Vector transmission regulates immune control of Plasmodium virulence

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Defining mechanisms by which Plasmodium virulence is regulated is central to understanding the pathogenesis of human malaria. Serial passage of Plasmodium through rodents1–3, primates4 or humans5 increases parasite virulence, suggesting that vector transmission regulates Plasmodium virulence within the mammalian host. In agreement, disease severity can be modified by vector transmission6–8, which is assumed to ‘reset’ Plasmodium to its original character. However, direct evidence that vector transmission regulates Plasmodium virulence is lacking. Here we use mosquito transmission of serially blood passed (SBP) Plasmodium chabaudi to interrogate regulation of parasite virulence. Analysis of SBP P. c. chabaudi before and after mosquito transmission demonstrates that vector transmission intrinsically modifies the asexual blood-stage parasite, which in turn modifies the elicited mammalian immune response, which in turn attenuates parasite growth and associated pathology. Attenuated parasite virulence associates with modified expression of the pir multi-gene family. Vector transmission of Plasmodium therefore regulates gene expression of probable variant antigens in the erythrocytic cycle, modifies the elicited mammalian immune response, and thus regulates parasite virulence. These results place the mosquito at the centre of our efforts to dissect mechanisms of protective immunity to malaria for the development of an effective vaccine.

The definitive host for mammalian Plasmodium is the anopheline mosquito. Within this vector, a complex series of developmental events, including fertilization and meiosis, culminates in invasion of the salivary glands by infective sporozoites, which are transmitted to the mammalian host through mosquito bite. Sporozoites deposited in the dermis migrate to the liver, invade hepatocytes and undergo further developmental processes before the release of merozoites that invade erythrocytes. The subsequent erythrocytic cycle is entirely responsible for the morbidity and mortality associated with malaria. The complexity of the Plasmodium life cycle has led to much of the basic biology of the blood-stage infection being studied in isolation, with in vivo experiments largely initiated through direct injection of infected erythrocytes. However, serial blood passage of Plasmodium increases parasite virulence9–11, suggesting that regulation of Plasmodium virulence is an inherent consequence of vector transmission12. This could result indirectly from vector control of inoculum size or the passage of large parasite populations through extreme bottlenecks, although these consequences of mosquito transmission are not thought to be major determinants of disease severity13,14. Alternatively, vector transmission may regulate Plasmodium virulence by intrinsically modifying the parasite and its interaction with the mammalian host. In this context, the immune response elicited by Plasmodium influences disease severity15, and can therefore dictate parasite virulence. The interrelationship between the vector, parasite and mammalian immune system could thus underpin the pathogenesis of malaria.

To study regulation of Plasmodium virulence we developed routine mosquito transmission of SBP P. c. chabaudi, a rodent malaria parasite that has many characteristics associated with the pathogenesis of human infection16. This allowed us to directly compare SBP parasites before and after vector transmission. Accordingly, mice were infected with SBP P. c. chabaudi AS either by injection of parasitized erythrocytes (pE) or mosquito bite (see Methods). Following mosquito transmission, asexual blood-stage parasite growth was attenuated (Fig. 1a), and a low-grade, recrudescent infection with extended chronicity was established (Supplementary Fig. 1). Attenuated parasite growth in the erythrocytic cycle was not influenced by dose (ref. 9 and Supplementary Fig. 2) or, importantly, by the pre-erythrocytic stages of infection, as attenuated parasite growth was similarly observed when mice were injected with pE derived from recently mosquito-transmitted (MT) parasite lines (Fig. 1b). Similar results were observed with cloned parasites derived from SBP P. c. chabaudi AS (Supplementary Fig. 3), and with the hypervirulent P. c. chabaudi CB (Supplementary Fig. 4). Mosquito transmission therefore attenuated the asexual blood-stage parasite. As expected, serial blood passage of MT P. c. chabaudi AS rapidly increased parasite growth (Supplementary Fig. 5). Mice infected with P. c. chabaudi AS through mosquito bite did not show the severe hypothermia, cachexia or hepatic cellular damage that was observed during the acute phase of infection in mice injected with SBP parasites, although they still showed severe anaemia despite attenuated parasite growth (Fig. 1c–f). Mosquito transmission therefore reduced disease severity in the mammalian host. Despite attenuated parasite growth and reduced pathogenicity, MT P. c. chabaudi AS elicited robust, long-term protection to reinfection with homologous or heterologous blood-stage parasites (Fig. 1g and Supplementary Fig. 6). Thus, vector transmission regulates the virulence of Plasmodium by intrinsically modifying the asexual blood-stage parasite, without influencing the capacity of the mammalian host to acquire robust immunity to reinfection.

The pathogenesis of malaria is complex and influenced by the mammalian immune system; dysregulated immune reactions can directly promote severe disease17, whereas an appropriate response can enhance parasite clearance without promoting pathology18. The immune response induced by Plasmodium can therefore define its virulence. Throughout the erythrocytic cycle the spleen is the major anatomical site associated with the developing immune response19,20, and mice infected with P. c. chabaudi AS through mosquito bite developed marked splenomegaly with rapid recruitment of inflammatory monocytes (Supplementary Figs 7 and 8). Importantly, following mosquito transmission there was enhanced expansion of activated CD8+ T cells, which present malaria-specific antigens and stimulate CD4+ T-cell proliferation17, in the acute phase of infection (Fig. 2a and Supplementary Fig. 9). Correspondingly, the magnitude of the effector CD4+ T-cell response, which orchestrates innate and adaptive immune control of blood-stage parasite growth17, was also enhanced following mosquito transmission, and the memory CD4+ T-cell population showed a predominantly effector memory phenotype (Fig. 2b and Supplementary Fig. 10). Infection with MT P. c. chabaudi AS also increased the magnitude of the class-switched malaria-specific antibody response, a central component of erythrocytic immunity17.
Parasite growth and pathogenicity are, in part, determined by host susceptibility. Infection of susceptible mouse strains with *P. c. chabaudi* AS through mosquito bite causes severe disease and death, demonstrating that vector transmission does not limit the potential virulence of the asexual blood-stage parasite. We therefore addressed whether attenuated parasite virulence in *C57BL/6* mice infected with MT *P. c. chabaudi* AS was a consequence of the transformed host immune response. Immunodeficient mice were infected with SBP *P. c. chabaudi* AS, or with an equivalent number of pE derived from a recently MT line. Disruption of the innate and adaptive immune responses, through depletion of CD4$^+$ (open bars) and CD8$^+$ (closed bars) dendritic cells (a), and effector (T_E) (open bars) and memory (T_M) (closed bars) CD4$^+$ T cells (b) in spleens of *C57BL/6* mice injected with $10^5$ SBP Pcc AS or infected with Pcc AS through mosquito bite. Data presented as arbitrary units (AU) relative to hyper-immune plasma. e, Plasma cytokine concentration in *C57BL/6* mice injected with $10^5$ SBP Pcc AS or infected with Pcc AS through mosquito bite. (n = 3–5 mice per group per time-point; data presented as mean ± s.e.m.).
adaptive immune responses elicited by, and directed against, the blood-stage parasite. Vector transmission of \textit{Plasmodium} thus intrinsically modifies the asexual blood-stage parasite, which in turn modifies the elicited mammalian immune response, which in turn regulates parasite virulence.

Defining parasite gene expression in the erythrocytic cycle after vector transmission is thus central to understanding the pathogenesis of malaria. We therefore performed genome-wide RNA sequencing on \textit{P. c. chabaudi} AS, directly comparing blood-stage parasites before and after mosquito transmission. This allowed us to identify a set of \textit{Plasmodium} virulence genes that direct the elicited mammalian immune response (Fig. 4). Vector transmission modified expression of approximately 10% of the entire genome in the late trophozoite stage parasite (Supplementary Tables). The majority of genes upregulated following mosquito transmission encoded exported proteins, with the potential to access and modulate the mammalian immune system. Importantly, parasite gene expression was most intensely regulated within the sub-telomeric large multi-gene families, with preferential regulation of the \textit{pir} multi-gene family (termed \textit{cir} in \textit{P. c. chabaudi}) (Supplementary Fig. 12). Out of 200 \textit{cir} genes, 123 (61.5%) were differentially expressed following mosquito transmission, with 114 \textit{cir} genes (57%) upregulated. Furthermore, the most upregulated gene following serial blood passage was identified as the most highly expressed \textit{cir} gene (PCHAS_110030) in mice infected with \textit{SBP P. c. chabaudi} AS (ref. 17, Fig. 4 and Supplementary Fig. 12). Serial blood passage therefore selected for dominant \textit{cir} gene expression, whereas mosquito transmission revoked the selected expression hierarchy and promoted a generalized increase in \textit{cir} expression across the parasite population. We therefore uncovered a direct association between \textit{pir} gene expression and \textit{Plasmodium} virulence, and demonstrate that vector transmission regulates expression of probable antigenic variants\textsuperscript{18}, as proposed previously\textsuperscript{19,20}. Vector transmission of \textit{Plasmodium} thus regulates parasite gene expression in the erythrocytic cycle and, consequently, regulates immune control of \textit{Plasmodium} virulence.

Vector transmission will inherently regulate \textit{Plasmodium} virulence within the mammalian host. Recombination of distinct parasite genotypes within the mosquito is likely to be fundamental for the evolution of virulence\textsuperscript{21}. The results of this study reveal that vector transmission also regulates \textit{Plasmodium} virulence by modifying parasite gene expression, and therefore the mammalian immune response, in the erythrocytic cycle. This is probably the outcome of a combination of distinct regulatory processes acting at multiple stages of the parasite life cycle, in both the mosquito vector and the mammalian host. It is therefore important to delineate the timing and mechanism(s) of regulation of parasite gene expression, in the context of the complete \textit{Plasmodium} life cycle, to understand the molecular regulation of parasite expression levels for each gene are also shown (inner circles). Sepia wedges highlight genes whose products are predicted to be exported, or otherwise accessible to the mammalian immune system.
virulence. Attenuation of parasite virulence following mosquito trans-
mission associates with modified expression of the pir multi-gene family,
which is conserved from rodent to human Plasmodium13. Importantly,
vector transmission of cultured Plasmodium falciparum similarly modi-
fies the composition and frequency of var gene expression23. Regulation
of antigenic variants by vector transmission is therefore universal19,20,22,
and vector transmission will therefore universally regulate immune con-
trol of Plasmodium virulence. The interrelationship between the vector,
parasite and mammalian immune system thus underpins the pathogen-
esis of malaria.

METHODS SUMMARY

P. c. chabaudi AS and CB were mosquito transmitted (MT) and cloned at
the University of Edinburgh, UK, and sent to NIMR in 1978 and 1982, respectively.
Parasites were serially blood passed (SBP) through mice 26–32 times before use
in this study. To initiate infections with SBP parasites, mice were injected intra-
peritoneally (i.p.) or intravenously (i.v.) with 10^7–10^8 pE derived from cryopre-
erved stocks. Alternatively, SBP parasites were transmitted through Anopheles
stephensi and mice were infected by mosquito bite, with an estimated 9.15 infective
bites per mouse9. We therefore directly compared SBP parasites before and after
mosquito transmission. The first 52 h of infection initiated by mosquito bite was
required to complete the pre-erythrocytic stages12; the erythrocytic cycle thus
begins on day 2 post-infection. To bypass the pre-erythrocytic stages, and control
the dose initiating the blood-stage infection, mice were injected i.p. or i.v. with
10^7–10^8 pE derived from recently MT parasite lines that were just one blood
passage from mosquito transmission (unless otherwise stated). The course of
infection was monitored on thin blood smears by enumerating the percentage
of erythrocytes infected with asexual parasites (parasitaemia). The limit of detection
for patent parasitaemia was 0.01% infected erythrocytes. To determine chroni-
city of infection, 100 µL blood was sub-inoculated into RAG-KO mice; absence of
parasitaemia in recipient mice after 14 days indicated clearance of infection in
donor mice. Rechallenge studies were initiated ≥ 90 days after the first infection,
when ≥ 95% C57BL/6 mice had naturally cleared blood-stage parasites.

Full Methods and any associated references are available in the online version of the paper.

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1. Dearsly, A. L., Sinden, R. E. & Self, I. A. Sexual development in malarial parasites: gametocyte production, fertility and infectivity to the mosquito vector. Parasitology 100, 359–368 (1990).

2. Mackinnon, M. J. & Read, A. F. Selection for high and low virulence in the malaria parasite Plasmodium chabaudi. Proc. R. Soc. Lond. B 266, 741–748 (1999).

3. Yoeli, M., Hargreaves, B., Carter, R. & Walliker, D. Sudden increase in virulence in a strain of Plasmodium berghei yoelii. Ann. Trop. Med. Parasitol. 69, 173–178 (1975).

4. Hartley, E. G. Increased virulence of Plasmodium cynomolgi basinelli in the rhesus monkey. Trans. R. Soc. Trop. Med. Hyg. 63, 411–412 (1969).

5. Chin, W., Contacos, P. G., Collins, W. E., Jeter, M. H. & Alpert, E. Experimental mosquito-transmission of Plasmodium knowlesi to man and monkey. Am. J. Trop. Med. Hyg. 17, 355–358 (1968).

6. Alger, N. E., Branton, M., Harant, J. & Silverman, P. H. Plasmodium berghei NK65 in the inbred A/J mouse: variations in virulence of P. berghei demes. J. Protozool. 18, 596–601 (1971).

7. Knowles, G. & Walliker, D. Variable expression of virulence in the rodent malaria parasite Plasmodium yoelii yoelii. Parasitology 81, 211–219 (1980).

8. Mackinnon, M. J., Bell, A. & Read, A. F. The effects of mosquito transmission and population bottlenecks on virulence, multiplication rate and rosetting in rodent malaria. Int. J. Parasitol. 35, 145–153 (2005).

9. Spence, P. J., Jarrar, W., Lévy, P., Nahrendorf, W. & Langhorne, J. Mosquito transmission of the rodent malaria parasite Plasmodium chabaudi. Malar. J. 11, 407 (2012).

10. Glynn, J. R., Collins, W. E., Jeffery, G. M. & Bradley, D. J. Infecting dose and severity of falciparum malaria. Trans. R. Soc. Trop. Med. Hyg. 89, 281–283 (1995).

11. Langhorne, J., Ndungu, F. M., Sponaas, A. M. & Marsh, K. Immunity to malaria: more questions than answers. Nature Immunol. 9, 725–732 (2008).

12. Stephens, R., Cullenot, R. L. & Lamb, T. J. The contribution of Plasmodium chabaudi to our understanding of malaria. Trends Parasitol. 28, 73–82 (2012).

13. Spence, P. J. & Langhorne, J. T-cell control of malaria pathogenesis. Curr. Opin. Immunol. 24, 444–448 (2012).

14. del Portillo, H. A. et al. The role of the spleen in malaria. Cell. Microbiol. 14, 343–355 (2012).

15. Sponaas, A. M. et al. Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells. J. Exp. Med. 203, 1427–1433 (2006).

16. Jain, V. et al. Plasma IP-10, apoptotic and angiogenic factors associated with fatal cerebral malaria in India. Malar. J. 7, 83 (2008).

17. Lawton, J. et al. Characterization and gene expression analysis of the cirmulti-gene family of Plasmodium chabaudi chabaudi (AS). BMC Genomics 13, 125 (2012).

18. Cunningham, D., Lawton, J., Jarrar, W., Preiser, P. & Langhorne, J. The pir multigene family of Plasmodium: antigenic variation and beyond. Mol. Biochem. Parasitol. 170, 65–73 (2010).

19. Brannan, L. R., McLean, S. A. & Phillips, R. S. Antigenic variants of Plasmodium chabaudi AS and the effects of mosquito transmission. Parasite Immunol. 15, 135–141 (1993).

20. McLean, S. A., Phillips, R. S., Pearson, C. D. & Walliker, D. The effect of mosquito transmission of antigenic variants of Plasmodium chabaudi. Parasitology 94, 443–449 (1987).

21. Manske, M. et al. Analysis of Plasmodium falciparum diversity in natural infections by deep sequencing. Nature 487, 375–379 (2012).

22. Peters, J. et al. High diversity and rapid changeover of expressed var genes during the acute phase of Plasmodium falciparum infections in human volunteers. Proc. Natl Acad. Sci. USA 99, 10689–10694 (2002).

Supplementary Information is available in the online version of the paper.

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Author Contributions P.J.S., W.J. and J.L. designed the study. P.J.S., W.J., P.L., L.C. and T.B. performed the experiments. P.J.S. and A.J.R. analysed the data. M.S. and M.B. provided project management. P.J.S. wrote the manuscript.

Author Information RNA-seq datasets have been deposited in ArrayExpress with accession number E-ERAD-95. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.L. (jlangho@nimr.mrc.ac.uk).
METHODS

Mice. Inbred wild-type, major histocompatibility complex class II knockout (MHC II KO)23 and recombination activation gene 1 knockout (RAG KO)23 C57BL/6 mice were bred under specific pathogen-free conditions at NIMR. All experiments were performed in accordance with UK Home Office regulations (PPL 80/2358) and approved by the ethical review panel at NIMR. Experimental mice were age- and sex-matched, housed under reverse light conditions (light 19:00–07:00, dark 07:00–19:00) at 20–22 °C, and had continuous access to mouse breeder diet and water. Measurements of clinical pathology were taken at 16:00. Core body temperature was measured with a rectal thermometer; body weight was calculated relative to a baseline measurement taken on day –2, and erythrocyte density was determined on a VetScan HMII haematology system (Abaxis). To measure liver enzymes, plasma was analysed on a VetScan Chemistry Analyzer, using a Mammalian Liver Profile reagent rotor (Abaxis).

Enumeration of blood-stage parasites by real-time PCR. Whole blood was isolated 20h after liver merozoite egress, when parasites were at the late trophozoite stage of development and within the first cycle of schizogony. Total RNA was extracted by acid guanidium thiocyanate-phenol-chloroform extraction25, and reverse transcribed by PCR at 42 °C using 75 U MuLV reverse transcriptase and 2.5 μM random hexamer primers (both Applied Biosystems) per sample. Parasites were quantified by real-time PCR, comparing P. c. chabaudi AS 18S ribosomal RNA copy number between samples and a standard curve of PPE prepared at the late trophozoite stage of development. The reaction mix contained TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM forward primer (5′-AACGATTAATAGAAGCAATCACCTCACT-3′), 300 nM reverse primer (5′-GGGAGGTGTTGTTAGCATGTATGGC-3′) and 50 nM probe (5′-E6AM-CATATGTTACCTTCTCTTTT-3′). Real-time PCR amplification was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), with a temperature profile as follows: 50 °C for 2 min, followed by 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Parasite numbers were determined per 100 μl whole blood; total circulating parasites were then calculated for each mouse based on their weight and, therefore, their estimated circulating blood volume.

Flow cytometry. Single-cell suspensions of splenocytes were prepared, erythrocyte lysed, and cells enumerated on a haemocytometer. Cells were stained with Flow cytometry.

Antibodies and cytokines. Malaria-specific antibodies were measured in plasma by enzyme-linked immunosorvent assay. 96-well PolySorp plates (Nunc) were coated with 50 μg ml⁻¹ parasite lysate prepared from P. falciparum isolated from C57BL/6 mice infected with SBF P. c. chabaudi AS; twofold serial dilutions of plasma from uninfected and hyper-immune mice were used as negative and positive controls, respectively, for experimental samples; alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, IgG2c, IgG2b and IgG3 (all from SouthernBiotech) were used for detection. Samples were developed with 1 mg ml⁻¹ 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) and attenuation was measured at 405 nm. Antibody concentrations are presented as arbitrary units (AU) relative to hyper-immune plasma. Cytokines were measured in plasma by LEGENDplex Luminex custom assay (BioLegend).

Plasmodium RNA preparation. C57Bl/6 mice were infected with SBF P. c. chabaudi AS through injection of infected erythrocytes, or through mosquito bite. Parasites were isolated at exactly 20 h into the seventh cycle of the blood-stage infection, at the late trophozoite stage of development, as follows. Whole blood was depleted of leukocytes by Plasmodipur filtration (EuroProxima); erythrocytes were centrifuged at 400 g for 10 min and lysed with 0.15% (v/v) saponin (Sigma). Samples were centrifuged at 1,000g for 5 min and washed with PBS; parasites were resuspended in TRizol (Life Technologies) and snap-frozen on dry ice. We prepared three biological replicates of SBF P. c. chabaudi AS from eight mice each, and two biological replicates of MT P. c. chabaudi AS from 30 mice each. RNA was extracted as described26, resuspended in water and DNA removed with a TURBO DNA-free Kit (Applied Biosystems), according to the manufacturer’s instructions. RNA quantity/quality was determined on an Agilent 2100 Bioanalyzer RNA 6000 Nano chip.

Amplification-free RNA-seq libraries. PolyA+ transcripts were selected from 10 μg total RNA using Sera-Mag Oligo(dT)-coated Magnetic Particles (Thermo Scientific). RNA was diluted with water to a volume of 130 μl and fragmented to approximately 200 nucleotides using Covaris Adaptive Focused Acoustics technology (settings: 5% duty cycle; intensity 5; 200 cycles per burst for 60 s). The RNA was ethanol-precipitated and resuspended in 10 μl water. First-strand cDNA was synthesized with Random Hexamer primers and SuperScript II Reverse Transcriptase (Life Technologies), following the manufacturer’s instructions. Second-strand cDNA synthesis, end repair and 3′-tailling were performed using the NEBNext mRNA library kit for Illumina (New England Biolabs), eluting in a final volume of 15 μl. Sequencing templates were prepared by mixing 15 μl cDNA, 5 μl 33 μM adapters (based on the published adapter27 with the addition of barcode sequences; oligonucleotides supplied by Integrated DNA Technologies), 25 μl Quick Ligation buffer and 5 μl Quick DNA ligase (both from New England Biolabs) and incubating for 15 min at 25 °C. Excess adapters were removed with two rounds of clean up with 50 μl of Agencourt AMPure PX Beads (Beckman Coulter). Final libraries were eluted in 30 μl water, visualized on an Agilent Bioanalyzer 2100 High Sensitivity DNA chip and quantified by qPCR. A pool of the five indexed libraries was sequenced on an Illumina HiSeq2000, with 100-bp paired-end reads.

Analysis of RNA expression. Paired-end RNA sequencing reads were mapped to the P. c. chabaudi AS reference genome (September 2012 release: ftp://ftp.sanger. ac.uk/pub/pathogens/P_chabaudi/SampleData/2012/12/malaria/). We independently added ‘glideosome’ as a sub-category. To conduct differential gene expression analysis between SBF and MT P. c. chabaudi AS we used DSEseq29; the three SBF P. c. chabaudi AS replicates were compared against the two MT P. c. chabaudi AS replicates to determine genes upregulated in blood-stage parasites following mosquito transmission, and vice versa to determine genes upregulated following serial blood passage. In both cases a corrected P value cutoff of 0.01 was applied. The resulting gene lists were categorised into ‘c$’ (based on published annotation30), ‘Pc’ (based on GenDB annotation by Ulrike Boehme at the Wellcome Trust Sanger Institute, with minor reannotation), ‘exported’ (based on known biology or ExportPred prediction), ‘Other known function’ and ‘Unknown function’. For those genes within the category ‘Other known function’, we sub-categorised genes based on enriched biological process GO terms using TopGO31; a P value cutoff of 0.01 was applied. We independently added ‘gliodesome’ as a sub-category.

23. Madsen, L. et al. Mice lacking all conventional MHC class II genes. Proc. Natl Acad. Sci. USA 96, 10338–10343 (1999).
24. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68, 869–877 (1992).
25. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction: twenty-six-months on. Nature Protocols 1, 581–585 (2006).
26. Kyes, S., Pinches, R. & Newbold, C. A simple RNA analysis method shows Kinetoplastid RNA-Seq. Proc. Natl Acad. Sci. USA 107, 96, 23223–23228 (2010).
27. Trapnell, C., Pachter, L. & Salzberg, S. L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111 (2009).
28. Anders, S. & Huber, W. Differential expression analysis for sequence count data. Genome Biol. 11, R106 (2010).
29. Sargeant, T. J. et al. Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Nature Methods 9, 291–295 (2012).
30. Alexa, A., Rahnenfuhrer, J. & Lengauer, T. Improved scoring of functional groups exportPred30 prediction), ‘Other known function’and ‘Unknown function’. For those genes within the category ‘Other known function’, we sub-categorised genes based on enriched biological process GO terms using TopGO; a P value cutoff of 0.01 was applied. We independently added ‘gliodesome’ as a sub-category.

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