimenez et al. 2012; Pal et al. 2014). In humans, Tah1 is replaced by RPAP3 (RNA polymerase II-associated protein 3), a 665 amino acid residue protein containing an uncharacterized N-terminal domain, two in tandem TPR domains, followed by an unstructured region that binds to PIH1D1, and an α-helical RPAP3-specific C-terminal domain (Maurizy et al. 2018) (Fig. 3a).

Recently, cryo-electron microscopy structures of the yeast and human R2TP complexes were solved (Rivera-Calzada et al. 2017; Tian et al. 2017; Martino et al. 2018) (Fig. 3b, c). In yeast R2TP, the Pih1-Tah1 complex binds to the domain II of the Rvb1/2 hetero-hexamer (Fig. 3b). Interestingly, the human R2TP structure revealed that the RPAP3-specific C-terminal domain binds to the domain III of RUVBL2, while RPAP3 TPR and N-terminal domains, as well as PIH1D1, were found in the proximity of domain II of RUVBL1/2(Fig. 3c).

While there is some information on human and yeast R2TP complexes, little is known about the R2TP complex of *P. falciparum*. Figure 3a shows a comparison of the domain structure of *P. falciparum*, human and yeast R2TP subunits. Three RUVBL genes have been identified encoding for RUVBL proteins in the *P. falciparum* genome (Gangwar et al. 2009): PF3D7_0809700 (PfRUVBL1), PF3D7_1106000 (PfRUVBL2), and PF3D7_1362200 (PfRUVBL3). Sequence analysis has shown that PfRUVBL1 and PfRUVBL2 proteins are closer to yeast and human Rvb1/RUVBL1, whereas PfRUVBL3 is closer to Rvb2/RUVBL2 (Ahmad et al. 2012, 2013; Ahmad and Tuteja 2013a, 2013b; Sen et al. 2018). The similarity between PfRUVBL3 and yeast Rvb2 has been demonstrated, since it was able to complement Rvb2 functions in yeast (Sen et al. 2018). Interestingly, PfRUVBL1 has a longer N-terminal region in comparison with its human and yeast orthologues, yet its functional role is unknown (Ahmad and Tuteja 2013a). Furthermore, Walker A, Walker B, and R-finger motifs were found to be conserved in all three RUVBL proteins in the

malaria parasite (Ahmad et al. 2012; Ahmad and Tuteja 2013a, 2013b; Sen et al. 2018).

PfRUVBL1 has been shown as an active ATPase in the absence and, more robustly, in the presence of single-stranded DNA (ssDNA). It was found to have DNA unwinding activity in the 5′ to 3′ direction, thus exhibiting robust helicase activity (Ahmad and Tuteja 2013a). In contrast, PfRUVBL2 showed ATPase activity only in the presence of ssDNA and a weak helicase activity (Ahmad and Tuteja 2013b). PfRUVBL3 has been found as hexamers and also had ATPase activity but, unlike PfRUVBL1 and PfRUVBL2, PfRUVBL3 ATPase was stimulated by double-stranded DNA (dsDNA) and the recombinant protein did not show helicase activity (Ahmad and Tuteja 2013b; Sen et al. 2018). The deletion of PfRUVBL3 domain II increased the ATPase activity of the protein, suggesting a regulatory role for this domain (Sen et al. 2018). Immunoprecipitation assays revealed that PfRUVBL2 and PfRUVBL3 are part of the same complex, but not PfRUVBL1 (Ahmad and Tuteja 2013b). In fact, the interaction between PfRUVBL3 and PfRUVBL2 positively modulated the ATPase and 5′ to 3′ helicase activities of PfRUVBL2 (Ahmad and Tuteja 2013b). Surprisingly, another activity of PfRUVBL3 was identified. Instead of having helicase activity, PfRUVBL3 displayed ATPase-dependent dsDNA cleavage activity exerted by domain II (Sen et al. 2018). This novel activity was inhibited by doxorubicin, a known inhibitor of eukaryotic topoisomerase type II. This finding has to be further verified. PfRUVBL3 was found interacting with PfMYST, an essential histone acetyltransferase for the intraerythrocytic stage of *P. falciparum* (Miao et al. 2010; Sen et al. 2018). Therefore, it was proposed that PfRUVBL3 may alter DNA topology during histone modification and, as a result, affecting the regulation of transcriptional processes (Sen et al. 2018).

As mentioned above, in the mammalian host, *P. falciparum* has a complex life cycle consisting of exoerythrocytic and intraerythrocytic stages. The latter consists of ring-stage trophozoites, mature trophozoites that undergo schizogony, and merozoites that are released from schizonts to infect new erythrocytes (Seraphim et al. 2014). PfRUVBL1 was found to localize mainly in the nucleus, with minor expression observed in the cytoplasm of parasites in the ring stage, mature trophozoite and merozoites (Ahmad and Tuteja 2013a). PfRUVBL2 was also found primarily in the nucleus of ring-stage and mature trophozoites. However, as soon as mature trophozoites start nuclear division, PfRUVBL2 localization partially changes to cytoplasmic. When merozoites are released from schizonts, PfRUVBL2 becomes mainly nuclear again (Ahmad and Tuteja 2013b). PfRUVBL3 was initially found being expressed only in schizonts and merozoites, localizing in the nucleus (Ahmad et al. 2012). Nevertheless, recent work has shown that PfRUVBL3 is expressed in all the intraerythrocytic stages in *P. falciparum* and that its localization changes over the course of the parasite development. PfRUVBL3 localized in the parasitophorous vacuole during the ring stage, being shuttled to the nucleus in the mature trophozoite stage. During schizogony, PfRUVBL3 was found in punctate structures at the nuclei periphery (Sen et al. 2018).

Little is known about PfRPAP3 and PfPIH1D1. In previous bioinformatics analysis, a gene encoding for a TPR-containing protein was identified as being RPAP3 (Ahmad et al. 2013). The protein encoded by the PF3D7_1434300 gene contains three TPR domains in its 564 amino acid-length structure, similarly to human RPAP3 (665 amino acid residues). However, in addition to the absence of the RPAP3-specific C-terminal domain (Maurizy et al. 2018), a detailed literature survey revealed that this gene, in fact, encodes for the Hsp70/Hsp90-organizing protein (Hop) (Gitau et al. 2012; Zininga et al. 2015; Silva et al. 2019). Hence, so far there is no experimentally verified RPAP3 protein in *Plasmodium*.

The PF3D7_1235000 gene has been identified in *P. falciparum* as being PIH1D1 (PfPIH1D1) (Ahmad et al. 2013). Further analysis revealed that PfPIH1D1 is 161 amino acid residues longer than its human counterpart and 107 amino acids residues longer than yeast Pih1 (see Fig. 3a). Sequence alignment showed that, besides a predicted CS domain, PfPIH1D1 has an extra C-terminal tail. Thus far, cell biology, biochemical, and structural studies have not been reported for *P. falciparum* PIH1D1 protein. The functional role of PfPIH1D1 and PfRPAP3 is still unknown. In addition, stage-specific mechanisms and functional association between PfRUVBL1, PfRUVBL2, and PfRUVBL3, as well as which of them interact with PfPIH1D1 to form the *P. falciparum* R2TP complex remains unclear.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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