A pigment-deficient mosquito line with fluorescent salivary glands enables in vivo imaging of individual *Plasmodium* sporozoites

Dennis Klug^{1,#}, Katharina Arnold^{1,2}, Eric Marois¹ and Stéphanie A. Blandin¹

¹Université de Strasbourg, CNRS UPR9022, INSERM U1257, Institut de Biologie Moléculaire et Cellulaire, 67000 Strasbourg, France

²Current address: German Cancer Research Institute (DKFZ), 69120 Heidelberg, Germany

to whom correspondence should be addressed:
dklug@unistra.fr

Abstract

Plasmodium parasites are the causative agent of malaria, a major health burden in sub-Saharan Africa. A key step in the transmission process of *Plasmodium* is the colonization of the salivary glands of the female *Anopheles* mosquito by the parasite sporozoite stage. How sporozoites recognize and invade the salivary glands is still poorly understood. Here we generated salivary gland reporter lines in the African malaria mosquito *Anopheles coluzzii* using salivary gland-specific promoters of the genes encoding anopheline antiplatelet protein (AAPP), the triple functional domain protein (TRIO) and saglin (SAG). The observed expression pattern of the DsRed and roGFP2 fluorescent reporters revealed lobe-specific activity of these promoters within the salivary glands, restricted either to the distal lobes or the middle lobe. We characterized four mosquito lines (AAPP-DsRed, AAPP-roGFP2, TRIO-DsRed and SAG-EGFP) in terms of localization, expression strength and onset of expression, as well as potential influences of the expressed fluorescent reporters on the infection with *Plasmodium berghei* and salivary gland morphology. Furthermore, using crosses with a pigmentation deficient *yellow(-)* mosquito line, we demonstrate that our salivary gland reporter lines represent a valuable tool to study the process of salivary gland colonization by *Plasmodium* parasites in live mosquitoes.

Introduction

Vector borne diseases have been a major health burden in the history of mankind. An efficient way to counteract diseases transmitted by blood sucking arthropods is to prevent transmission itself by reducing the number of vectors in the environment or by preventing vectors from biting humans in risk areas. As a consequence of malaria control interventions, approximately 663 million clinical malaria cases could be averted between 2000 and 2015. The most effective measure turned out to be the use of insecticide treated bed nets which prevented an estimated 68% of cases (Bhatt *et al.*, 2015). Still, preventive measures are counteracted by the very effective transmission of vector borne pathogens, which hampers eradication approaches. This is especially true for the causative agent of malaria, a protist named *Plasmodium*, which evolved highly specific adaptations to ensure efficient transmission between mosquitoes and human.

After infection of the mosquito midgut through the uptake of a blood meal from an infected host, *Plasmodium* parasites form a cyst, called oocyst, below the basal lamina of the midgut epithelium where they undergo multiple rounds of DNA replication. Rapid cell division, called schizogony, then follows, leading to the generation of several thousand of sporozoites per oocyst. These cells are released from the cysts and travel from the midgut to the salivary glands and, after being deposited in the skin during a blood meal, traverse the dermis, enter blood capillaries and reach the human liver. The sporozoites' distinctive cell polarization and crescent shape, in combination with high motility, allows them to „drill“ through tissues. In addition, sporozoites are highly deformable enabling passing through densely packed environments. An important feature of sporozoites is their long survival time, up to several weeks, in mosquito tissues (Mayne, 1922).

While it is believed that the invasion process of the salivary glands by sporozoites that have egressed from oocysts is rapid (~1-10 minutes), *in vivo* data supporting this assumption is lacking (Douglas *et al.*, 2015). Still, the time a sporozoite needs to reach and successfully invade the glands is an important parameter, because at this timepoint, a mosquito is considered infective to humans. Moreover, the very quick invasion of the salivary glands suggests that sporozoites have specific receptors that recognize ligands exposed on the gland surface. Up to date only two mosquito proteins, saglin and circumsporozoite protein (CSP) binding protein (CSPBP), have been described to support salivary gland invasion of sporozoites (Ghosh and Jacobs-Lorena, 2009; Wang *et al.*, 2013). Knockdown experiments of both proteins have shown that sporozoite numbers in the salivary glands are reduced

compared to controls, indicating a lower invasion rate. However, overexpression of saglin in the distal lobes of *Anopheles stephensi* did not lead to an increase in the number of salivary gland resident sporozoites, and immunofluorescence-staining using anti-saglin antibodies indicated that this protein is expressed in the middle lobe of each salivary gland (O’Brochta *et al.*, 2019). This observation is puzzling since sporozoites strongly prefer to invade the distal lobes, with sporozoite invasion of the smaller middle lobe being less prominent or even completely absent (Wells and Andrew, 2019). Thus, the relation between saglin and sporozoites may be more complex than a simple ligand-receptor interaction. Microscopic studies of infected glands revealed that sporozoites form large accumulations in organized „stacks“ inside cavities of acinar cells. The astonishing degree of order of these structures raises the question whether sporozoites can recognize each other. Alternatively, specifically preferred invasion sites may lead to accumulations at these entry points. However, since receptor-ligand interactions between sporozoites and acinar cells are still undefined, no specific entry site has been identified thus far.

Proteins secreted in the saliva of blood-sucking arthropods have been a long-term focus of interest. Saliva proteins often possess vasodilating, anti-coagulating and anti-inflammatory properties which counteract host responses and enable efficient blood feeding (Ribeiro and Francischetti, 2003). One protein specifically found in *Anopheles gambiae* saliva is TRIO (triple functional domain protein) that shares low homology to *Drosophila melanogaster* TRIO (Francischetti *et al.*, 2002, Arcà *et al.*, 2005, Dragovic *et al.*, 2018). Interestingly TRIO expression increases in the presence of *P. berghei* sporozoites (Dragovic *et al.*, 2018). The anopheline antiplatelet protein (AAPP) has anti-thrombotic functions and prevents collagen-induced platelet aggregation (Yoshida *et al.*, 2008; Hayashi *et al.*, 2012). Consistently, transgenic co-expression of an AAPP-specific single chain antibody blocking AAPP function negatively affects blood feeding *in vivo* (Islam *et al.*, 2019). The region upstream of the *aapp* gene was the first promoter used to drive salivary gland-specific transgenes in the Asian malaria mosquito *Anopheles stephensi* (Yoshida and Watanabe, 2006). Here we compare the promoter activities of the regions upstream of the *aapp*, *trio* and *saglin* genes in African *Anopheles coluzzii* mosquitoes and we test potential applications of the generated reporter lines to investigate sporozoite migration.

Results

Generation of transgenic mosquito lines with salivary gland-specific reporter expression and low pigmentation

To generate salivary gland reporter lines, the 5' upstream sequences of *saglin* (AGAP000610), *aapp* (AGAP009974) and *trio* (AGAP001374) were used to drive the expression of DsRed and hGrx1-roGFP2 from transgenic constructs integrated in the X1 docking line (**Fig. 1A, Fig. S1**) (Vолоhonsky *et al.*, 2015). The fluorescence reporter hGrx1-roGFP2 was chosen because it bears the advantages of EGFP and enables the measurement of the oxidation level of the intracellular glutathione pool by ratiometric quantification (Gutscher *et al.*, 2008). We used the *aapp* promoter sequence from *Anopheles coluzzii* orthologous to the one used to drive salivary gland-specific transgene expression in *Anopheles stephensi* (Yoshida and Watanabe, 2006). For both *saglin* and *trio*, we defined putative promoter regions according to the genomic context. In addition, as the selected *saglin* promoter region was very short (220bp, **Fig. S2**), we also used CRISPR/Cas9 to integrate a fluorescence cassette encoding EGFP into the endogenous *saglin* locus, replacing the coding sequence, to study *saglin* promoter activity in its native genomic context. For this, site directed knock-in mutagenesis was performed by injecting a plasmid encoding three guide RNAs targeting *saglin* and carrying a repair template marked with 3xP3-EGFP into mosquito eggs from a line with germline-specific expression of Cas9 (*vasa-Cas9*), generating a *sag(-)KI* line (**Fig. 1B, S3A**). To place the *EGFP* gene directly under control of the *saglin* promoter, the 3xP3 promoter used to selected transgenic mosquito larvae was flanked by lox sites to enable its excision by Cre recombination (**Fig. S3E, Material & Methods**). Similarly, we generated a knockin (KI) into the *yellow* gene that encodes a protein required for the synthesis of black pigments in insects (Hunt and Bridges, 1916; Wittkopp, True and Carroll, 2002). For this, we used a plasmid with guide RNAs specific for *yellow* and corresponding homology arms flanking a fluorescence cassette expressing EGFP surrounded by lox sites (**Fig. S3B**). Through crossing of salivary gland reporter lines with low pigmented *yellow(-)KI* mosquitoes, we aimed to improve the fluorescence signal of the expressed reporter and extend the range of applications, e.g. for *in vivo* imaging. Genotyping by PCR confirmed the successful generation of the salivary gland reporter lines *aapp-DsRed*, *aapp-hGrx1-roGFP2*, *trio-DsRed* and *sag-DsRed* (**Fig S1**) as well as the mosquito lines *sag(-)KI* and *yellow(-)KI* (**Fig S3C,D**). Loss of the 3xP3 promoter in *sag(-)EX* mosquitoes (mosquitoes lacking the 3xP3 promoter after *Cre* mediated excision)

was confirmed by PCR in the progeny of a cross between the *sag(-)KI* line and a mosquito line expressing *Cre* recombinase in the germline (**Fig. S3E**) (Volohonsky *et al.*, 2015).

Lobe-specific transcriptional activation by *sag*, *aapp* and *trio* promoter sequences

Dissection of female salivary glands revealed a highly specific expression pattern of DsRed, EGFP and hGrx1-roGFP2 driven by *sag*, *aapp* and *trio* promoter sequences inside salivary glands (**Fig. 1C**). While no fluorescence was observed for DsRed driven by the cloned 5' upstream sequence of the *saglin* gene in dissected salivary glands (**Fig. S3A**), promoter activity was detected in the line where *EGFP* replaced the *saglin* gene (**Fig. 1C**), indicating that essential transcriptional elements localize outside of the intergenic sequence between *AGAP000609* and *AGAP000610* (*saglin*) (**Fig. S3B**). Interestingly, fluorescence signals in glands of female *aapp-DsRed*, *aapp-hGrx1-roGFP2* mosquitoes were restricted to the distal lobes, while female *trio-DsRed* and *sag(-)EX* mosquitoes displayed fluorescence exclusively in the middle lobe (**Fig. 1C**). This difference became especially evident when crossing *aapp-hGrx1-roGFP2* mosquitoes with *trio-DsRed* mosquitoes giving rise to a trans-heterozygous offspring displaying hGrx1-roGFP2 expression in distal lobes combined with DsRed expression in the middle lobe (**Fig. 1C**). Similarly, when crossing *aapp* and *trio* DsRed reporter lines, the entire salivary gland was red fluorescent in the F1 progeny (**Fig. 1C**). Expression of *trio-DsRed* and *aapp-DsRed* was so strong that the respective parts of the salivary gland appeared pink colored even in brightfield microscopy (**Fig. 1C**). Of note, fluorescent reporter expression in the salivary glands of *aapp-DsRed*, *aapp-hGrx1-roGFP2* and *trio-DsRed* mosquito colonies was restricted to females while no fluorescence signal could be observed in this organ in males (**Fig. S4A**). Imaging of *aapp-DsRedP*, *aapp-hGrx1-roGFP2* and *trio-DsRed* pupae during development revealed no distinct fluorescence signal in the head part where the salivary glands are located (**Fig. S4B**). Still, DsRed expression was visible in the salivary glands of *aapp-DsRed* females that had been dissected directly after emergence (**Fig. 2A**), indicating that the *aapp* promoter is already active in pharate adults. In contrast, no DsRed expression was observed in the glands of freshly hatched *trio-DsRed* females (**Fig. 2A**). To evaluate the expression profile over time we performed a time-course experiment by dissecting and imaging salivary glands of *trio-DsRed* and *aapp-DsRed* females every day. DsRed expression guided by *aapp* and *trio* promoters increases steadily until day 5 after hatching (**Fig. 2B**). The strongest increase in fluorescence in *aapp-DsRed* females was observed from day three to four, and from day four to five in *trio-DsRed* females (**Fig. 2B**). The increase in DsRed in the salivary glands of *aapp-DsRed* and *trio-DsRed* females could

also be followed in transmitted light based on the pink coloration of the distal lobes (**Fig. S4C**). In *Anopheles stephensi*, AAPP expression was shown by qRT-PCR to be upregulated upon blood feeding (Yoshida and Watanabe, 2006). We followed DsRed fluorescence levels in the salivary glands of *aapp-DsRed* and *trio-DsRed* sibling mosquitoes that were fed on blood or kept on sugar (**Fig. 2C**). Our results confirm that the *aapp* promoter is induced upon ingestion of a blood meal while there was no difference between blood-fed and sugar-fed in females expressing the *trio-DsRed* transgene.

Subcellular localization of fluorescent reporters and impact on salivary gland morphology

During first microscopic examinations using low magnification, different intracellular localizations of the fluorescent proteins DsRed, EGFP and hGrx1-roGFP2 were observed (**Fig. 1C**), which was confirmed by confocal microscopy. DsRed expressed in the salivary glands of *aapp-DsRed* and *trio-DsRed* mosquitoes appeared spotty and localized in vesicles within the cytoplasm of the acinar cells, while hGrx1-roGFP2 and EGFP expressed in the glands of *aapp-hGrx1-roGFP2* and *sag(-)KI* mosquitoes displayed a cytoplasmic localization (**Fig. 3A**). Due to the anatomy of the secretory cells, which form an inward cavity into which saliva is secreted, the DsRed-positive vesicles localize mainly at the outer rim of the salivary gland. In contrast, localization of hGrx1-roGFP2 and EGFP was homogenous in the cytoplasm and appeared to be also nuclear (**Fig. 3A**). We also observed morphological alterations. While salivary glands of *aapp-DsRed*, *trio-DsRed* and *sag(-)KI* females were indistinguishable from wild type (*G3*), the apex of the distal lobes in *aapp-hGrx1-roGFP2* expressing salivary glands appeared shrunken and we detected a strangulation between the apex and the remainder of the lobe (**Fig. 3B,C**). Acinar cells in the apex of the lobes that displayed the highest expression levels of hGrx1-roGFP2, appeared thinned and atrophied, and presented an inflation of the salivary duct in the apex region (**Fig. 3A**).

Natural variation of DsRed expression in *trio-DsRed* mosquitoes

During the generation of the salivary gland reporter lines and their subsequent outcrossing we observed that most L1 mosquito larvae carrying the *trio-DsRed* transgene displayed DsRed expression in the eyes (**Fig. S5A**), which was absent in the two other reporter lines *aapp-DsRed* and *aapp-hGrx1-roGFP2*. Interestingly, a few L4 stage larvae of the *trio-DsRed* line even displayed strong ubiquitous DsRed expression (**Fig. S5A**). At the pupal stage, DsRed expression was detected in the ocellus, an eye spot close to the main compound eye, the

antennae and/or throughout the body. The observed expression patterns were highly variable and ranged from completely non-fluorescent pupae to pupae expressing DsRed in all three tissues (**Fig. S4B, S6A**). In adult mosquitoes, DsRed expression was less distinct compared to pupae although complete body fluorescence was occasionally observed in both males and females, with the strongest expression of DsRed in the salivary glands of females and the palps and antennae of males (**Fig. S5B**). Dissections of adult mosquitoes displaying whole body fluorescence at the pupal stage revealed strong and sex-independent DsRed signals in Malpighian tubules, an insect organ that performs functions similar to the vertebrate kidney (**Fig. S5C**). Of note, fluorescence in tubules strictly correlated to the high pupal fluorescence phenotype and was never observed in mosquitoes hatched from low fluorescent pupae (**Fig. S5D**). We next investigated sex-specificity of each fluorescence pattern at the pupal stage and inheritance of the high and low fluorescent *trio-DsRed* transgene. Non-fluorescent pupae were always females, while fluorescent antennae were observed exclusively in male pupae (**Fig. S6B**). In contrast, the presence of a fluorescent ocellus or body fluorescence was sex-independent (**Fig. S6B**). Quantification of the observed expression pattern revealed that a fluorescent ocellus, either alone or in combination with body fluorescence, was most frequently observed (**Fig. S6C**). The ratio of pupae that displayed body fluorescence to those that did not was close to 50% (**Fig S6C**). To follow the inheritance pattern of high and low body fluorescence, pupae were separated according to their fluorescence phenotype and intercrossed. Quantification of pupal DsRed expression in the F1 generation revealed a strong enrichment for whole body fluorescence (~80%) if high pupal body fluorescence was selected in the previous generation. In contrast, no pupae with high body fluorescence were observed in the offspring of mosquitoes hatched from low fluorescent pupae (**Fig. S6D**). This result indicates a form of Mendelian inheritance, with the locus conferring high body fluorescence being dominant over that conferring low body fluorescence. To investigate this further, virgin female pupae of the F1 generation displaying high pupal DsRed expression were crossed with wild type males. Ten single female families were created which were all screened for pupal body fluorescence. All families except family 8 displayed a 1:1 distribution for high and low pupal body fluorescence (**Fig. S6E**). Family 8 displayed exclusively high pupal body fluorescence, indicating that the founder female was homozygous for the locus conferring high body fluorescence, while all other females were heterozygous for the „low“ and the „high“ *trio-DsRed* loci. Notably, we detected high DsRed signals in the salivary glands of all adult females in families 5, 6, 7, 8 and 10 (other families were not investigated), regardless of whether individuals hatched from pupae displaying high or low body fluorescence.

Genotyping of females homozygous for low *trio-DsRed* or heterozygous for WT/high *trio-DsRed* revealed integration of the transgene into the X1 locus as a single copy (**Fig. S7**).

Impact of reporter expression on infection with *Plasmodium berghei*

To test if the expression of a fluorescent reporter in the salivary glands alters parasite development in the mosquito, we infected the three reporter lines *trio-DsRed*, *aapp-DsRed* and *aapp-hGrx1-roGFP2* as well as the parental line *G3* with the rodent malaria parasite *Plasmodium berghei* line $\Delta p230p$ -GFP that constitutively expresses EGFP at high levels in all developmental stages (Manzoni *et al.*, 2015). Parasite counts observed in reporter lines varied from 118 to 142 oocysts and were not significantly different from the control (121 oocysts) (**Fig. 4A**). The average number of salivary gland sporozoites at day 17-18 after infection was 8.200 to 17.400 sporozoites per mosquito in salivary gland reporter lines vs 9.600 in *G3* controls (**Fig. 4B**). An average of 10.000 sporozoites is what is usually observed in our laboratory, while numbers can fluctuate from 5.000 to more than 20.000 sporozoites per mosquito even in non-transgenic lines, indicating no significant difference between the *G3* control and reporter lines. To normalize the counts of salivary gland sporozoites relative to differences in oocyst numbers, we calculated the number of sporozoites that had successfully invaded the salivary glands per oocyst. Again, no significant difference was detected between reporter lines (86-146 sporozoites per oocyst) and *G3* control (133) mosquitoes (**Fig. 4C**). These results indicate that fluorescence expression in the salivary glands does not affect parasite development in mosquitoes or salivary gland infection with sporozoites.

Salivary gland reporter lines as a tool to investigate sporozoite biology / invasion of the salivary glands

We next investigated whether our highly fluorescent salivary gland reporter lines could facilitate *in vivo* imaging of *Plasmodium* development through the cuticle of a living mosquito using the same *P. berghei* line $\Delta p230p$ -GFP (**Fig. 5A**). This line is bright enough to make parasites visible through the mosquito cuticle, allowing pre-sorting of infected mosquitoes based on fluorescence in the mosquito midgut, the salivary glands or in the wing joint, a localization where hemolymph sporozoites tend to accumulate. Still, light absorption by the pigmented cuticle and light scattering by chitin generate signal losses reducing image quality and resolution. To reduce light absorption, we crossed *aapp-DsRed* mosquitoes, displaying highly fluorescent salivary glands without altered morphology, with *yellow(-)KI* mosquitoes, a loss-of-function line with low cuticle pigmentation (**Fig. 5A**). Yellow is a

protein required for the synthesis of black pigments in insects, its absence renders the cuticle pale and yellowish (Hunt and Bridges, 1916; Wittkopp, True and Carroll, 2002). As expected, *yellow(-)-aapp-DsRed* mosquitoes displayed a low pigmented cuticle in combination with highly fluorescent salivary glands (**Fig. 5B**). The decrease in pigmentation greatly increased the observed GFP signal visible through the cuticle, notably in strongly pigmented areas that appear almost black in WT mosquitoes (**Fig. 5C**). We next imaged mosquitoes infected with *P. berghei* $\Delta p230p$ -GFP between day 17 and day 20 after infection, when sporozoites have already invaded the salivary glands. In addition, mosquitoes were pre-sorted for GFP fluorescence in the salivary glands (**Fig. S8A**) as well as in the wing joint (not shown) to confirm the presence of sporozoites. While pre-sorting, we observed that the salivary glands in a subset of mosquitoes (less than 1 out of 5) localized very close to the cuticle (**Fig. S8B,C**). To facilitate *in vivo* imaging, we further selected mosquitoes positive for sporozoites and with cuticular localization of at least one salivary gland. Subsequently the legs of selected mosquitoes were removed and mosquitoes were glued on a microscopy slide. To prevent squeezing of mosquitoes, cover slips were prepared with small plasticine feet at each corner, and fixed with nail polish to prevent shifting of the cover against the microscopy slide (**Fig. S8D**). Confocal imaging enabled to visualize precisely single sporozoites through the cuticle close to the salivary gland as well as subcellular structures, like the cavities of acinar cells, of the gland itself (**Fig. 6A**). Live imaging revealed that a considerable number of sporozoites persist in tissues close to the gland and sometimes perform active movement (**Fig. 6B,C, Movies 1 and 2**). We also observed single sporozoites moving actively through tissues in a back and forth gliding manner (**Fig. 6B, Movie 3**) or making contact with the salivary gland although no invasion event could be monitored (**Fig. 6D**).

Discussion

Mosquito salivary glands have long interested entomologists, parasitologists, virologists and microbiologists because they are a key organ in the transmission of vector borne diseases. Still, the host-pathogen interactions that allow the specific recognition and colonization of this organ by different pathogens has been understudied, probably in part because of the relative difficulty to access and visualize these processes in comparison to midgut invasion. The reporter lines we generated in this study represent valuable tools to circumvent some of these issues: they allow (1) to easily visualize the glands, even in brightfield or DIC microscopy,

(2) to distinguish distal and middle lobes of the salivary glands, and (3) to image single sporozoites in proximity to the salivary glands *in vivo*.

We used the promoters of three salivary gland genes, *aapp*, *trio* and *saglin*, to drive fluorescent reporter expression in *A. coluzzii* mosquitoes. The different fluorescence patterns obtained in the salivary glands of these lines show that the proteomes of middle and distal lobes are diverse and most likely contribute different saliva components. In addition, the variable fluorescence intensity in the distal lobes of both *aapp* lines (stronger towards the apex) point towards an even more complex expression pattern along the lobe. Interestingly the *trio* and the *saglin* promoters were only active in the middle lobe, which, for *saglin*, is coherent with immunofluorescence studies performed in *A. stephensi* (O’Brochta *et al.*, 2019). This observation is puzzling since both proteins have been shown to either influence salivary gland colonization or transmission of *Plasmodium* sporozoites (Ghosh and Jacobs-Lorena, 2009; Dragovic *et al.*, 2018) while parasites prefer to invade the distal lobes (Wells and Andrew, 2019) that lack activity of *saglin* and *trio* promoters. A possible explanation is that the site of expression of both proteins is not the site where they interact with parasites.

The absence of reporter expression in mosquitoes expressing DsRed controlled by the *saglin* 5’ upstream sequence is likely due to the shortness of the cloned promoter sequence. The *saglin* gene localizes in the middle of an array of four genes believed to be salivary gland-specific (Francischetti *et al.*, 2002) and separated by short intergenic regions (**Fig. S1**). Regulatory sequences controlling transcription are likely located upstream and/or downstream of this array and might be shared by all four genes.

Notably, the reporter lines display different subcellular localizations of hGrx1-roGFP2, EGFP and DsRed. While all expressed fluorescence, proteins lack any targeting signal which could explain a different localization, only hGrx1-roGFP2 and EGFP localized within the cytoplasm. In contrast, DsRed displayed a vesicular pattern in both middle (*trio*) and distal lobes (*aapp*), while a DsRed2 variant driven by the *aapp* promoter in *A. stephensi* mosquitoes was shown to be cytoplasmic (Yoshida and Watanabe, 2006). The difference in localization between DsRed and other fluorescent reporters points to the presence of a cryptic trafficking signal in DsRed recognized by the acinar cells of *A. coluzzii*. Indeed, although we did not investigate their nature, we also observed the presence of vesicles in the salivary glands of wild type mosquitoes with similar distribution and morphology and displaying weak red autofluorescence (**Fig. S3A**). DsRed could potentially be trafficked to this or a similar compartment explaining its unique localization. Differences in salivary gland morphology between *aapp*-DsRed and *aapp*-hGrx1-roGFP2 expressing mosquitoes are likely due to the

expression of human Glutaredoxin 1 (hGrx1) and not of roGFP2, a protein derived from EGFP with 2 amino acid changes. Indeed, no change in morphology was detected in *sag(-)EX* mosquitoes expressing EGFP alone in the middle lobe. Still, EGFP appeared less expressed in the *sag(-)EX* compared to hGrx1-roGFP2 in *aapp-hGrx1-roGFP2* (**Fig. 1**) and we cannot exclude that the acinar cells of the middle and distal lobes react differently to the expression of the respective reporters or that the cells show different responses to roGFP2 and EGFP. Despite its influence on cell morphology the salivary glands of hGrx1-roGFP2 expressing mosquitoes retained their integrity even with high mosquito age and apoptosis of cells expressing the fluorescent reporter was not observed.

We also report a surprisingly large panel of fluorescence patterns in mosquitoes expressing the *trio-DsRed* transgene. The *trio* promoter activated transcription of DsRed in the salivary glands of all females (but not in males), but also in ocelli, antennae and Malpighian tubules as well as whole body in some but not all mosquitoes, independently of their sex and with a strict correlation between strong DsRed expression in pupal body and adult Malpighian tubules. The fact that *trio* expression was reported to be salivary gland-specific (Dragovic *et al.*, 2018) suggests that the region we selected upstream of *trio* does not contain all regulatory elements that control its natural expression, or that the genomic context of the X1 locus may influence its expression. Up to date, the number of available promoters able to drive ubiquitous expression of transgenes in *Anopheles* mosquitoes is limited (Adolfi *et al.*, 2018), thus replacing DsRed in a selected highly fluorescent line could provide a new means to ubiquitously express transgenes. By sorting and crossing according to the observed pupal fluorescence phenotype, it was possible to separate alleles conferring high and low pupal body fluorescence, which allowed the production of a colony enriched with the high pupal fluorescence phenotype and a colony showing only low pupal body fluorescence, especially in the ocelli. This observation pointed towards a genetically encoded difference which is inherited in a Mendelian manner rather than a transient (epi)genetic modification. Genotyping confirmed the presence of a single copy of the transgene in the X1 locus in all types of mosquitoes. Since fluorescent ocelli, antennae and whole body expression were only observed in the *trio-DsRed* population, we propose that the different DsRed expression patterns are locus-specific effects caused by interactions between the selected *trio* promoter sequence and regulatory elements within sequences flanking the transgene, or in trans. The transgene integration into the X1 locus may also have occurred with slight alterations that could influence DsRed expression or a subset of mosquitoes may have inherited a second copy of the transgene integrated in a different locus causing different locus-specific expression effects.

However, DsRed positive ocelli and antennae were observed in both high and low fluorescence populations, which is most likely explained by a variable DsRed expression from the transgene inserted at X1. While the observed DsRed expression patterns in larvae and pupae are helpful in the laboratory to select transgenic individuals it also urges caution in interpreting results as it clearly shows that transcription is contextual.

Intravital imaging has revealed the dynamics of parasite development inside its vertebrate host (Sturm *et al.*, 2006; Wang *et al.*, 2020). While optical windows in mice is a well implemented method (De Niz *et al.*, 2019), *in vivo* imaging through the cuticle of insects is scarcely performed, mostly because it is difficult to achieve good resolution by imaging through the insect cuticle that scatters and absorbs light very efficiently. Recent studies have explored the possibility to circumvent this issue through the application of clearing protocols on infected mosquitoes to improve resolution and enable imaging through whole mosquitoes (de Niz *et al.*, 2019; Mori, Hirai and Mita, 2019). However, while these improvements are remarkable, imaging of cleared specimens only represents snapshots whereas mapping of dynamic processes would be desired. Imaging of tissues *ex vivo* in combination with the use of hemolymph-like media allows the observation of dynamic processes (Klug and Frischknecht, 2017). Still, imaging *ex vivo* samples can only be performed for a limited amount of time and artifacts due to the artificial tissue environment cannot be excluded. A step forward towards intravital imaging was made recently by developing a protocol that combines feeding of dyes relevant for imaging with blood feeding prior to sample preparation (Trisnadi and Barillas-Mury, 2020). Due to the inflation of the mosquito abdomen directly after feeding, cuticle plates open up and create temporary optical windows that can be exploited for microscopy. Still, in this case, mosquitoes were decapitated and tightly flattened between microscopy slide and cover slip, thus allowing for optimal imaging but maintaining *in vivo* conditions only for a limited time. To demonstrate the use of the salivary gland reporter lines for imaging purposes, we crossed *aapp-DsRed* mosquitoes with the low pigmented mosquito line *yellow(-)KI* deficient for black pigment synthesis. In a subset of mosquitoes, salivary glands localized very closely to the cuticle allowing the recognition of subcellular structures. Combining brighter cuticle with cuticular positioning of the salivary glands within the mosquito thorax was instrumental to achieve visualization of single sporozoites through the cuticle and we were able to get an insight into sporozoite behaviour *in vivo*. It is generally believed that sporozoites, after they have been released from the oocyst, are passively transported to the salivary glands and engage in active motility only upon contact with acinar cells (Douglas *et al.*, 2015). Our *in vivo* observations indicate that

sporozoites can perform active motility even when not in contact with the salivary gland (Movie 1, 2 and 3). Interestingly, a significant number of sporozoites appeared trapped in close proximity to the gland. These sporozoite accumulations are already known from other sites of the mosquito circulatory system (Hillyer, Barreau and Vernick, 2007). Thus sporozoite localization in the salivary gland area, for example through the detection of GFP, does not necessarily represent a measure for successfully invaded sporozoites (Fig. 6A). Here we provide a proof of concept of *in vivo* salivary gland imaging, and we hope that the method can be further improved to gain a better insight of the invasion of salivary glands by sporozoites. The injection of fluorescent beads or dyes could be used as vitality measure to monitor the heartbeat of the mosquito while, at the same time, highlighting contact areas with hemolymph and salivary glands. Also we did not exhaust the repertoire of possible genetic modifications to decrease pigmentation of the cuticle. Deletion of the genes *tan* and *ebony*, two enzymes which regulate the homeostasis of the pigment precursors dopamine and N- β -alanyldopamine, in combination with the deletion of *yellow* could potentially further decrease cuticle pigmentation (Wittkopp, True and Carroll, 2002; True *et al.*, 2005). Another possibility could be the overexpression of arylalkylamine-N-acetyltransferase, an enzyme converting dopamine into the sclerotizing precursor molecule N-acetyldopamine, which was shown to affect pigmentation in different insect species by enhancing the production of colorless components of the cuticle (Osanai-Futahashi *et al.*, 2012). A key improvement for *in vivo* imaging would be an increase in the number of mosquitoes displaying ideally positioned salivary glands. Preliminary monitoring experiments showed that the position of the gland does not significantly change over time in adult mosquitoes indicating that positioning occurs during metamorphosis. Should salivary gland positioning be genetically determined, selective breeding may help to improve the ratio of mosquitoes with cuticular salivary glands. Taken together we hope that the transgenic lines developed in this study can contribute to improve *in vivo* imaging and salivary gland dissections in *Anopheles* mosquitoes, and enable real-time imaging of *Plasmodium* and other vector borne pathogens.

Materials & methods

Animals

Transgenesis was performed in the *Anopheles coluzzii* strain X1 (Volohonsky *et al.*, 2015) or in a strain expressing Cas9 from the X1 locus under control of the *vasa* promoter

(transgenesis plasmid: Addgene # 173670) and introgressed into the *Ngousso* genetic background. Wild type *Anopheles coluzzii* strains *Ngousso* and *G3* were used as controls. CD1 mice purchased from Charles River or from our own colonies were used for both mosquito maintenance through blood meals, and for parasite maintenance and mosquito infections. For infections with rodent malaria parasites, we made use of the *Plasmodium berghei* line $\Delta p230p-GFP$ derived from the wild type background *P. berghei* ANKA (Manzoni *et al.*, 2015).

Breeding of mosquitoes

Anopheles coluzzii mosquitoes were kept in standard conditions (27°C, 75-80% humidity, 12-hr/12-hr light/dark cycle). Larvae were hatched in pans of osmosed water and fed with pulverized fish food (TetraMin, Melle, Germany). Adult mosquitoes were maintained on a 10% sucrose solution. To propagate colonies, four to seven day old mosquitoes were blood fed for 10-15 minutes on anesthetized mice and allowed to lay eggs on wet filter paper 2-3 days later. For mosquito blood feeding, female CD-1 mice (> 35g) were anesthetized with a mixture of Zoletil (42.5 mg/kg) and Rompun (8.5 mg/kg) in 0.9% NaCl solution.

Construction of salivary gland reporter transgenes

The 5'upstream sequences of *saglin* (primers P9 and P10), *aapp* (primers P1 and P2) and *trio* (primers P7 and P8) were amplified from genomic DNA of *G3* mosquitoes isolated with the Blood & Tissue Kit (Qiagen) as shown in **File S1**. DsRed (primers P5 and P6 for *aapp/trio*; P11 and P12 for *saglin*) and hGrx1-roGFP2 (primers P3 and P4) were amplified from the plasmids pJET-DsRed-SV40 and pDSAP_actin-hGrx1-roGFP2 (donated by Raquel Mela-Lopez). Primers are shown in **Table S1**. PCR was performed using Phusion polymerase according to the manufacturer recommendations (ThermoFisher Scientific) and subcloned in the pJET vector using the CloneJET PCR Cloning Kit (ThermoFisher Scientific). The 5'upstream sequence of the *saglin* gene was fused to the *DsRed* gene by a Phusion mediated assembly PCR (TerMaat *et al.*, 2009) before cloning into pJET. After sequence verification, assembly blocks were fused into the destination vector pDSAP (Volohonsky *et al.*, 2015, Addgene # 62293) using Goldengate cloning and generating pDSAP-aapp-DsRed, pDSAP-aapp-hGrx1-roGFP2, pDSAP-trio-DsRed and pDSAP-saglin-DsRed.

Construct cloning for site-directed mutagenesis into *yellow* and *saglin* genes

For the design of guide RNAs (gRNAs), the coding sequence of *saglin* as well as the first exon of the *yellow* gene were manually searched for PAMs (NGG), with N representing any nucleotide. Candidate gRNA sequences were investigated for potential off-target effects using <http://www.rgenome.net/cas-designer/> (Cas-OFFinder) (Bae, Park and Kim, 2014). We selected gRNAs starting with a G considering the transcription start from the U6 promoter and differing by ≥ 3 nucleotides from putative off-target sequences in the *A. coluzzii* genome. For each knockin construct, linkers encoding the protospacer motif of three gRNAs (**Table S1**) were generated by annealing two primers ordered from IDT DNA and cloned using *BbsI* into pKSB-sgRNA (Addgene #173671—3) vectors containing the U6 snRNA polymerase III promoter (AGAP013557), the invariable sequence of a standard crRNA-tracerRNA fusion (Jinek et al., 2012), and the RNA polIII TTTT terminator. The three complete gRNAs blocks were then assembled into a single pKSB-sgRNA destination plasmid by GoldenGate cloning using different but matching *BsaI* overhangs. For the construction of repair templates, regions of homology on the 5' side (*saglin*: primers P29 and P30; *yellow*: primers P19 and P20) and on the 3' side (*saglin*: primers P31 and P32; *yellow*: primers P21 and P22) of the region targeted by the gRNAs were amplified from *Ngousso* genomic DNA and subcloned in the pJET vector, generating plasmids pJET-5'saglin, pJET-5'yellow, pJET-3'saglin and pJET-3'yellow. After sequence validation, all building blocks were fused in the correct order in the destination plasmid pENTR using GoldenGate assembly together with a building block containing either the fluorescence cassette pKSB-Lox3xP3Lox-EGFP or pKSB-Lox3xP3-EGFP-Lox, generating pENTR-Saglin;Lox3xP3Lox-EGFP and pENTR-Yellow;Lox3xP3-EGFP-Lox. Primers are shown in **Table S1**.

Transgenesis of *A. coluzzii*

All plasmids used for DNA injections were purified using Endofree kits (Qiagen). Freshly laid eggs of the docking line X1 or the *vasa-Cas9* line were injected as described (Fuchs, Nolan and Crisanti, 2013; Pondeville et al., 2014; Volohonsky et al., 2015). For Φ C31-integrase mediated transgenesis a mix of three plasmids (pDSAP-aapp-DsRed, pDSAP-aapp-hGrx1-roGFP2 and pDSAP-trio-DsRed) (~160 ng/ μ L) and the integrase helper plasmid carrying the Vasa2- Φ C31-Integrase gene (Addgene # 62299, ~200 ng/ μ L) diluted in 0.5x phosphate buffered saline (0.1 mM NaHPO₄ buffer, 5 mM KCl, pH 6.8) was injected into approximately 500 embryos. The plasmid pDSAP-saglin-DsRed was injected in the same way but into a different batch of eggs. For site-directed knock-in mediated by Cas9, the

plasmid solution (pENTR-Saglin;Lox3xP3Lox-EGFP or pENTR-Yellow;Lox3xP3Lox-EGFP) (~200-300 ng/μL) was diluted in PBS supplemented with 1 μM Scr7 to inhibit non-homologous end joining. Each construct was injected in a separate batch of approximately 500 eggs obtained from *vasa-Cas9* females.

Salivary gland reporter lines *sag-DsRed*, *trio-DsRed*, *aapp-DsRed* and *aapp-hGrx1-roGFP2*

Following egg micro-injection, surviving pupae were sorted according to sex, hatched in separate cages (G0 generation) and crossed with ~100 mosquitoes of the other sex from a mosquito line expressing CFP from the X1 docking site that was used as a balancer of the new, non-fluorescent but puromycin resistant transgenic insertions at the same locus. Neonate larvae from this cross were treated with puromycin (0.05 mg/mL). G1 pupae were subsequently sex sorted and crossed with CFP positive mosquitoes to expand the population. Adult puromycin resistant G2 females were sorted according to the observed fluorescence phenotype (DsRed or roGFP2) in their salivary glands, reflecting the expression of the *trio-DsRed*, *aapp-DsRed* or *aapp-hGrx1-roGFP2* transgenes and crossed to CFP balancer males. For *sag-DsRed* females, sorting was not necessary, as transgenesis was performed separately by injection of the pDSAP-saglin-DsRed plasmid alone. Puromycin-resistant G3 larvae were raised, intercrossed and the G4 offspring was sorted by COPAS (Marois et al., 2012) for the absence of CFP to select lines homozygous for *sag-DsRed*, *trio-DsRed*, *aapp-DsRed* and *aapp-hGrx1-roGFP2*. Lines were then raised without puromycin treatment. Single females of all generated colonies were genotyped by PCR to monitor correct integration of the transgene into the docking site (**Fig. S1A,B**). Successfully genotyped mosquito colonies were expanded and used to perform experiments.

***yellow(-)KI* and *sag(-)KI* lines**

Pupae obtained from plasmid injected eggs were sorted according to sex and crossed with 100 *Ngousso* wild type partners. G1 neonate larvae were screened for the expression of EGFP using a Nikon SMZ18 fluorescence Stereomicroscope. Selected transgenic larvae were sex sorted at the pupal stage and crossed with an excess of wild type partners. The G2 progenies (from a single positive G1 female for *sag(-)KI* and from 10 positive individuals for *yellow(-)KI*) were COPAS sorted to enrich for the integrated transgene and to remove the *vasa-Cas9* transgene (marked with DsRed). The generated *sag(-)KI* colony was kept in equilibrium with the wild type allele, while the *yellow(-)KI* colony was completely homozygotized for the

transgene by selecting for mosquitoes with a yellow body color. Homozygous *sag(-)KI* mosquitoes were crossed with the C2S line expressing Cre in the germline (Volohonsky *et al.*, 2015) for Cre recombinase-mediated excision of the lox cassette to generate *sag(-)EX* mosquitoes.

Genotyping of mosquito lines

Genotyping was performed on genomic DNA extracted from single mosquitoes of *aapp-DsRed*, *aapp-hGrx1-roGFP2*, *trio-DsRed*, *sag(-)KI*, *sag(-)EX* and *yellow(-)KI* colonies. Genotyping PCRs were performed using GoTaq Green Mastermix (Promega) (**Fig. S1B**), Phusion polymerase (ThermoFisher Scientific) or the Phire Tissue Direct PCR Master Mix (ThermoFisher Scientific) (**Fig. S2 and S7**) according to manufacturer recommendations. See **Table S2** for genotyping primers.

Mosquito infection and evaluation of parasite numbers

Parasites were maintained as frozen stocks at -80°C or by passage between mice. For this, blood was taken by heart puncture from a donor mouse with a parasitaemia of 3-5%, diluted to 2.10^7 parasitized erythrocytes in 100 µL 0.9% NaCl that were injected intravenously into a naïve mouse. Parasitemia was monitored via flow cytometry (Accuri C6, BD biosciences) three days later. Mosquitoes were fed for 10-15 min on anesthetized mice with 2.5 – 3.5% parasitemia. Blood fed females were sorted and kept on 10% sucrose at 21°C and 50-70% humidity. Midguts and salivary glands were dissected in PBS under a SZM-2 Zoom Trinocular stereomicroscope (Optika). Images were acquired with the Nikon SMZ18 Stereomicroscope (without coverslip) or with a Zeiss LSM 780 confocal microscope (sealed coverslip). To count oocysts, midguts were dissected 7-8 days post infection. Fluorescence images of infected midguts were processed using the watershed segmentation plugin (Soille and Vincent, 1990) and oocysts were subsequently counted using the „analyze particles“ function implemented in Fiji (Schindelin *et al.*, 2012). To count salivary gland sporozoites, salivary glands were collected in PBS 17-18 days post infection and ground with a plastic pestle for one minute. The number of sporozoites was measured using a Neubauer hemocytometer under a light microscope (Zeiss).

Fluorescence imaging, fluorescence measurements and image analysis

Samples imaged using the Nikon SMZ18 Stereomicroscope were usually acquired using the respective fluorescence filter to visualize expression of DsRed or roGFP2/EGFP combined with brightfield to visualize the whole specimen. Images were taken using a 63-fold magnification objective (NA 1.4) on a Zeiss LSM 780 confocal microscope (**Fig. 3A**), a Zeiss Axiovert 200 fluorescence microscope (**Fig. S2A**) or using an iPhone 6 for the mounted mosquitoes prepared for microscopy in **Fig. S8D**. All other images as well as images used for fluorescence measurements were acquired with a Nikon SMZ18 Stereomicroscope. For fluorescence quantifications, images were acquired in the DsRed channel and settings were kept the same for all experiments. For each salivary gland, only a single lobe was measured by analyzing a square of 51×51 pixels covering the central part of the fluorescent area to determine the mean fluorescence intensity. The image processing for all experiments was performed with Fiji (Schindelin *et al.*, 2012).

In vivo imaging

For *in vivo* imaging experiments, infected *yellow(-)-aapp-DsRed* mosquitoes were pre-screened between days 17 - 21 post infection for the presence of hemolymph sporozoites (hinge of the wing, GFP fluorescence) and salivary gland sporozoites (salivary gland, GFP fluorescence) as well as for cuticular positioning of the salivary gland (DsRed signal) (**Fig. S8B**). After removing their legs, mosquitoes were glued on a microscopy slide using small amounts of UV-light reactive glue (Bondic Pocket) and covered with a coverslip prepared with plasticine feet at each corner to prevent a squeezing of the sample. Corners of the coverslip were coated with nail polish to prevent sliding. Imaging was performed on a Zeiss LSM 780 confocal microscope with GaAsP detector and Metamorph acquisition software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Data sets were either tested with a one-way ANOVA or a Student's t test. A value of $p < 0.05$ was considered significant.

Data availability

All data have been made available within this manuscript. Transgenic mosquito lines as well as plasmids are available upon request.

Ethics statement

Experiments were carried out in conformity with the 2010/63/EU directive of the European Parliament on the protection of animals used for scientific purposes. Our animal care facility received agreement #I-67-482-2 from the veterinary services of the département du Bas Rhin (Direction Départementale de la Protection des Populations). The use of animals for this project was authorized by the French ministry of higher education, research and innovation under the number APAFIS#20562- 2019050313288887 v3. The generation and use of transgenic lines (bacteria, mosquitoes, parasite) was authorized by the French ministry of higher education, research and innovation under the number 3243.

Funding

This work was supported by the Laboratoire d'Excellence (LabEx) ParaFrap (grant LabEx ParaFrap ANR-11-LABX-0024), by the Equipement d'Excellence (EquipEx) I2MC (grant ANR-11-EQPX-0022), by the ERC Starting Grant N°260918, and by funding from CNRS, Inserm, and the University of Strasbourg. DK is funded by a DFG postdoctoral fellowship (KL 3251/1-1).

Acknowledgements

We thank Amandine Gautier for help with rearing mosquitoes and technical assistance during infections with *P. berghei*, Sarra Manai for help with experiments, Jean-Daniel Fauny for assistance during microscopy and the whole mosquito immune responses (MIR) team for assistance with mosquito breeding and pupae collection.

Authors contributions

DK designed and performed experiments, analyzed data, made figures and wrote the draft. KA cloned constructs, performed initial experiments and analyzed data. EM performed transgenesis of mosquitoes. SB edited the draft and helped with conceptualization of experiments. All authors edited and approved the manuscript for publication.

Competing interests

The authors declare no competing interests.

References

- Adolfi, A. et al.** (2018) ‘Multi-tissue GAL4-mediated gene expression in all *Anopheles gambiae* life stages using an endogenous polyubiquitin promoter’, *Insect Biochemistry and Molecular Biology*. doi: 10.1016/j.ibmb.2018.03.005.
- Bae, S., Park, J. and Kim, J. S.** (2014) ‘Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases’, *Bioinformatics*. doi: 10.1093/bioinformatics/btu048.
- Berghammer, A. J., Klingler, M. and Wimmer, E. A.** (1999) ‘A universal marker for transgenic insects’, *Nature*. doi: 10.1038/46463.
- Bhatt, S. et al.** (2015) ‘The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015’, *Nature*. doi: 10.1038/nature15535.
- Douglas, R. G. et al.** (2015) ‘Active migration and passive transport of malaria parasites’, *Trends in Parasitology*, pp. 357–362. doi: 10.1016/j.pt.2015.04.010.
- Dragovic, S. M. et al.** (2018) ‘Immunization with AgTRIO, a Protein in *Anopheles* Saliva, Contributes to Protection against *Plasmodium* Infection in Mice’, *Cell Host and Microbe*. doi: 10.1016/j.chom.2018.03.008.
- Engler, C. and Marillonnet, S.** (2014) ‘Golden Gate cloning’, *Methods in Molecular Biology*. doi: 10.1007/978-1-62703-764-8_9.
- Francischetti, I. M. B. et al.** (2002) ‘Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector *Anopheles gambiae*’, *Journal of Experimental Biology*.
- Fuchs, S., Nolan, T. and Crisanti, A.** (2013) ‘Mosquito transgenic technologies to reduce plasmodium transmission’, *Methods in Molecular Biology*, 923, pp. 601–622. doi: 10.1007/978-1-62703-26-7_41.
- Ghosh, A. K. and Jacobs-Lorena, M.** (2009) ‘*Plasmodium* sporozoite invasion of the mosquito salivary gland’, *Curr Opin Microbiol*, 12. doi: 10.1016/j.mib.2009.06.010.
- Gutscher, M. et al.** (2008) ‘Real-time imaging of the intracellular glutathione redox potential’, *Nature Methods*. doi: 10.1038/nmeth.1212.

- Hayashi, H. et al.** (2012) ‘Anopheline anti-platelet protein from a malaria vector mosquito has anti-thrombotic effects in vivo without compromising hemostasis’, *Thrombosis Research*. doi: 10.1016/j.thromres.2011.09.015.
- Hillyer, J. F., Barreau, C. and Vernick, K. D.** (2007) ‘Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel’, *International Journal for Parasitology*. doi: 10.1016/j.ijpara.2006.12.007.
- Hunt, T. M. and Bridges, C. B.** (1916) *Sex Linked Inheritance in Drosophila*, Carnegie Institution of Washington.
- Islam, A. et al.** (2019) ‘Anopheline antiplatelet protein from mosquito saliva regulates blood feeding behavior’, *Scientific Reports*. doi: 10.1038/s41598-019-39960-2.
- Klug, D. and Frischknecht, F.** (2017) ‘Motility precedes egress of malaria parasites from oocysts’, *eLife*, 6.
- Lee, J. H. et al.** (1996) ‘Sequential amplification of cloned DNA as tandem multimers using class- IIS restriction enzymes’, *Genetic Analysis - Biomolecular Engineering*. doi: 10.1016/S1050-3862(96)00164-7.
- Manzoni, G. et al.** (2015) ‘A rapid and robust selection procedure for generating drug-selectable marker-free recombinant malaria parasites’, *Scientific Reports*, 4(1), p. 4760. doi: 10.1038/srep04760.
- Mayne, B.** (1922) ‘How Long Does a Mosquito Retain Malaria Parasites?’, *Public Health Reports (1896-1970)*. doi: 10.2307/4576379.
- Mori, T., Hirai, M. and Mita, T.** (2019) ‘See-through observation of malaria parasite behaviors in the mosquito vector’, *Scientific Reports*. doi: 10.1038/s41598-019-38529-3.
- Natarajan, R. et al.** (2001) ‘Fluorescent Plasmodium berghei sporozoites and pre-erythrocytic stages: A new tool to study mosquito and mammalian host interactions with malaria parasites’, *Cellular Microbiology*, 3(6), pp. 371–379. doi: 10.1046/j.1462-5822.2001.00117.x.
- De Niz, M. et al.** (2019) ‘3D imaging of undissected optically cleared Anopheles stephensi mosquitoes infected with Plasmodium parasites’, *bioRxiv*. doi: 10.1101/682054.

- De Niz, M. et al.** (2019) ‘Intravital imaging of host–parasite interactions in skin and adipose tissues’, *Cellular Microbiology*. doi: 10.1111/cmi.13023.
- O’Brochta, D. A. et al.** (2019) ‘Is Saglin a mosquito salivary gland receptor for *Plasmodium falciparum*?’ , *Malaria Journal*. doi: 10.1186/s12936-018-2634-5.
- Osanai-Futahashi, M. et al.** (2012) ‘A visible dominant marker for insect transgenesis’, *Nature Communications*. doi: 10.1038/ncomms2312.
- Pondeville, E. et al.** (2014) ‘Efficient Φ C31 integrase–mediated site-specific germline transformation of *Anopheles gambiae*’, *Nature Protocols*, 9(7), pp. 1698–1712. doi: 10.1038/nprot.2014.117.
- Ribeiro, J. M. C. and Francischetti, I. M. B.** (2003) ‘Role of Arthropod Saliva in Blood Feeding: Sialome and Post-Sialome Perspectives’, *Annual Review of Entomology*. doi: 10.1146/annurev.ento.48.060402.102812.
- Schindelin, J. et al.** (2012) ‘Fiji: an open-source platform for biological-image analysis’, *Nature Methods*, 9(7), pp. 676–682. doi: 10.1038/nmeth.2019.
- Soille, P. and Vincent, L. M.** (1990) ‘<title>Determining watersheds in digital pictures via flooding simulations</title>’, in *Visual Communications and Image Processing ’90: Fifth in a Series*. doi: 10.1117/12.24211.
- Sturm, A. et al.** (2006) ‘Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids.’, *Science (New York, N.Y.)*, 313(5791), pp. 1287–1290. doi: 10.1126/science.1129720.
- TerMaat, J. R. et al.** (2009) ‘Gene synthesis by integrated polymerase chain assembly and PCR amplification using a high-speed thermocycler’, *Journal of Microbiological Methods*. doi: 10.1016/j.mimet.2009.09.015.
- Trisnadi, N. and Barillas-Mury, C.** (2020) ‘Live In Vivo Imaging of *Plasmodium* Invasion of the Mosquito Midgut ’, *mSphere*. doi: 10.1128/msphere.00692-20.
- True, J. R. et al.** (2005) ‘*Drosophila tan* encodes a novel hydrolase required in pigmentation and vision.’, *PLoS genetics*, 1(5). doi: 10.1371/journal.pgen.0010063.
- Volohonsky, G. et al.** (2015) ‘Tools for *Anopheles gambiae* Transgenesis’, *G3 (Bethesda)*; *Genes|Genomes|Genetics*, 5(6), pp. 1151–1163. doi: 10.1534/g3.115.016808.

- Wang, J. et al.** (2013) ‘Anopheles gambiae circumsporozoite protein--binding protein facilitates Plasmodium infection of mosquito salivary glands’, *J Infect Dis*, 208. doi: 10.1093/infdis/jit284.
- Wang, L. T. et al.** (2020) ‘A Potent Anti-Malarial Human Monoclonal Antibody Targets Circumsporozoite Protein Minor Repeats and Neutralizes Sporozoites in the Liver’, *Immunity*. doi: 10.1016/j.immuni.2020.08.014.
- Wells, M. B. and Andrew, D. J.** (2019) ‘Anopheles salivary gland architecture shapes plasmodium sporozoite availability for transmission’, *mBio*. doi: 10.1128/mBio.01238-19.
- Wittkopp, P. J., True, J. R. and Carroll, S. B.** (2002) ‘Reciprocal functions of the Drosophila Yellow and Ebony proteins in the development and evolution of pigment patterns’, *Development*.
- Yoshida, S. et al.** (2008) ‘Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito’, *Blood*. doi: 10.1182/blood-2007-06-097824.
- Yoshida, S. and Watanabe, H.** (2006) ‘Robust salivary gland-specific transgene expression in Anopheles stephensi mosquito’, *Insect Molecular Biology*. doi: 10.1111/j.1365-2583.2006.00645.x.

Figure 1. Lobe-specific reporter expression observed in female *aapp-DsRed*, *aapp-hGrx1-roGFP2*, *trio-DsRed* and *sag(-)EX* mosquitoes.

A) ΦC31 integrase-mediated transgene insertion. The *aapp*, *trio* and *saglin* promoter sequences were fused to *DsRed* or *hGrx1-roGFP2* within the pDSAP vector which enables chemical selection of mosquito larvae by puromycin. Transgenes were integrated by ΦC31 integrase-mediated recombination into the X1 line harbouring an *attP* site on chromosome 2L. **B)** Cas9-mediated knockin of a fluorescence cassette in the *saglin* gene (AGAP000610). Upstream and downstream sequences of *saglin* were cloned into the pENTR vector flanking a fluorescence cassette expressing EGFP under the control of the 3xP3 promoter. Three guides targeting *saglin* controlled by *U6* promoters were encoded on the same pENTR vector. Transgenesis was performed in mosquitoes with germline-specific expression of Cas9 (*vasa-Cas9*). **C)** Fluorescent reporter expression in *trio-DsRed*, *aapp-DsRed*, *aapp-hGrx1-roGFP2* and *sag(-)EX* expressing mosquito females in comparison to wild type (*G3*). Representative

799 images of salivary glands are shown in brightfield beside an image of the same gland in the
800 relevant fluorescence channel. Red arrows in brightfield images highlight the pink parts of
801 *aapp-DsRed* and *trio-DsRed* salivary glands in the absence of fluorescence excitation
802 indicating the high expression of DsRed. The illustration on the right of each panel indicates
803 the genotype of the gland as well as the observed fluorescence pattern. Scale bars: 250 μ m.
804

Figure 2. Onset, strength and inducibility by blood feeding of reporter expression in *aapp-DsRed* and *trio-DsRed* mosquitoes.

A) Images of salivary glands dissected directly after mosquitoes emerged. DsRed expression was observed in *aapp-DsRed* but not in *trio-DsRed* expressing salivary glands. Scale bar: 250 μ m. **B)** Mean fluorescence intensity measurements of dissected *aapp-DsRed* and *trio-DsRed* salivary glands from day one to day five after hatching. Data were normalized for the highest exposure time. Each dot represents a single gland (2-5 glands / timepoint) and the bars, mean values. * $p < 0.05$, Mann Whitney test. **C)** The blood meal inducibility of the *trio* and the *aapp* promoters was assessed by providing sibling females either with a blood meal or sugar solution. Approximately 24h later, salivary glands were dissected, imaged and the mean DsRed fluorescence intensity was quantified. Data pooled from three independent experiments. Same representation and statistical analysis as in (B), ** $p = 0.0015$.

Figure 3. Different subcellular localizations of the expressed DsRed, EGFP and hGrx1-roGFP2 fluorescent reporters.

A) Differential interference contrast (DIC) and confocal fluorescence images of distal (*aapp-DsRed*, *trio-DsRed*) or middle (*trio-DsRed*, *sag(-)EX*) salivary gland lobes. Different z-sections (top, middle and bottom) of the same glands are displayed in the relevant fluorescence channel. The „neck“ regions of the *aapp-DsRed* and *aapp-hGrx1-roGFP2* expressing lobes are indicated by white arrows. Note that DIC and fluorescence images were

taken with a Hamamatsu Orca Flash 4.0 V1 and a Yokogawa CSU X-1 camera, respectively. Images between different channels are therefore not completely matching although depicting the same salivary gland. The illustration on the left depicts the genotype and corresponding expression pattern. Scale bars: 20 μ m. **B)** Brightfield images of female wild type (*G3*), *aapp-DsRed* and *aapp-hGrx1-roGFP2* salivary glands. The „neck“ is indicated by a red asterisk and the measured diameter by a black line. Scale bars: 250 μ m. **C)** Diameter measurements of the distal lobe neck region of *aapp-DsRed* and *aapp-hGrx1-roGFP2* in comparison to wild type (*G3*). The number of measurements is indicated above each genotype. Note that only the diameter of one distal lobe per gland was measured. Data represent ≥ 3 independent experiments. *** $p < 0.0001$, one-way-ANOVA (Bonferroni's Multiple Comparison Test).

Figure 4. Oocyst densities and salivary gland colonization in *aapp-DsRed*, *aapp-hGrx1-roGFP2* and *trio-DsRed* mosquitoes infected with *P. berghei*.

Oocyst (A) and salivary gland sporozoite (spz) (B) counts in infected *aapp-DsRed*, *aapp-hGrx1-roGFP2* and *trio-DsRed* females in comparison to *wild type* (*G3*) females. Data pooled from three biological replicates. The total number of dissected mosquitoes is given above each genotype. C) Number of salivary gland sporozoites (spz) per oocyst for all three reporter lines in comparison to *wild type* (WT: *G3*). The invasion rate was calculated using the data shown in (A) and (B). Each dot represents a single mosquito (A) or the result of a counting (B and C), the median is indicated by a bar. All data were tested for significance using a one-way-ANOVA (Kruskal-Wallis test). ns: not significant ($p > 0.05$).

Figure 5. Salivary gland reporter lines as tool for *in vivo* imaging.

A) Genetic models used for *in vivo* imaging. Mosquitoes carrying the *aapp-DsRed* transgene were crossed with *yellow(-)KI* mosquitoes displaying reduced black pigmentation. Infections were carried out using the highly fluorescent *P. berghei* line $\Delta p230p-GFP$ constitutively expressing EGFP (Manzoni *et al.*, 2015). **B)** Pigmentation phenotype and salivary gland-specific DsRed expression of *yellow(-)-aapp-DsRed* mosquitoes in comparison to wild type (WT: *Ngousso*). Brightfield and DsRed expression of the same field of view. Scale bar: 0.5 mm. **C)** Images of the abdomen of infected *yellow(-)KI* and wild type (*Ngousso*) mosquitoes. Brightfield and GFP expression of the same field of view. White arrows point towards oocysts visible through strong pigmented areas of the cuticle of *yellow(-)KI* mosquitoes. Scale bar: 250 μ m.

Figure 6. Confocal imaging of sporozoite salivary gland interactions in live mosquitoes.
A) Thorax view of a *yellow(-)-aapp-DsRed* female mosquito infected with *P. berghei* *Δp230p-GFP*. Differential interference contrast (DIC) image on the left and the GFP signal on the right. Note that the DsRed expression in the salivary glands is strong enough to be visible in the GFP channel. Red arrows point towards sporozoites and the dashed line in the DIC image indicates the position of the salivary gland as seen in the GFP channel. Scale bar: 20 μm. **B)** Zoom on a group of sporozoites sequestered in close proximity to the salivary gland in the area boxed as (a) in the previous image (A). DIC image and a time series of the GFP signal with 6 s between frames. Red arrows point towards sporozoites displaying movement. Scale bar: 10 μm. **C,D)** Accumulation of sporozoites close to the salivary gland. **C** and **D** show the same salivary gland with altered focus and shifted field of view. DsRed signal (left) to indicate the position of the salivary gland (SG), and time series of the GFP signal with 8 s between frames. The red arrow points towards sporozoites displaying active movement (C) or towards a sporozoite in contact with the salivary gland (D). Scale bar: 10 μm. Note that imaging was performed with a Hamamatsu Orca Flash 4.0 V1 camera for A) and B) or a Yokogawa CSU X-1 for C) and D) using a 63-fold magnification objective (N.A. 1.4).

Figure S1. Genotyping of the salivary gland reporter lines *aapp-DsRed*, *aapp-hGrx1-roGFP2* and *trio-DsRed*.

A) Schematic representation of salivary gland reporter transgenesis cassettes inserted on chromosome 2L. Fusion of *attB* and *attP* sites generate *attL* and *attR* sites after integration. The *Lox* site upstream of the transgene is a remnant of the fluorescence cassette initially required to select the docking line X1. Note that the illustration is not drawn to scale. **B)** Genotyping of generated transgenic mosquito colonies in comparison to *wild type* (WT: *G3*) or the parental line X1. Five different analytical PCRs were performed. The 5'INT and 3'INT PCRs amplify the integration borders upstream and downstream of the transgene, respectively. The presence of the puromycin resistance cassette was tested (OpiE2-Puro PCR). To ensure integration of the relevant fluorescence cassette, the PCRs *aapp-DsRed*, *aapp-hGrx1-roGFP2*, *trio-DsRed* and *sag-DsRed* were performed. In addition, a PCR control amplifying the 5'UTR of the *yellow* gene on chromosome X was included. Genotyping primers and amplified sequences are indicated with arrowheads and thin black lines in the scheme (A). The red asterisk marks an unspecific band only observed in the control X1.

Figure S2. The intergenic sequence between AGAP000609 and AGAP000610 (*saglin*) displays no promoter activity.

A) Images of salivary glands from a *sag-DsRed* and a wild type female (*Ngousso*). Dissected glands were stained with Hoechst 33342 to detect the nuclei of acinar cells. The dotted black line in *DsRed* images indicates the outline of the salivary gland lobe observed in DIC. Scale bar: 20 μ m. **B)** Genomic context of *saglin* (AGAP000610) with neighboring genes. The length of intergenic sequences is indicated above the scheme and the sequence tested for promoter activity located between AGAP000609 and *saglin* is shown. The stop codon TAA of AGAP000609 and the start codon of *saglin* are highlighted in black. The length of the cloned sequenced intergenic sequence was 220 bp (without stop codon of AGAP000609 and start codon of *saglin*). Note that the illustration is not drawn to scale.

Figure S3. Genotyping of *saglin(-)KI*, *saglin(-)EX* and *yellow(-)KI* mosquitoes.

Saglin(-)KI (A) and *yellow(-)KI* (B) mosquitoes were generated by injecting embryos expressing Cas9 (*vasa-Cas9*) with plasmids carrying repair templates that contain a Lox3xP3Lox-EGFP or a Lox3xP3-EGFP-Lox cassette flanked with upstream and downstream sequences of the respective targeted locus, in combination with three guide RNAs specific for the *saglin* (AGAP000610) or *yellow* gene (AGAP000879). Genotyping of the 5' and 3' integration borders revealed successful integration of the transgenes into *saglin* (C) and *yellow* (D). The red asterisk marks an unspecific PCR product observed in the wild type control (WT: *Ngousso*). E) To investigate the native expression of the *saglin* promoter, the 3xP3 promoter

929 initially used to select transgenic *sag(-)KI* mosquito larvae was removed by Cre-mediated
930 excision. The loss of the *3xP3Lox* sequence in *sag(-)EX* mosquitoes was confirmed by PCR.
931

Figure S4. Expression driven by *trio* and *aapp* promoters is female- and adult-specific and the fluorescent reporter accumulates after the emergence of adults.

A) Fluorescence and brightfield images of female / male pairs of *trio-DsRed*, *aapp-DsRed* and *aapp-hGrx1-roGFP2* mosquitoes. Note that all depicted mosquitoes were bred in synchrony and were seven day old (+/- 1 day). Scale bar: 0.5 mm. **B)** Fluorescence and brightfield images of *aapp-DsRed*, *trio-DsRed* and *aapp-hGrx1-roGFP2* pupae in comparison

to *wild type* (*G3*). Dotted circles indicate the putative position of the salivary glands. *Trio-DsRed* pupae with low and high DsRed body expression are shown for comparison. The red arrows indicate DsRed positive ocelli observed in most *trio-DsRed* pupae. Scale bar: 0.5 mm. C) Brightfield images of *aapp-DsRed* and *trio-DsRed* female salivary glands from day 1 to day 5 after hatching (a single image per day and per line). All images were acquired using the same settings. Scale bar: 250 μ m.

Figure S5. DsRed expression in Malphigian tubules (MT) of *trio-DsRed* mosquitoes is linked to ubiquitous DsRed expression in larvae, pupae and adults.

A) *Trio-DsRed* L1 and L4 larvae were classified as *trio-DsRed low fluo* or *trio-DsRed high fluo* according to their body DsRed fluorescence intensity. Red arrowheads point towards the DsRed positive eyes observed in the majority of larvae. Scale bar L1: 250 μ m. Scale bar L4: 0.5 mm. **B)** Whole body fluorescence of *trio-DsRed high fluo* male and female mosquitoes. From top to bottom: brightfield, inverted DsRed fluorescence signal displayed in black and white and in red on black. Note that images were stitched from two separate images showing either the head or the abdominal part. Scale bar: 0.5 mm. **C)** Representative images of male and female midguts dissected from mosquitoes that displayed high body fluorescence at the pupal stage in comparison to *wild type* (WT: *G3*). Malphigian tubules (MT) and midguts (MG) are indicated by black arrows. Scale bar: 0.5 mm. **D)** Correlation of high body fluorescence at the pupal stage with DsRed expression in malphigian tubules (MT). Data were collected from two different mosquito generations. The number of analyzed mosquitoes is indicated above each column.

Figure S6. *trio-DsRed* pupae display high variability in DsRed expression.

A) Representative images of DsRed expression patterns in *trio-DsRed* pupae. A subpopulation of pupae displayed high body DsRed expression (images in red frames) while remaining pupae showed no DsRed body expression (black frames). Some pupae displayed fluorescent ocelli and/or fluorescent antennae. The presence of DsRed expression in body, ocelli and antennae occurred in different combinations that are represented by rectangles with different shades and frames. Scale bar: 0.5 mm **B)** Fluorescence patterns in pupae in relation to their sex. Non-fluorescent pupae were exclusively female while pupae with fluorescent antennae were exclusively male. Data pooled from four generations (≥ 20 pupae per generation). **C)** Proportion of each fluorescence pattern in relation to the whole population. Shading and framing of the different patterns as in (A). **D)** Proportion of pupae with high and low body fluorescence in the *trio-DsRed* colony (F0) and after intercrossing individuals displaying high or low pupal body fluorescence (F1). The number of analyzed individuals is given above each column. **E)** Females hatched from pupae with high body fluorescence analyzed in (D, high fluo F1) were kept separately and crossed to wild type males (*Ngousso*). The progeny of single females was evaluated at the pupal stage for body fluorescence (same color code as D, dashed line indicates 50% of the population). Females of families 5, 6, 7, 8 and 10 were also evaluated for salivary gland fluorescence which was present in all specimens.

Figure S7. Genotyping of *trio-DsRed high fluo* in comparison to *trio-DsRed low fluo*.

Genotyping of a female mosquito homozygous for the *trio-DsRed low fluo* transgene taken from the colony generated by crossing fluorescence negative pupae (see Fig. S6D) in comparison to a female mosquito heterozygous for the *trio-DsRed high fluo* transgene and the wild type allele obtained from family 1. An illustration of the modified X1 locus containing the *trio-DsRed* transgene in comparison to the unmodified wild type allele is shown on top. Genotyping primers and expected products are shown as black arrows and lines, respectively. The length of the expected PCR products is indicated below the gel images. Note that for the heterozygous female carrying the *trio-DsRed high fluo* transgene the smaller PCR fragment amplifying the unmodified locus dominates while the long PCR fragment representing the transgene containing allele is visible as a faint band at the upper edge (marked by a red arrow). The *OpiE2-Puro* PCR to verify the presence of the transgene gave several unspecific bands. PCRs were performed on genomic DNA obtained from single mosquitoes.

Figure S8. Selection and sample preparation of mosquitoes for *in vivo* imaging.

A) Images of an infected mosquito with sporozoites (Spz, GFP channel) inside salivary glands and salivary gland (SG, red channel) localization close to the cuticle. GFP and DsRed images show the mosquito neck region squared in the brightfield image (top). **B)** Positioning of the salivary gland varies between mosquitoes, either deep (rows 1 & 2) or close to the cuticle (rows 3 & 4). Brightfield (left) and DsRed (right) images of the same field of view. Images were acquired with the same settings. Scale bar: 250 μ m. **C)** Quantification of salivary gland positioning according to the fluorescence pattern shown in (B). Two mosquito batches were evaluated per time point post emergence (p.e.). The number of evaluated mosquitoes is indicated above each column. **D)** Sample preparation before imaging. Legless mosquitoes were glued on a microscopy slide and covered with a cover slip underlaid with orange plasticine to avoid squeezing. Corner areas were subsequently coated with teal-colored nail polish to prevent the cover slip from moving.

Movie 1. Sporozoites display active motility inside the mosquito.

Movie showing three sporozoites imaged in close proximity to the salivary gland. Sporozoites appear to be attached to a larger vessel and observed movements might be a mixture of active motility influenced by passive hemolymph flow. Imaging performed with a Hamamatsu Orca Flash 4.0 V1 camera using a 63x (NA 1.4) objective. GFP signal of a 3 min movie with 3 seconds between frames.

Movie 2. Sporozoites form accumulations close to the salivary gland.

Movie showing an accumulation of sporozoites close to the salivary gland directly below the cuticle. Several sporozoites display active back and forth movements. Imaging was performed with a Yokogawa CSU X-1 camera using a 63x (NA 1.4) objective. The left side of the movie displays the DsRed signal (salivary gland) while the right side shows the GFP signal (sporozoites) in black on white. Two min movie with 2 seconds between frames.

Movie 3. Sporozoite crawls through tissue by employing back and forth gliding.

Movie showing an actively moving sporozoite below the cuticle. The sporozoite was captured in proximity to the gland. By actively moving back and forth the sporozoite crawls slowly forward reaching eventually deeper tissues layers. Imaging was performed with a Yokogawa CSU X-1 camera using a 63x (NA 1.4) objective. The left side of the movie displays the DsRed signal (salivary gland) while the right side shows the GFP signal (sporozoites) in black on white. Two min movie with 2 seconds between frames.

Supplementary Table S1.

List of primers used for cloning plasmids to generate salivary gland reporter lines as well as *yellow(-)KI* and *sag(-)KI* lines. Nucleotides highlighted in green represent the cutting site for *BsaI*. The guanine written in white on a black background indicates an undefined position of the *BsaI* cutting site which can be any nucleotide. The nucleotides marked in red represent the overhangs needed for assembly of the GoldenGate building blocks. Sequences written in black define primer binding sites and nucleotides highlighted in orange represent overhangs to allow fusion of DNA sequences by assembly PCR.

Primer	Sequence (5' to 3')	Description
P1	GGTCTCGATCCCTTTTCTTTACCCTTTGT AACACGCTAATAACG	aapp_5'UTR_forward
P2	GGTCTCGCTCTGTCGTTTTTTTGTGTTGTG GTATTATTTCACTG	aapp_5'UTR_reverse
P3	GGTCTCGAGAGATGGCTCAAGAGTTTGTG AACTGCAAAATC	hGrx1-roGFP2_forward
P4	GGTCTCGAAGCTTACTTGTACAGCTCGTC CATGCCG	hGrx1-roGFP2_reverse
P5	GGTCTCGAGAGATGGTGCGCTCCTCCAAG AACG	DsRed_aapp/trio_forward
P6	GGTCTCGAAGCTACAGGAACAGGTGGT GGCGGC	DsRed_aapp/trio_reverse
P7	GGTCTCGATCCCTTTCTGAGGTGATCTTTC GAAAAGATCG	trio_5'UTR_forward
P8	GGTCTCGCTCTGTAAGTGGGAGCAGATTG ATTTTATGG	trio_5'UTR_reverse
P9	GGTCTCGATCCCTTTCATGCGTACAATGTG TATGATCACTG	saglin_5'UTR_forward
P10	CGTTCTTGGAGGAGCGCACCATTATAAGT GAACCTTATGCAAACCTTCCCTG	saglin_5'UTR_reverse

1071	P11	CAGGGAAGGTTTGCATAAGGTTCACTTATA	DsRed_saglin_forward
1072		AATGGTGCCTCCTCCAAGAACG	
1073	P12	GGTCTCGAAGCCTACAGGAACAGGTGGTGG	DsRed_saglin_reverse
1074		CGG	
1075	P13	CCTTGTAAGCGTGCCTGGTGGCCGC	Yellow_gRNA1_fw
1076	P14	AAACGCGGCCACCAGGCACGCTAC	Yellow_gRNA1_rev
1077	P15	CCTTGCGACTACGTGCCCCACGAA	Yellow_gRNA2_fw
1078	P16	AAACTTCGTGGGCACGTAGTCGCC	Yellow_gRNA2_rev
1079	P17	CCTGGAGAACAAGCTGTTCGTCT	Yellow_gRNA3_fw
1080	P18	AAACAGACGAACAGCTTGTCTCC	Yellow_gRNA3_rev
1081	P19	GGTCTCGAACAAGAATTGAACGCCCAAGGTACGGCTG	Yellow_5'UTR_fw
1082	P20	GGTCTCACCCCGCTTCTGCCACTAAGGAACTTCTGTG	Yellow_5'UTR_rev
1083	P21	GGTCTCGAAGACGTGTGTCCTGGAAGTGTGATTATCG	Yellow_3'UTR_fw
1084	P22	GGTCTCGAAGCGTAGCGTGCCTGGTGGCCGCCGG	Yellow_3'UTR_rev
1085		AACGCTTTCCCTGCAGCGTAC	
1086	P23	CCTTGCGTTCAAGTCATCTCGAGC	Saglin_gRNA1_fw
1087	P24	AAACGCTCGAGATGACTTGAACGC	Saglin_gRNA1_rev
1088	P25	CCTTGCCGCAGTCCTCGCGGCTGG	Saglin_gRNA2_fw
1089	P26	AAACCCAGCCGCGAGGACTGCGGC	Saglin_gRNA2_rev
1090	P27	CCTGAAGTTTGCAACGTTTGCGA	Saglin_gRNA3_fw
1091	P28	AAACTCGCAAACGTTGCAAACCTTC	Saglin_gRNA3_rev
1092	P29	GGTCTCGAACAATGGGTTCACCTCGCTGTTAGACTGTG	Saglin_5'UTR_fw

1093 **P30** GGTCTCAATGGTTATAAGTGAACCTTATGCAAACCTTCCCTG Saglin_5'UTR_rev

1094 **P31** GGTCTCGAAGAGCGTCGGCGACGGTCTAAAAG Saglin_3'UTR_fw

1095 **P32** GGTCTCGAAGCGTTCAAGTCATCTCGAGCCGG Saglin_3'UTR_rev
1096 CTTCTTCTGCTGCCGCAGAAAC

1097

1098 **Supplementary Table S2.**

1099 List of primers used for genotyping. Note that some primers used for construct cloning have
1100 also been used for analytical PCRs.
1101

1102	Primer	Sequence (5' to 3')	Binding site
1103	P33	GGTCTCGAACAGAATTGAACGCCCAAGGT	yellow_5'_forward
1104		ACGGCTG	
1105	P34	GGTCTCACCCCGCTTCTGCCACTAAGGAAC	yellow_5'_reverse
1106		TTCTGTG	
1107	P35	CCCGTCATGAGTTCAAGCCTGAA	X1_forward_1
1108	P36	CCCGGTCTCAAATTGCCGCGGTGACCGTC	attB_reverse
1109		GA	
1110	P37	CAAGCACCTTTATACTCGGTGGCCTC	OpiE2_forward
1111	P38	TTCCAGGAAGGCGGGCACCCC	PuroR_reverse_1
1112	P39	ACTGCAACCCATTCACTG	X1_reverse
1113	P40	CAGGGAAGGTTTGCATAAGGTTCACTTATA	DsRed_forward
1114		AATGGTGCGCTCCTCCAAGAACG	
1115	P41	CCTCTACAAATGTGGTATGGCTGA	SV40_reverse
1116	P42	GGTCTCGATCCCTTTCATGCGTACAATGTGT	sag_forward
1117		ATGATCACTG	
1118	P43	CCTTGAAGATTGGAATCCATCCAT	X1_forward_2
1119	P44	GGTCTCGATCCCTTTTCTTTTACCCTTTGTAAC	aapp_forward
1120		ACGCTAATAACG	
1121	P45	GGTCTCGATCCTTTTCTGAGGTGATCTTTCGA	trio_forward
1122		AAAGATCG	
1123	P46	GGTCTCGAGAGATGGCTCAAGAGTTTGTGAA	hGrX1-roGFP2_forward
1124		CTGCAAAATC	
1125	P47	GCTTATAAGAGCGCTACGGGTGTAC	Sag_5'_fw_genotyping
1126	P48	CTGTCCCTTCAGCTTCACCAGCTC	Sag_3'_rev_genotyping
1127	P49	GAACGACAGGTGAGCTGACAGTAAC	Yellow_5'_fw_genotyping

1128	P50	GATTTGGCGAGATGGTTTGTGCGAC	Yellow_3'_rev_genotyping
1129	P51	GAGCTCGAGTTCAGGCACCGGGCTTGC	PuroR_reverse_2
1130	P52	CTTCGGGCATGGCGGACTTG	EGFP_reverse
1131	P53	CAGCCATACCACATTTGTAGAG	SV40_forward
<hr/>			
1132			

1133 **Supplementary File S1.**

1134 Promoter sequences of the *trio*, *saglin* and *aapp* genes used to generate the mosquito lines
1135 *aapp-DsRed*, *aapp-hGrx1-roGFP2*, *sag-DsRed* and *trio-DsRed*. Nucleotides highlighted in
1136 red are part of the primer sequence used to amplify the promoter sequence. Sequences with a
1137 grey background indicate the 5'UTRs of the *trio* and *aapp* transcript, respectively.

1138
1139 ***aapp* promoter sequence (AGAP009974, 1673 bp):**

1140
1141 **CTTTTCTTTTACCCTTTGTAACACGCTAATAACG**AAAGACCTCCTGCGTGTTAGTACG
1142 TGGTGAGGATGATGCGTTTTAGCCTAGCTAGTATTCAAAAAACCTATTAATGCAACAATTAC
1143 AGTGTGAGGTTACAAAAATGGAATTAATTAAACACACTGCTCAACACAGCCATCGTGCTGCAT
1144 CGTGCTTTGTTGAGGCGCAACAGTTAAATTGTAAGTGCAACCACCGGTATAGAGAGCTGCTT
1145 TTGGCAAATGATATGGAATTGTGCTAAATTATGTAATGTTTTTTTTTAACTAACTCGTTTACC
1146 CGTTAATGGTGGAAATGTTTGCAGGTGCGTAGATTAATTTTTTTTTTAAATGTGCAAATGGAGAA
1147 AACAGGATAACGCAAAAGTATTTCCCTCGCTGTAGTAATAGGCTATTATTTATGTACGCTCTT
1148 TATGTGACCCCGCTAGTGTGGCTGTATTTTATATCTGTAGGTAAATGACGCGCTATGTATT
1149 GTAATAAAATTGGACAAACATTTGCATGTACGATCTGTTAGCTTAGAGAATAAGTATTTTTTC
1150 TTTTTTCGCTACATTTCTCTAAATTCGACCGACGATGACTGTTTTAGGTGTTTTTTCGTAACCT
1151 TGATCAAGGTAACACCTTTTCTTGGCGTAACGTCCTACGCGGACATGCCGGCCGGCCTTATT
1152 CAGGCTTTCGAGACTTAATTCATTACCACGTAGCCGGATAAGTCCAATCCTTGCACAACGGG
1153 GGAACGGGTGTCCATTTTCAAGGCTTGGGCCCATGACGGGCATGTTATTGAGTCGTTTCGAGTTG
1154 ACGACTGTACCATGGGACCGCCCCATTTTTTGGCAGTCCATTGATCGTTACGTCGTTTTACGT
1155 CTACTGAAGGATGTCCGTTATCAGGTCAAGTCCAGCATAGATTAAAGTCTCCCATACGAAGGA
1156 TTGATTAGCTCGAGACTTAATGTTATATACAAAATTCCTTATACATGTTCTAGATCAGGATTG
1157 TTGCGCTACACTGTCTTCCATGTCGATCTTCTATATTCAGTTTTTGGCACAGTGATCGTCAC
1158 TGTAATCTCAATAGATTTGAATTTCAATACTTCTATGGGAGGAGGATGAGTAGAAAGGTGTC
1159 CAAAGCGCTGGATCTATGGCTGCAACTCCTCATGCTTGTAAGCACCCGACAGCCAATTCGAT
1160 CTACCTGACCTGATGCGGTATTCGAACATAAAGCACAGATCAAGCACTGTTTTCACACGATCT
1161 GAAGCACTGTTCTACTGCAAAGATAAGTGCATATTAGTTATTTTTTCATACCGATCTTCACTG
1162 CGACGGTTTAGCGATCTTCATTATTTTCCACCTTCACCCCTCAACCCAGGGAGTGCGCTTTT
1163 CCCCGTCGAGATAAAATTACTAAATGTAAACTTTGCATGCAATTATGTAGCTGTATGGTAAG
1164 CAAATAAAAATCTTTCTCCTATTGCAACACGTCGCTTGTTTGCAGCGGATATTTGCCACACA
1165 GTAAAGCCCGATCGCCTGTGCATTGTGTTTAAACACGTGCTGCAAGCTGTATCAATACCGATC
1166 GATTGTATGATTCCACGATCTTCGTGTGGTATAAAAACGGCGCTGAACAGCAGTAAAGATCA
1167 TCACCTTACATTTGTTTTATTCTCCACGGAGT**CAGTGAAATAATACCACAACAACAAAAAAC**
1168 **G**
1169

***trio* promoter sequence (AGAP001374, 1537 bp):**

TTTTCTGAGGTGATCTTTCGAAAAGATCGATCGACCGACCCCCAACGGCAACTCTCCC
TCGGATGACCTCAGCCCAGAATAGGCATTCTTTCAGCAAGTCATTCTATCATCCCTTTTACA
TCTTACGTTACTCCAAAAAAGAGAAGCTTTCACCCCAAAGCTTTCGTTGTGGTGCAGCTT
TCGATGGTGATCGCGATCACGGTACCACACGTACGTTTGTGTGCTACTCTCGCTCACCGTTT
TCTTCGATTTTCGTGTCTCGGGTACCGTTTGCTCGGTGCGGTAGTGCTGCGAAGAAAGCCGTT
GGCCGAGAGAATAATGCTCACTTCGGCTGCACGCAACTCGCCGTGCCACAGCCGTGGAAGGG
GTAGGTTGGATGGTTCGGGGTCGGGACCGCAACGCCGAAGTGAACGCCGGCGTGCCAGCGAAC
GATCGCACCCCGGCACAGTCCAAGTGCCTGCTGGGCGCGCTCGGCGGACGGTTCTCGGTGT
GTGCGCCCCCTTTGGTGCTCATCCCTCATATCTCATCTCATCCTTTCGCGCTGCTCGCGGTT
CCCGCAGAACCACGCGCGAGAGGGAGTTGCGCGAACCCAGGGAGAGCCCGACACCTGGGCAGC
GTCCAGGCGGACCTGTTTCGGGAACGGTTCGCGGTGTATAAACTCGATTCCCGCCCCGCCGTTA
GGTAACAGTCCATTGTGTAACGTTGCCGGGATCGCGAGCCATCCTGCAGGAACACAGCCGTG
CGAGTTTGTGTGCGCGTGTATTTTGCTTACAAGCAAGTGAGCAGCAATCAGTGAGAACAAACA
TCTAAACAGAAAAACAAACAGTGAAGTACAGTGCTGTGGAGCAGTGGAATTTGGTGTTAAT
TGAAGAGCTTTGTACATCGATCACACGGACAACACGCACGTGGAGAAAGTTCTGCTGCGGT
GCGGGTTACTTGCGTACACCACATTAAACATTGTGTGTGGCATTAAATAAATACTACTCGC
GCCCTCCCCTCAGCTGCTCTCGCGGGCACGCCCGATGCCAGAGGTCGCCCCGACCGGACATT
GAACGTGGAAGAAGAAAGTTGTCGGTTGTTTACACTTCCGTCCTGAGTGGTGTGTGGCTA
CCGTTTGTGACCGTTGGTGAAATAAAGTAACATAACCCCTCCCGAGGGCAGCAGCGACAATGA
CCTCCGGAAATTCGATAACAATACGTAAGTTACTTTTCATTTTTTTGCAAATTAAATTACCTCG
TGATAATTGAGTGGAGTGAGTAGGTACAGGTTGTTAACGCGTCTTTTGTGTTAGTTCGGCAAT
CCGATCCGAAGATGAGAGGAGATTGCCTCTGATGATGATGTTATCATGATTGCTTGTGTAA
CGACGCAAAGATTCTCAGAGAACAACCTCTCCCAACATAATTAATCTTGTTGAAGGGTCTGT
AAACACGTGGTGGGATATCTTACAACCTGAACCAATATTCGACCGATAGTATAAAGTCCTTCG
CACCATCGATGCGTTTACCATTTTCCCATAAAATCAATCTGCTCCAGTTACA

***sag* promoter sequence (AGAP000610, 220 bp):**

CTTTCATGCGTACAATGTGTATGATCACTGATGACAAAGCGCTTGGGTGGGCGCTTGC
TGCTGAAGCGTGGCTGACAAATGGGGGGGGGGGGGGCTTGAACAAATATTTGCACACACGAT
AATGCTCTTGTGTTGCCATGCGCTGACGGAAGACAAATCGCTGCAAGCGACGGCTCAAACCTG
CTCAAACAGGGAAGGTTTGCATAAGGTTCACTTATAA