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Anopheles gambiae SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*

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We report on a phylogenetic and functional analysis of genes encoding three mosquito serpins (SRPN1, SRPN2 and SRPN3), which resemble known inhibitors of prophenoloxidase-activating enzymes in other insects. Following RNA interference induction by double-stranded RNA injection, knockdown of *SRPN2* in adult *Anopheles gambiae* produced a notable phenotype: the appearance of melanotic pseudotumours, which increased in size and number with time, indicating spontaneous melanization and association with an observed lifespan reduction. Furthermore, knockdown of *SRPN2* strongly interfered with the invasion of *A. gambiae* midguts by the rodent malaria parasite *Plasmodium berghei*. It did not affect ookinete formation, but markedly reduced oocyst numbers, by 97%, as a result of increased ookinete lysis and melanization.

Keywords: malaria; serpin; innate immunity; *Anopheles*; *Plasmodium*
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INTRODUCTION

Anopheline mosquitoes are obligatory vectors of human and other mammalian malaria parasites of the genus *Plasmodium*. Although the means for malaria control exist, many are compromised or inadequately implemented, and the disease burden is still increasing (Greenwood & Mutabingwa, 2002). An innovative strategy to reduce malaria transmission would be the genetic or chemical control of wild mosquito vector populations, to make them inhospitable for the parasite (Christophides *et al*, 2004). Candidate molecular targets that affect parasite development in the mosquito are thus needed. Recent work has established that innate immunity is the principal determinant of the vectorial capacity of the mosquito: *Anopheles* immune reactions account for substantial parasite losses during invasion of the mosquito midgut, but often allow immune escape and parasite transmission.

Anopheles gambiae, the primary malaria vector in Africa, is a principal target of vector-control strategies and serves as a model for other malaria vectors. The availability of its genome sequence (Holt *et al*, 2002) has created unprecedented opportunities for mosquito research. A comparative genomic analysis of putative immune gene families between *Anopheles* and *Drosophila* (Christophides *et al*, 2002) showed that core immune signalling components are well conserved, whereas components implicated in recognition or effector mechanisms are extensively divergent. Thus, innate immunity seems to be under strong diversifying selection, possibly to meet new challenges in species-specific eco-ethological niches. Subsequent functional analysis has identified a few *A. gambiae* factors that regulate development of the murine malaria parasite *Plasmodium berghei*, both positively and negatively (Blandin *et al*, 2004; Osta *et al*, 2004).

Conceptually, negative immunomodulators, protective of the parasite, would be ideal targets for an anti-malaria strategy: their absence or inactivation could lead to increased immune responses, thereby reducing parasite numbers. One well-known class of such negative regulators are serpins, members of a large family of serine protease inhibitors present in all higher eukaryotes as well as in some viruses. Typically, serpins are suicide inhibitors: a target protease cleaves the serpin at its so-called scissile bond (designated P_1–P_1), followed by marked conformational changes, covalent linkage and irreversible inactivation of the protease (Potempa *et al*, 1994). In humans, serpins are involved in numerous functions, including blood clotting and fibrinolytic cascades, inflammation and complement activation, extracellular matrix maintenance or remodelling and apoptosis (Gettins, 2002). Some insect serpins negatively regulate the Toll signalling pathway and the prophenoloxidase (PPO) activation pathway, both central to innate immune reactions against bacteria (Kanost, 1999; Ligoxygakis *et al*, 2002). An *A. gambiae* serpin (SRPN10) is induced by *P. berghei* infection and may regulate apoptosis of invaded midgut cells (Danielli *et al*, 2003, 2005). Here, we report on phylogenetic, genetic and molecular studies that identify the presence of SRPN2 as necessary for successful *P. berghei* oocyst development in *A. gambiae*. This is the first inhibitory serpin associated with the control of mosquito vectorial capacity.

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RESULTS AND DISCUSSION

SRPN1 and SRPN2 resemble known PPAE inhibitors

To identify *A. gambiae* SRPNs that regulate immune reactions important for *Plasmodium* development, we first performed a detailed phylogenetic analysis of all known full-length arthropod serpins. The steadily increasing number of expressed sequence tag (EST) and complementary DNA sequences in the public domain allowed us to improve the earlier annotation of 15 SRPNs in the genome of *A. gambiae* (Christophides *et al*, 2002). We discovered another serpin gene, denoted as ENSANGG00000024665 in the second release of the ENSEMBL genome annotation (Hubbard *et al*, 2005), and named it *SRPN16*. Expression of the protein-coding regions of all *Anopheles* SRPNs was verified by reverse transcription–PCR (RT–PCR; data not shown). We then used the deduced amino-acid sequences, together with all available full-length arthropod inhibitory serpin sequences (as predicted by conservation of sequence motifs required for function), to reconstruct their phylogenetic relationships by Baysian inference (Fig 1A; also see supplementary information online).

SRPN1–3 cluster together with all PPO-activating enzyme (PPAE)-inhibiting serpins known from other insects, with high posterior probability, indicating that one or more of these *Anopheles* SRPNs could function as inhibitors of the melanization cascade. Phylogenetic analysis (Fig 1A,B) identified them as members of an orthologous group including the *Drosophila* melanization-related spn27A and the two known lepidopteran PPAE-inhibiting serpins. SRPN1–3 were also identified as 1:1 orthologues of three corresponding *Aedes aegypti* serpins (Fig 1A) that are represented in the available collection of yellow fever mosquito BAC end and cDNA sequences (http://www.tigr.org/tdb/e2k1/aabe). Thus, it seems that an ancestral *PPAE* inhibitor gene underwent two consecutive duplications after the divergence of mosquitoes from sandflies and before the divergence of *Aedes* and *Anopheles*, giving rise to SRPN1–3. Consistent with this hypothesis is the close physical clustering of *SRPN1–3* in a 10.4 kb fragment in the chromosomal subdivision 2L-23D.

Target specificity of inhibitory serpins is controlled by the sequence and tertiary structure of their reactive centre loop, which allows binding and cleavage by target proteases. It is known that P1–P1′ residues (NK) in the *Drosophila melanogaster* spn27A (De Gregorio *et al*, 2002; Ligoxygakis *et al*, 2002), the lepidopteran *Manduca sexta* sp3 (Zhu *et al*, 2003) and *Hyphantria cunea* serpin (Park *et al*, 2000) sequences match the activation cleavage site of the conspecific PPOs. The hypothesis that this sequence match allows serpin inhibition of the respective PPAEs is consistent with both the ability of all three serpins to control haemolymph-derived melanization, and with *in vitro* studies on *Manduca* sp3 (Zhu *et al*, 2003). The NK P1–P1′ residues are conserved both in SRPN1 and SRPN2 and in eight out of the nine *Anopheles* PPOs (with the exception of PPO9; Fig 1C). Therefore, we considered SRPN1 and SRPN2 as potential inhibitors of melanization in *A. gambiae*.

SRPN2 knockdown causes spontaneous melanization

To test this hypothesis, serpin function was analysed by reverse genetics. Adult female *A. gambiae* were injected with double-stranded RNAs (dsRNAs) targeting *SRPN1*, *SRPN2* or *SRPN3*, and changes in messenger RNA levels were assessed by quantitative RT–PCR, using mRNA levels in double-stranded green fluorescent protein (dsGFP)-injected control mosquitoes for calibration. Reduction was significant in every case (unpaired *t*-test, $P \leqslant 0.03$), and ranged from 50% for *SRPN1* and *SRPN2* to 78% for *SRPN3* (Fig 2A). Similar results were obtained using alternative dsRNAs targeting different regions of SRPN1 and SRPN2 (data not shown). In our experience, RNA analysis underestimates the extent of gene silencing. Indeed, immunoblot analysis of haemolymph samples from dsRNA-treated adult females, using SRPN1- and SRPN2-specific antibodies, showed a complete absence of SRPN1 or SRPN2 proteins 4 days after their dsRNA treatment (Fig 2B). This result verified the effective knockdown of SRPN expression and showed target specificity of the dsRNA treatment.

Although no visible effects were detected for the SRPN1 and SRPN3 knockdowns, SRPN2-depleted mosquitoes developed widespread melanotic masses, often referred to as melanotic (pseudo)tumours (Sparrow, 1978; Fig 3A), either attached to the body wall (Fig 3Ba) or floating in the haemolymph. Melanization was associated with several tissues, including the midgut, ovaries and pericardial cells (Fig 3Bb). Melanotic masses were visible 2 days after dsRNA injection and progressively increased in size, reaching 100% prevalence by day 4 (Fig 3C). Furthermore, the SRPN2 knockdown reduced significantly the lifespan of adult mosquitoes compared with the dsGFP-treated controls, causing a threefold decrease in survival by day 10 post-injection (Fig 3D).

As SRPN2 depletion causes spontaneous melanization, we conclude that SRPN2 normally inhibits the melanization response. The phenotype of SRPN2 depletion is more severe than that of the spn27A-null mutant in *D. melanogaster* (De Gregorio *et al*, 2002; Ligoxygakis *et al*, 2002). We speculate that differences in lifestyle and encountered parasites or pathogens make melanization and its control more crucial for the physiology of adult mosquitoes than of flies. This supposition may also explain the threefold higher number of mosquito PPOs and their differential expression profiles during development (Christophides *et al*, 2002).

SRPN2 knockdown affects *P. berghei* infection

To assess the potential effects of silencing *SRPN1*, *SRPN2* or *SRPN3* on parasite development, we infected susceptible *A. gambiae* 4 days after dsRNA injection with the *P. berghei* GFP-CON transgenic parasite strain (Franke-Fayard *et al*, 2004). This parasite expresses GFP constitutively at all life stages and facilitates the quantitative monitoring of infection by fluorescence. Infection levels were scored by the number of oocysts per midgut 7 days post-infection (for a review on *Plasmodium* life stages in the mosquito, see Sinden, 1999). Table 1 summarizes and statistically evaluates the results of several independent experiments. RNA interference (RNAi) targeting SRPN1 and SRPN3 had no significant effect on the prevalence of infection or parasite load, compared with the control (*U*-test, $P = 0.55$ and 0.97, two tailed). In contrast, the *SRPN2* knockdown showed marked and statistically significant effects ($P < 2^{-6}$): infection prevalence was reduced by 55% and parasite load by 97%.

To address the question of whether this 32-fold loss of parasites occurs before, during or after midgut invasion and to show the dynamics of underlying processes, invading parasites were scored by confocal microscopy at 24 h, 36 h, 48 h, 72 h and 7 days post-infection (Fig 4; supplementary Fig S2 online) in midguts dissected and completely freed of the blood meal. Three

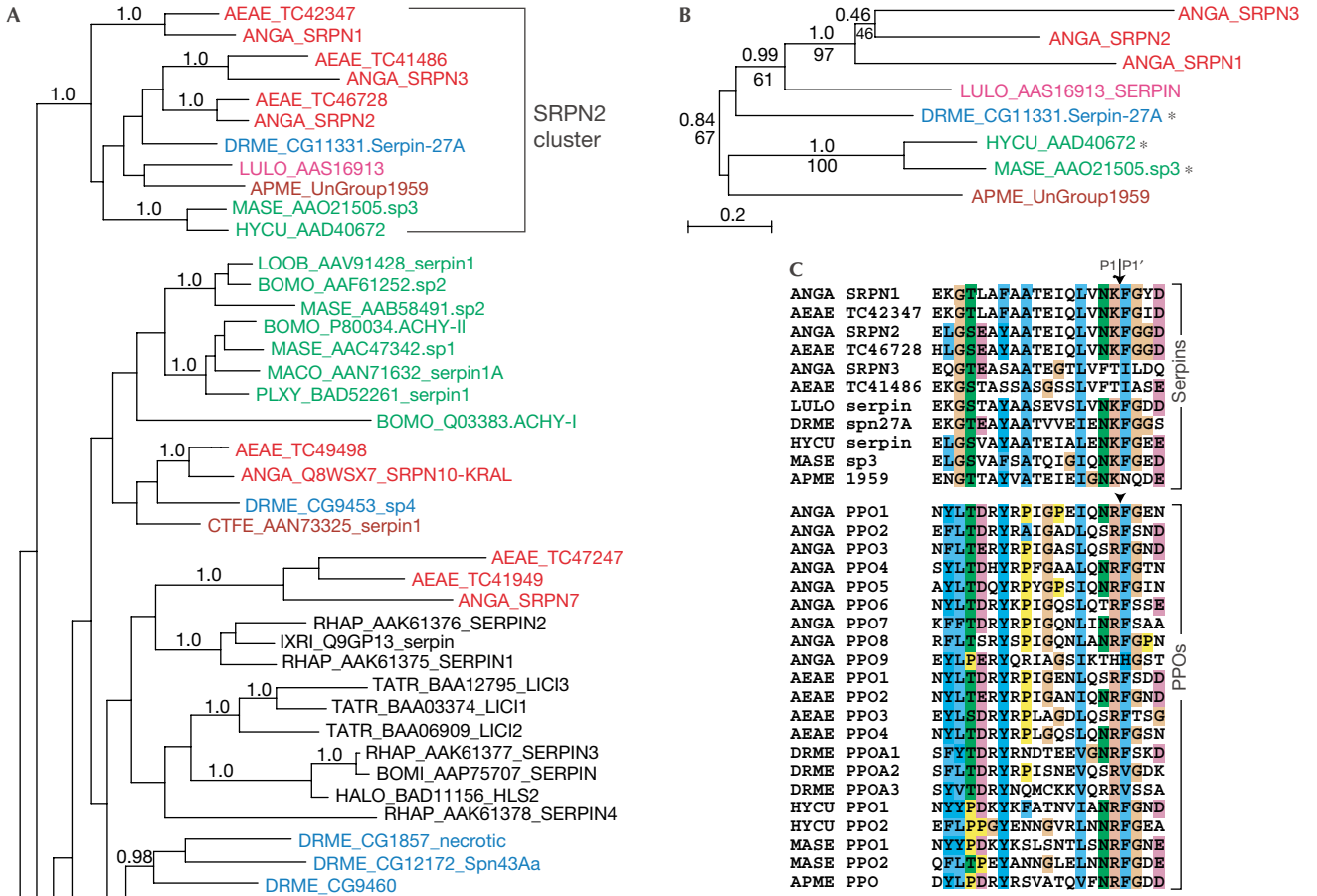

Fig 1 | Phylogenetic analysis of arthropod serpins. (**A**) Baysian inference of phylogenetic relationships of arthropod serpins. SRPN1–3 form an orthologous group with other insect serpins that are known negative regulators of prophenoloxidase (PPO)-activating enzymes (PPAEs). Red entries correspond to mosquitoes, pink to other Nematocera, blue to *Drosophila melanogaster*, green to Lepidoptera, brown to other insects and black to Crustacea and Chelicerata. The tree was estimated from 179 aligned residues. Sequence alignment, species names and accession numbers are given in the supplementary information online. (**B**) Phylogenetic analysis of the PPAE-inhibitory serpins. Proteins that have been shown biochemically to inhibit PPAEs are marked with an asterisk. The tree was estimated from 264 aligned residues. (**C**) Sequence similarities of reactive centre loops (P1–P1′ cleavage sites; arrow) of SRPN2-like serpins (SRPN2 cluster in (**A**)) with the activation cleavage sites of PPOs from the same species (arrowhead). Note the strong similarities in residues flanking the serpin and corresponding PPO sites, and the resemblance of the neighbouring amino-acid environments.

phenotypic classes of parasite were distinguishable (Fig 4A, insets). The first, GFP+ class combined three features: GFP fluorescence (a marker for living parasites; Blandin *et al*, 2004); staining with monoclonal P28 antibody (which detects a *P. berghei* surface molecule of zygotes, ookinetes and early oocysts; Winger *et al*, 1988); and normal parasite shape (banana-shaped ookinetes, mainly at 24 h and rarely at 36 h, and round oocysts from 36 h onwards; white arrows and arrowheads in

Fig 4B; supplementary Fig S2 online). Class 2 parasites (P28-only; white asterisks in Fig 4B and supplementary Fig S2 online) showed P28 staining, but no GFP fluorescence, and often had irregular shapes, accompanied by membrane blebbing and nuclear condensation, indicative of lysis. Class 3 (melanized) parasites showed a melanin sheath under light microscopy (Fig 4A, inset), and were only observed in the *SRPN2* knockdown mosquitoes.

The potential role of SRPN2 in these processes, and their temporal dynamics, can be summarized as follows. SRPN2 depletion does cause early death. Depletion reduces the number of GFP + parasites (relative to controls); this disparity is detectable by 24 h, statistically significant by 32 h (*U*-test, $P<0.01$) and continues thereafter. In control midguts, parasite death is also early, although less frequent: it begins before 24 h and comes to an end between 48 h and 72 h. Parasite lysis is also early, becoming prominent in both control and experimental midguts by 24 h. *SRPN2*-knockdown-associated melanization begins by 24 h and continues to 48 h. SRPN2 depletion does not cause significant clearance before invasion: overall parasite numbers do not differ significantly between experimental and control midguts during the peak of invasion (24 h).

Ultimately, 97% of all invading parasites are lost in *SRPN2* knockdown compared with control mosquitoes: 18% are melanized and 79% are lysed and cleared.

Taken together, our results have established that SRPN2 dampens melanization and melanotic pseudotumour formation, and that its presence protects parasites during invasion and development on the midgut basal surface. The mechanisms of this syndrome remain to be explained. Given the close phylogenetic relationship of SRPN2 with known PPAE-inhibiting serpins, sequence identity at their P1–P1′ residues and similarity with the PPO activation site, it is highly probable that SRPN2 directly inhibits one or more PPAEs in *A. gambiae*. A parsimonious hypothesis would ascribe the remaining phenotypes to the same molecular function: for example, the predominant lytic loss of parasites in the absence of SRPN2 may result from overproduction and circulation of toxic by-products during melanin synthesis. An alternative, tenable hypothesis is that SRPN2 is implicated in further, independent modules of innate immunity; for example, serine protease cascades that directly induce parasite lysis. The *A. gambiae* genome encodes 305 serine proteases (Holt *et al*, 2002), of which 41 include CLIP domains, suggesting regulatory functions. Systematic reverse genetic and biochemical analysis will be required to analyse causally this and other mosquito immune syndromes.

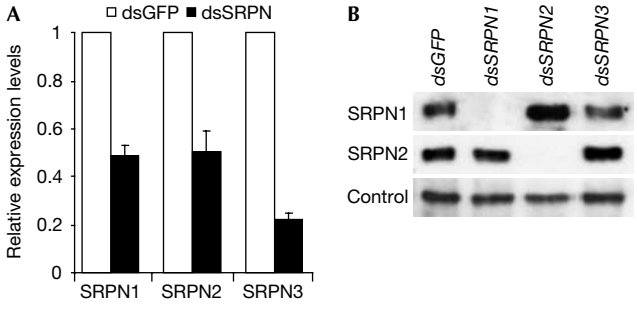

Fig 2 | RNA interference efficiency. (**A**) Expression levels were measured by quantitative RT–PCR 4 days after dsRNA injections, with dsGFP-treated samples as the calibrator for each treatment. (**B**) Immunoblot of 100 ng haemolymph proteins isolated from mosquitoes treated with *dsSRPN1*, *dsSRPN2* or *dsGFP* as a control. The same blot was probed with rabbit anti-SRPN1 antibody (1:1,000), stripped and re-probed with rabbit anti-SRPN2 antibody. Loading control was detected with anti-TEP1 antibody (1:3,000).

METHODS

Insect and parasite cultures, and infections. The *A. gambiae* G3 strain was reared according to Richman *et al* (1996). The *P. berghei* GFP-CON strain (Franke-Fayard *et al*, 2004) was passaged through CD1 or Balb/C mice and infections were performed using standard procedures (Sinden, 1996). To determine parasite prevalence and load, midguts were dissected 8 days post-infection and parasite morphology and number were determined by fluorescent light microscopy (Zeiss, Jena, Germany).

RNA isolation and cDNA synthesis. Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with deoxyribonuclease I. First strand cDNA syntheses were performed in a 20 μl reaction volume with 3 μg of total RNA, using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) and 200 U of Superscript Reverse Transcriptase II (Invitrogen). The quality of the *Anopheles* cDNAs was checked by PCR, using primers corresponding to the ribosomal protein S7, as described previously (Richman *et al*, 1996).

Real-time quantitative reverse transcription–PCR. Amplifications were performed with SYBR Green PCR mastermix and analysed using the ABI PRISM 7700 sequence detection system following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Expression levels were calculated by the relative standard curve method, as described in Technical Bulletin #2 of the ABI Prism 7700 Manual (Applied Biosystems), using (i) S7 as an endogenous reference and (ii) an exogenous calibrator

Table 1 | SRPN2 influences vectorial capacity for *Plasmodium berghei*

dsRNA	Number of experiments	Total number of midguts	Prevalence (%)	Parasite load	Range of oocyst numbers	*U*-test
Control	4	60	85	116	0–587	$P=0.55$
SRPN1	4	60	85	137	0–755	
Control	7	100	90	136	0–591	$P<2^{-6}$
SRPN2	7	100	41	4	0–60	
Control	4	51	86	140	0–614	$P=0.97$
SRPN3	4	51	88	103	0–395	

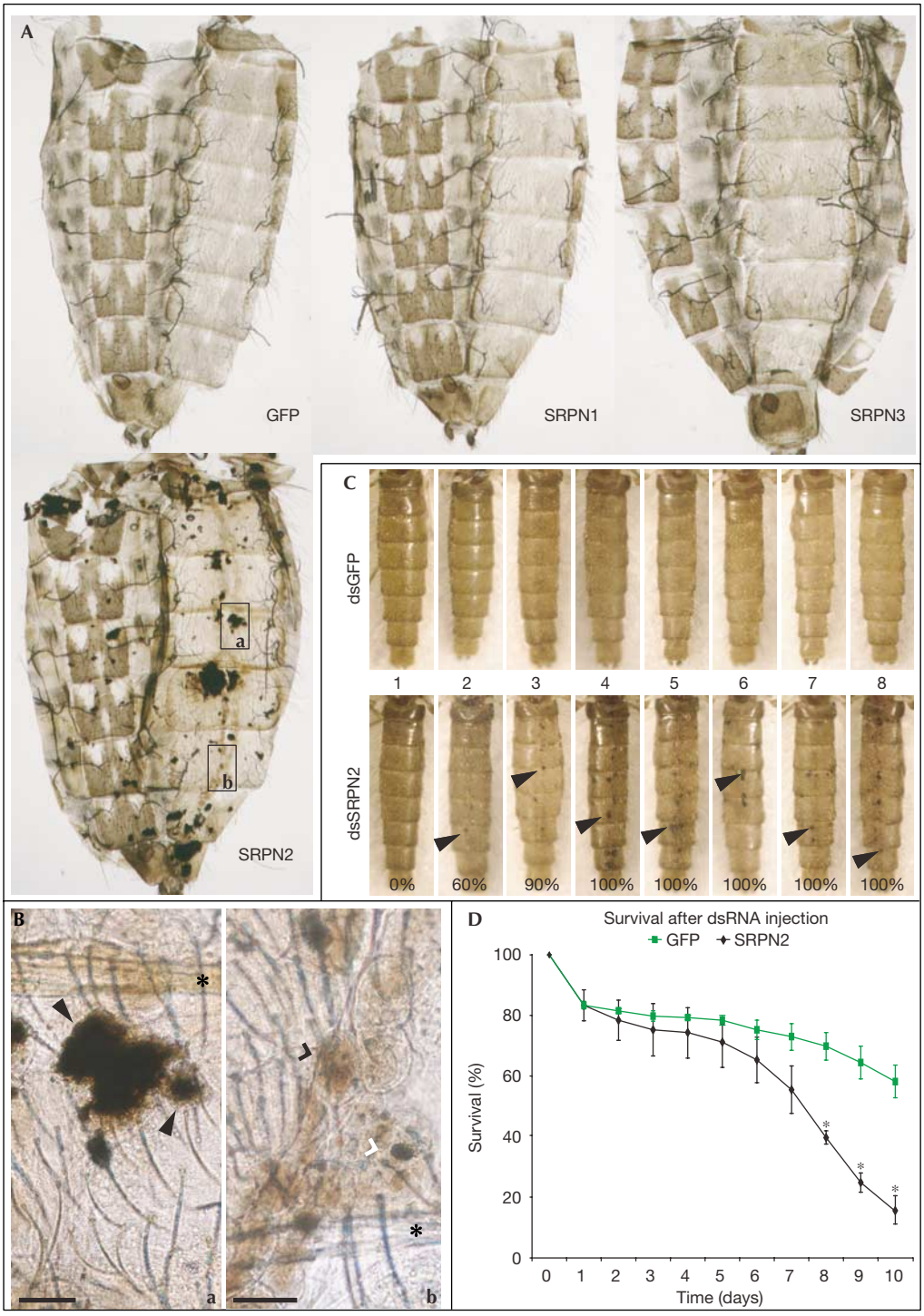

Fig 3 | SRPN2 RNA interference-induced phenotype in G3 female *Anopheles gambiae*. Adult females were injected with double-stranded RNA (dsRNA) against SRPN1, 2 or 3 and dsGFP as a control. (**A**) Dissected abdomens, 10 days after dsRNA injection, showed melanotic pseudotumours. (**B**) Magnified insets from (**A**). Pseudotumours were found attached to the body wall, possibly associated with the body fat (a; black arrowheads). Note the pale segment boundary (black asterisk). Pericardial cells (b) were also melanized, either diffusely (black open arrowhead) or localized (white open arrowhead). Scale bar, 50 μm. (**C**) Melanotic pseudotumours were first observed 2 days after dsRNA injection and reached 100% prevalence at 4 days (black arrowheads). (**D**) The graph shows relative percentages of surviving mosquitoes after dsRNA treatment targeting SRPN2 and GFP (green fluorescent protein; black and green, respectively), where the number of mosquitoes injected represented 100%. Asterisks indicate significant differences in survival (unpaired *t*-test, $P \leqslant 0.002$).

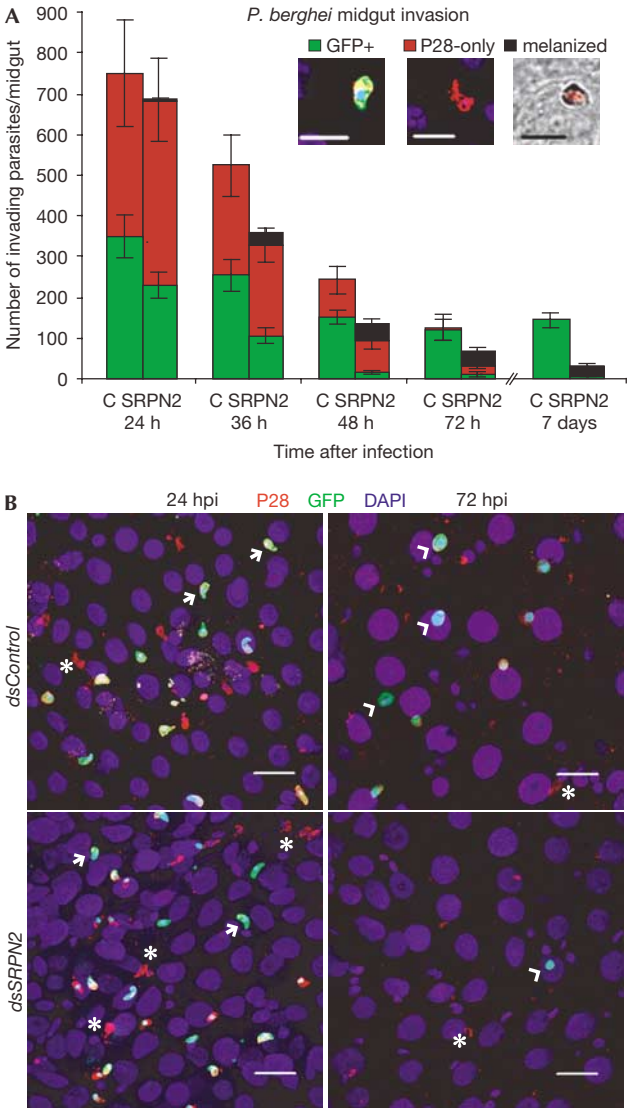

Fig 4 | Dynamics of ookinete to oocyst transition, and effects of *SRPN2* knockdown. Mosquitoes were injected 2 days after emergence with double-stranded RNAs targeting *SRPN2* or *GFP* (green fluorescent protein; control, C), and were infected 4 days post-infection with the *Plasmodium berghei* GFP-CON strain. (**A**) Bar graphs, see inset key, represent temporally changing GFP fluorescent parasites (green), parasites labelled with P28 only (red) and melanized parasites (black). Insets show confocal micrographs of these parasite classes (scale bar, 10 μm). Error bars represent standard error in the pooled data set of three independent biological experiments. (**B**) Three-dimensional projections of midguts dissected and stained with anti-P28 antibody (1:500) 24 h and 72 h after the infectious blood meal. hpi, hours post-infection. Living ookinetes (white arrows) and oocysts (white open arrowheads) appear green and yellow, as they are double labelled with anti-P28 antibody (red) and GFP (green). White asterisks indicate P28-only parasites that are in the process of lysis. DAPI, 4,6-diamidino-2-phenylindole. Scale bar, 20 μm.

appropriate to the experiment. Primers were designed using Primer Express™ software (Applied Biosystems). Primer pairs and sequences are listed in supplementary Table S1 online.

Double-stranded RNA production and injection. dsRNA was produced from plasmids pll6ds (GFP control; Levashina *et al*, 2001), pll1.1, 2.3 and 3.3 (SRPN1, SRPN2 and SRPN3, respectively) and injected as described previously (Blandin *et al*, 2002). Plasmids pll1.1, 2.3 and 3.3 were constructed as follows. *SRPN* cDNA was amplified using the following primer pairs: for SRPN1, 1.1f and 1.1r; for SRPN2, 2.3f and 2.3r; and for SRPN3, 3.3f and 3.3r (for sequences, see supplementary Table S1 online). Fragments were cloned into pGEMTEasy vector (Promega, Madison, WI, USA), creating clones pGEM1.1, 2.3 and 3.3. Subsequently, pGEM1.1 was cut with *Not*I and pGEM2.3 and 3.3 with *Eco*RI, and subcloned into pll10.

Anti-SRPN1 and anti-SRPN2 polyclonal antibody production. Chimeric proteins composed of glutathione-*S*-transferase (GST) fused to SRPN1 or SRPN2 partial gene product were produced from pGEXSRPN1 and pGEXSRPN2 expression vectors. SRPN1 and SRPN2 fragments, encoding amino acids 92–295 and 216–409, respectively (numbering according to ENSEMBL annotation), were amplified using cDNA from 2-day-old naive females and cloned into pGEMTeasy vector (for primer pairs and sequences, see supplementary Table S1 online). Subsequently, a *Bgl*II–*Sal*I fragment of SRPN1 and a *Bam*HI–*Sal*I fragment of SRPN2 were subcloned into the *Bam*HI–*Sal*I sites of the pGEX4T3 expression vector (Amersham Biosciences, Piscataway, NJ, USA). Recombinant GST–SRPN1 and GST–SRPN2 fusion proteins were expressed in the *Escherichia coli* BL21 (DE3) strain and purified from inclusion bodies using standard procedures. Two rabbits received primary immunization with 250 μg of recombinant protein in R730 adjuvant (RIBI Immunochem Research, Hamilton, MT, USA), which was boosted subsequently every 28 days. Terminal bleeds were obtained at week 18.

Western blotting. Haemolymph was collected from adult *A. gambiae* and blotted, as described previously (Danielli *et al*, 2000). Protein content was measured using a Bradford assay. SRPN1 and SRPN2 antisera were used at a 1:1,000 dilution and bound antibodies were detected with a 1:20,000 dilution of anti-rabbit IgG conjugated with horseradish peroxidase (Promega) and the Western Lightning Chemiluminescence Reagent Plus Kit (Perkin–Elmer, Wellesley, MA, USA).

Immunohistochemistry and confocal microscopy. Immunohistochemistry on dissected midgut tissues was performed as described previously (Danielli *et al*, 2000). Midgut tissues were incubated with primary antibody P28 (1:500; a kind gift from R.E. Sinden) followed by incubation with secondary anti-mouse Alexa546 antibody (Molecular Probes). Nuclei were stained with 4,6-diamidino-2-phenylindole. Midguts were mounted in Anti-Fade (Molecular Probes) and examined with an LSM510 META confocal microscope (Zeiss).

Phylogenetic analysis. For details, see supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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