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ORIGIN AND FUNCTION OF LOQS2, A MOSQUITO-SPECIFIC DOUBLE-STRANDED RNA-BINDING PROTEIN

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“If there’s something you don’t understand, learn to understand it”

- Hange Zoë

“It is not ‘impossible’, it is just ‘hard’.”

- Bokuto Kotarou

“Give up on your dreams and die.”

- Levi Ackerman

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A-to-I	Adenosine-to-Inosine
aBravo	aedine broadly active antiviral protein
ADAR	Adenosine deaminase acting on RNA
adj. p-value	adjusted p-value
Ae. aegypti	*Aedes aegypti*
Ae. albopictus	*Aedes albopictus*
Ago1/2/3	Argonaute 1/2/3
AMP	Antimicrobial peptides
An. gambiae	*Anopheles gambiae*
Arbovirus	Arthropod-borne virus
ATP	Adenosine Triphosphaste
Aub	Aubergine
Bel	Belle
BIC	Bayesian information criterion
bp	basepair
BSA	Bovine serum albumine
BTV	Blue tongue virus
C. *quinquefasciatus*	*Culex quinquefasciatus*
Cas9	CRISPR-associated protein 9
cDNA	complementary DNA
CHIKV	Chikungunya virus
COVID-19	Coronavirus disease-19
CP	Carboxypeptidase
CRISPR	Clustered regularly interspaced short palindromic repeats
CrPV	Cricket paralysis virus
D. *melanogaster*	*Drosophila melanogaster*
DALY	Disability-adjusted life years
Dcr-1/2	Dicer 1/2
DCV	Drosophila C virus
DDX3	DEAD-box helicase 3
DENV	Dengue virus
DHX9	DExH-box helicase 9
Dip1	Disconnect-interacting protein 1
DNA	Deoxyribonucleic acid
dpi	days post infection/injection
dsRBD	dsRNA-binding domain
dsRBP	dsRNA-binding protein
dsRNA	double-stranded RNA
E1	Envelope protein 1
EDTA	Ethylenediaminetetraacetic acid
endo-siRNA	endogenous siRNA
EtOH	Ethanol

exo-siRNA	exogenous siRNA
Exu	Exuperentia
FBS	Fetal bovine serum
FC	Fold-change
FDA	Food and Drug Administration
FDR	False discovery rate
FHV	Flockhouse virus
fmol	femtomol
Gb	Gigabase
gDNA	genomic DNA
GFP	Green fluorescent protein
GO	Gene Ontology
GSEA	Gene set enrichment analysis
h	hour
Het	Heterozygous
hPa	hectoPascal
HTV	Humaita-Tubiacanga virus
IKK	IκB kinase
IMD	Immune deficiency
ISV	Insect-specific virus
IVT	In vitro transcription
JAK	Janus activated kinase
JEV	Japanese encephalitis virus
kDa	kiloDalton
kg	kilogram
KO	Knock-out
LACV	La Crosse virus
LB	Luria-Berthani
Loqs	Loquacious
M	Molar
Mb	Megabase
mg	milligram
MgCl2	Magnesium chloride
µg	microgram
µL	microliter
µm	micrometer
min	min

miRNA	microRNA
mL	milliliter
mle	maleless
mM	millimolar
mRNA	messenger RNA
MS	Mass spectrometry
MSL	Male-specific lethal
MYA	Million years ago
NaCl	Sodium chloride
nanoLC-MS/MS	nano liquid chromatography-MS/MS
NaOH	Sodium hydroxide
nL	nanoliter
NLS	Nuclear localization signal
ns	not significant
NS1	Non-structural protein 1
nt	nucleotide
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBS-T	PBS-Tween 20
PCLV	Phasi Charoen-like phasivirus
PCR	Polymerase chain reaction
PGRP	Peptidoglycan recognition protein
piRNA	PIWI-interacting RNA
PIWI	P-element induced wimpy testis
PKR	Proteine kinase R
PTX	PBS-Triton X100
qPCR	quantitative PCR
RHA	RNA helicase A
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
RNAi	RNA interference
ROS	Reactive oxygen species
rpm	rounds per minute
rRNA	ribosomal RNA
RT	Reverse transcription
RVFV	Rift Valley fever virus
s	seconds
SDS	Sodium dodecyl sulfate
SFV	Seamliki Forest virus
sgRNA	single-guide RNA

SINV	Sindbis virus
siRNA	small-interfering RNA
ssRNA	single-stranded RNA
STAT	Signal transducers and activators of transcription
TAE	Tris-Acetate-EDTA
TE	Transposable element
Tris-HCl	Tris-Hydrochloric acid
tRNA	transfer RNA
Upd	Unpaired
US	United States
USD	US dollars
UTR	Untranslated region
v/v	volume/volume
vpiRNA	virus-derived piRNA
vsiRNA	virus-derived siRNA
VSR	Viral suppressor of silencing
w/w	weight/weight
WHO	World Health Organization
WNV	West Nile virus
WT	Wild-type
YFPnls	Yellow fluorescent protein with NLS
YFV	Yellow fever virus
ZIKV	Zika virus

Introduction

I. Mosquitoes, humanity's greatest nemesis

The mention of mosquitoes seems to always elicit the same type of response in people. The resentment towards this insect is such that it is often referred to as the number one public enemy, or even as our deadliest predator. This is a title that it seemed to have started earning for a while, when it went from being a simple nuisance, to being suspected of transmitting a deadly disease. Indeed, as early as 1717, Giovanni Maria Lancisi accused mosquitoes of being responsible for the transmission of malaria, in his book *De noxiis paladum effluviis*, proposing that "mosquitoes and other biting insects are the organisms that produce the fever after introducing the effluvia into the human body" (Klaassen *et al.*, 2011). It took more than 150 years for mosquitoes to indeed be proven guilty of this disease transmission, by the 1902 Nobel laureate Ronald Ross (Talapko *et al.*, 2019).

Although they started becoming a concern a few centuries ago, they have been around for a lot longer than that. It is estimated that mosquitoes originated some 226 million years ago (MYA), with some recently found fossils confirming that they were already present 100 to 110 MYA (Reidenbach *et al.*, 2009; Poinar *et al.*, 2020).

It is not known to which extent mosquitoes were present at the time, but nowadays, they are distributed all around the globe. Like for numerous insects, a large variety of mosquito species are found in tropical and subtropical regions, many of which are equally established in temperate areas. Mosquitoes are found in all countries, with the exception of Antarctica and a few islands. More than 3,500 mosquito species have been reported in all of these environments, and since many of these areas are not fully explored, it is estimated that at least a thousand more species remain to be discovered (Rueda, 2008).

One of the main characteristics of all these species, except for *Toxorhynchites* and a few others, is that females rely on the intake of blood to start their gonotrophic cycle and give birth to their progeny. The source of blood can vary greatly, depending on the mosquito species, from cattle and birds, to even reptiles and amphibian sometimes, and some species even developed a strong preference for humans (Rueda, 2008). Species such as *Anopheles gambiae*, *Aedes aegypti* and *Aedes albopictus* fall into that last category, as they are indeed highly to exclusively anthropophilic (Stone and Gross, 2018; Egid *et al.*, 2022).This preferential diet, and the disease transmission risk that comes with it, is what makes these species part of the 200 medically-relevant mosquito species (Reidenbach *et al.*, 2009).

This disease transmission risk is heightened by the behavioural habits of the mosquitoes, who usually live in close proximity to humans. Indeed, mosquitoes spend the first days of their life in the water, as larvae and then pupae. To allow for this first part of the development, females lay their eggs at the surface of the water, in a variety of aquatic environments, with each mosquito species having its preferences. Swamps, marshes and agricultural fields tend to be usual breeding sites for *Anopheles* and *Culex*, while *Aedes* mosquitoes most often lay eggs in tree-holes, and some species even adapted to be extend their breeding sites to artificial containers such as tires or tin cans

(Rueda, 2008; Chandrasegaran *et al.*, 2020). In newly urbanized area, water supply is oftentimes scarce, making it necessary to store it in large containers, which in turn become perfect breeding sites for *Aedes* mosquitoes, for example (Gubler, 2011).

This ability of mosquitoes to easily adapt to new environments, and to lay their eggs in a large variety of habitats, greatly participated in their spread around the world. Over these last decades, there was an extremely large expansion in the geographic distribution of *Ae. albopictus*, which was most likely introduced in temperate areas such as Europe through transportation of used tires, and largely through the trade of Lucky bamboos from Asia (Lambrechts *et al.*, 2010; Medlock *et al.*, 2012). Increase of aerial traffic also in part contributed to this spread, with the transportation of more than 2 billion people per year, travelling to and from areas where these species are endemic (Gubler, 2011). Lastly, a very important factor contributing to the domain expansion of these mosquitoes is the continuous rise in global temperatures, due to climatic change. This indeed allowed for very adaptable tropical and sub-tropical species to easily invade and establish themselves in more temperate areas, with *Ae. albopictus* now being found in much colder regions, such as Canada (Facchinelli *et al.*, 2023).

This widely uncontrolled expansion of the distribution of mosquitoes, and in particular readily adaptable species such as *Ae. albopictus* and *Ae. aegypti*, strongly correlated with an increase in the transmission of infectious diseases in the newly colonized areas, emphasizing the public health significance of these diseases.

II. Arboviruses represent an important threat to public health

These diseases transmitted by mosquitoes are referred to as vector-borne, and account for more than 17 % of all infectious diseases worldwide, according to the World Health Organization (WHO). The fatality for these diseases reaches almost 700,000 deaths each year, for which more than half can be imputed to malaria, a disease resulting from the transmission of a parasite from the *Plasmodium* genus by *Anopheles* mosquitoes, that mostly affect Africa nowadays (Talapko *et al.*, 2019).

Infectious diseases transmittable by mosquitoes can also be of viral origin, in which case, they are referred to as arthropod-borne viruses (arboviruses). Among those viruses, some are very well known worldwide, as it is the case for dengue virus (DENV). This virus is estimated to cause over 96 million symptomatic cases a year, in addition to 294 million asymptomatic infections. Considering the continuing expansion of the geographic distribution of the main vectors for this virus, *Ae. albopictus* and *Ae. aegypti*, it is now estimated that more than 3.9 billion people are at risk of getting infected by DENV, representing close to half of the world's population (Bhatt *et al.*, 2013; Messina *et al.*, 2019). For these reasons, among other, DENV was considered one of the 10 most important global health threats by the WHO, in 2019, before the arrival of coronavirus disease-19 (COVID-19).

Although representing one of the biggest health threats, DENV is not the only arbovirus of global importance. In the recent years, mosquito-transmissible viruses such as Yellow Fever virus (YFV), Zika virus (ZIKV), Chikungunya virus (CHIKV), as well as

West Nile virus (WNV) and Japanese encephalitis virus (JEV) have also gained a lot of attention (Carvalho and Long, 2021). As it is observed for DENV, infections by these viruses are mostly asymptomatic, which always makes it difficult to estimate the actual burden of the diseases. When symptomatic, the diseases usually present themselves by fever, headaches and malaises. These relatively non-life threatening symptoms in most infected patients, tend to explain the low mortality rates associated with these diseases, with around 40,000 yearly deaths imputed to DENV, for example, as compared to other infectious diseases, such as malaria (Bhatt *et al.*, 2013).

Nonetheless, these diseases still represent around 20 % of the global disease burden, which can largely be attributed to the long-term consequences of infection, rather than mortality (Carvalho and Long, 2021). Indeed, disabilities can result from an infection by the aforementioned viruses, that will affect the quality of life of the patients, as well as their life expectancy. The burden of a disease can thus also be assessed by an indicator called disability-adjusted life years (DALY), that takes these parameters into account, with one DALY corresponding to the equivalent of one year in full health lost to the disease, as described by the WHO. As an example, it was estimated in 2009, that the loss to DALY for DENV was approximating 700,000 a year (Murray and Lopez, 2013). Although lower for the other infectious diseases mentioned here, due to their lower infection rate, disabilities resulting from these infections can still be severe. ZIKV, for example has been shown to cause microcephaly in infants whose mother was infected during pregnancy, and also to trigger Guillain-Barré syndrome in some patients (Carvalho and Long, 2021). Usually resulting in fever and joint pain, an infection by CHIKV can sometimes lead to chronic arthritis, which can be very debilitating for patients, and which can last from several months to several years (Carvalho and Long, 2021). A final example of these long-term consequences is infection by WNV. This virus, transmitted by *Culex* mosquitoes whose primary source of blood is birds, can infect humans and other mammals, in which the disease can result in severe neurological complications (Carvalho and Long, 2021).

Although not accounted for in the usual measures of disease burdens, some of these arboviruses, such as WNV, Blue Tongue virus (BTV) and Rift Valley Fever virus (RVFV) can be responsible for large losses in other mammal species, such as horses and cattle (Carvalho and Long, 2021). These losses in turn affect productivity as well as livelihood of the populations that rely on these animals for their nourishment as well as their economy. In the Americas, studies have estimated that from 2000 to 2007, the aggregate annual cost of DENV approximated USD 2.1 billions, 60 % of which was related to indirect or productivity losses. Similar studies in South-East Asia have estimated these costs for DENV to reach USD 950 millions (Murray and Lopez, 2013). These already elevated costs, however, do not account for prevention and vector control. It is considered that *Aedes* species are among the costliest species in terms of invasion, considering that the cumulative costs related to damage control and management of the species are estimated to amount to USD 150 billion dollars, between 1970 and 2017 (Diagne *et al.*, 2021).

The economic burden of this rising spread of arboviruses is also heightened by the investment required for the research on the prevention and control of these diseases.

Although intensively studied for the past several decades, no specific treatments are available against these diseases, and very few vaccines are currently available. The US Food and Drug Administration (FDA) and the WHO only report available and approved vaccines against four of the previously mentioned mosquito-transmitted viruses, namely YFV, DENV and JEV, as well as a vaccine for veterinary use, against WNV (Carvalho and Long, 2021). These vaccines are mostly employed as a way to control damages and decrease severe outcomes and deaths related to the infection. The vaccine against YFV, which was developed more than 80 years ago, was instrumental in the control of haemorrhagic diseases induced by the virus, but the virus still cyclically emerges and re-emerges in numerous areas. In the case of DENV, the newly developed vaccine has been shown to protect individuals against all four serotypes, however with great variability in the efficiency depending on the serotype. This vaccine however is not to be used for prophylaxis in naïve individuals, as it could result in adverse effects, and is recommended for the protection of children and adults up to 45 years-old against reinfection by other DENV serotypes (Carvalho and Long, 2021). Of course, research on the production of treatments and vaccines against these and other arboviruses are still in progress, and encourages the study of new, innovative solutions to tackle the global threat of arboviral diseases. The use of insect-specific viruses (ISVs) as vectors for vaccines has been investigated in the past few years, as a way of increasing reactivity to epidemics by allowing for short (2 to 3 weeks) production time, and to increase the safety of vaccines, considering ISVs inability to replicate in vertebrate cells (Carvalho and Long, 2021).

This emphasizes the complexity of the struggle against arboviruses, and the difficulty faced by the scientific community in the development of efficient solutions to protect the populations. In the meantime, the control of these epidemics necessarily relies on alternative methods, most of which consist in the control of vector populations. To be efficient, these strategies require a deep understanding of the vectors in question, from their behaviour and ecological role to their biology and the factors that makes them efficient vectors in the first place.

III. Aedes mosquitoes are unique among mosquitoes

Only a few mosquito species, distributed between the *Culex* and *Aedes* genera, can vector these medically-important viruses. Among those, the ones that represent the greatest health burden, DENV, ZIKV, YFV and CHIKV, are transmitted by two vectors in particular, *Ae. aegypti* and *Ae. albopictus*. Their substantial contribution to the spread of these diseases makes these mosquitoes the main targets in the fight against arboviruses. In this context, the development of an efficient strategy mainly, if not only, relies on the extensive comprehension of these mosquitoes, and what differentiate them from other species which impact on public health is considerably lower.

A first important consideration about *Aedes* mosquitoes that makes them stand out, when compared to other mosquito and insect species is its exceptionally large genome. It is estimated that the genome of *Aedes* mosquitoes (based on the sequencing of *Ae. aegypti* as a reference species) nears 1.3 Gigabase (Gb) (Nene *et al.*, 2007; Matthews *et al.*, 2018). In comparison the sequenced genome of *Culex quinquefasciatus* is 579

Megabase (Mb), while that of *An. gambiae* only reaches 278 Mb, making *Aedes* genome around 2.5 and 5 times bigger than those of these two species, respectively (Arensburger *et al.*, 2010). Interestingly, despite this important difference in size, the number of genes predicted in *Ae. aegypti* (15,419) is not very different from that of *C. quinquefasciatus* (18,883) and *An. gambiae* (12,457), and 90 % of those genes have orthologues or homologues in various insect and vertebrate species (Waterhouse *et al.*, 2008; Arensburger *et al.*, 2010).

The most unique feature of *Aedes* mosquitoes, on a genetic point of view, is the exceptionally large proportion of transposable elements (TEs), which cover almost 50 % of the total genome size (Nene *et al.*, 2007; Matthews *et al.*, 2018). As a comparison, it is estimated that 45 % of the human genome is comprised of TEs (Ayarpadikannan and Kim, 2014), and in insects, these proportions are usually considerably lower, with 30 % and 11 % in *C. quinquefasciatus* and *An. gambiae*, respectively (Arensburger *et al.*, 2010). It is largely known that TEs are important drivers of evolution, as their integration in the genome can oftentimes promote genomic rearrangements, mostly through shuffling and recombination events, resulting in large-scale deletions of genomic segments, or gene duplications, for example (Ayarpadikannan and Kim, 2014; Bourque *et al.*, 2018). These elements can thus affect and modulate gene expression, when these genomic rearrangements affect promoters, enhancers, repressive elements and transcription factor binding sites (Ayarpadikannan and Kim, 2014; Bourque *et al.*, 2018).Thus, the presence of such a high percentage of TEs in the genome of *Ae. aegypti* necessarily shaped the evolution of this organism, and likely largely contributed to the development or improvement of the mosquito's adaptation mechanisms.

The adaptability of *Aedes* mosquitoes is also well known and remains one of the main characteristics that distinguishes them from other mosquito species. This allowed them to disperse throughout the world, and to establish colonies in areas with important variation in meteorological conditions, compared to their original habitats (Kraemer *et al.*, 2019). Although this dramatic spread has largely been helped by human activity, through trade, travel, urbanization and also climate change, the mosquitoes would not have been able to establish their colonies in these new territories, without this considerable adaptability (Gubler, 2011). While *Ae. aegypti* is still sensitive to lower, especially freezing temperatures, *Ae. albopictus* now thrives in temperate climates. Although adults very rarely survive the winter seasons in these temperate areas, females are able to lay desiccation-resistant eggs, allowing for delayed hatching, once the conditions are more favourable, even after a few months. In the case of *Ae. albopictus*, eggs can now even survive temperatures below zero degrees, thus increasing the climatic areas in which this mosquito can establish itself (Lwande *et al.*, 2020).

With an increase in the *Aedes* inhabited territories, unfortunately comes an increase in the spreading probability of arboviruses, since their distribution necessarily relies on the presence of their vector. As already established, *Ae. aegypti* and *Ae. albopictus* mosquitoes are known to transmit various arboviruses with great public health significance, such as DENV, ZIKV, CHIKV and YFV. These, however, are simply the examples that catch public and media attention the most, and the extent of viruses that these mosquitoes can transmit is greatly underestimated. It is currently estimated

that *Ae. albopictus* and *Ae. aegypti* have the ability to vector at least 22 arboviruses (Lwande *et al.*, 2020). In addition to the four viruses mentioned above, they also have the vectorial capacity for Sindbis virus (SINV), RVFV, JEV, and WNV (Schaffner *et al.*, 2013). These mosquitoes can also transmit Mayaro virus, Eastern equine encephalitis, Venezuelian equine encephalitis, La Crosse virus (LACV), Banna virus and Saint Louis encephalitis virus (Moore and Mitchell, 1997; Xia *et al.*, 2018). Additionally, the transmission ability of *Ae. albopictus* has been demonstrated experimentally for Ross River virus, Oropouche virus, Jamestown Canyon virus and Western encephalitis virus (Moore and Mitchell, 1997).

Although *Ae. aegypti* and *Ae. albopictus*, as well as other *Aedes* species, theoretically have the ability to get infected and potentially transmit all these viruses, their host-preference for blood intake greatly reduces the probability for them to get in contact with them in the wild. The impressive vectorial capacity of these mosquitoes however remains to be investigated, in order to comprehend the reasons behind this ability. It is likely that the co-evolution of these viruses with their vectors is mostly influenced by the virus itself, rather than by an adaptation from the mosquito. One great example of that is CHIKV, that evolved to improve its transmissibility by one of its vectors. A single amino acid change in the envelope 1 protein (E1) of this virus indeed led to an increase in adaptability and transmission by *Ae. albopictus*, compared to *Ae. aegypti* (Tsetsarkin *et al.*, 2007).

This evolution of viruses to adapt to their vector was likely largely shaped by their interaction with the immune system, that mounts a response to control their replication and dissemination, thus promoting the establishment or improvement of escape strategies by viruses.

IV. Multiple pathways participate in the antiviral defence

The interplay between arboviruses and mosquitoes starts with the fortuitous uptake of the virus during a blood-meal. Females, driven to feed on their preferred hosts enter in contact with these viruses, enabling the start of the cycle leading to its transmission to a new host.

Following ingestion of infected blood by the mosquito, the virus reaches the midgut, where it will follow its specific replication cycle, until its dissemination to the haemocoel. This allows the virus to further spread to other organs, where it will also further replicate, until it reaches the salivary glands. Viruses will once again replicate in this tissue, and at high enough levels, will be secreted in the saliva. Once this step is reached, mosquitoes will be able to transmit the viruses during their next blood meal, through the injection of their saliva in the host (Perveen *et al.*, 2023).

This process by which the virus reaches a point at which it can spread in new hosts is largely affected, mostly hindered but sometimes aided, by various factors in the mosquitoes at all stages, starting by their interaction with the midgut microbiome, to more virus-specific responses mounted by the mosquito's immune system.

1. Contribution of the midgut microbiota

One of the first encounters of viruses, as they reach the midgut in the blood, are bacteria from the natural intestinal microbiota of the mosquito. In this past decade, research has on numerous occasions demonstrated the importance of microbiota, as studies highlighted the effect of these micro-organisms on a multitude of biological processes ranging from immunity to gene expression (Jandhyala, 2015; Nichols and Davenport, 2021).

The specific biology of mosquitoes, and in particular their hematophagous nature amplifies the effect of these commensal bacteria and fungi. Indeed, it has been shown that the acquisition of blood by mosquitoes was associated to an increase of midgut bacterial colonization (Oliveira *et al.*, 2011). This would be largely explained by the presence in the blood of haemoglobin and hemes, which triggers a reduction of reactive oxygen species (ROS) in the midgut, thus promoting a favourable environment for bacterial growth (Oliveira *et al.*, 2011). As viruses enter the mosquito's organism simultaneously with blood, they are directly faced with this high bacterial concentration.

The encounter of viruses with this community can then have variable outcomes, seeing as interaction with some bacteria will lead to a hindrance in the replication of the virus, while others will promote further infection of the mosquito. In the case of *Ae. albopictus*, it has been shown that most bacteria identified in its natural microbiome significantly reduced infectivity of LACV, in *cellulo* (Joyce *et al.*, 2011). The mechanisms by which this happens has been partially elucidated a few years later, when studies demonstrated that bacteria of the *Chromobacterium* genus, naturally found in *Ae. aegypti* mosquitoes from the field, were able to produce aminopeptidases, that degrade the envelope protein of DENV, thus interfering with its ability to attach and fuse with the host cells (Ramirez *et al.*, 2014; Saraiva *et al.*, 2018). Other bacteria living in the midgut of *Ae. aegypti* throughout its whole life, such as *Serratia odorifera*, have been shown to have an opposite effect on the outcome of viral infection, by enhancing the susceptibility of the mosquitoes to DENV (Apte-Deshpande *et al.*, 2012).

Interestingly, other bacteria species, even when not commensal to the mosquito, can also affect viral infection when artificially integrated to the microbiome. This is the case with a strain of *Wolbachia*, whose introduction in *Ae. aegypti* has been shown to limit the infection and dissemination of viruses such as DENV and ZIKV (Moreira *et al.*, 2009).

The outcome of microbiota interaction with viruses is however not solely imputable to the bacteria themselves. Indeed, the increase of bacteria in the midgut following a blood meal is responsible for the priming of an immune response by the mosquito, which in turn largely contribute to the modulation of the further replication and dissemination of the virus (Oliveira *et al.*, 2011).

2. Immune deficiency (IMD) pathway

Among the main immune pathways in insects is the immune deficiency pathway (IMD), that plays an important role in the defence against bacteria, and especially Gram-negative bacteria (Georgel *et al.*, 2001).

Following the recognition of microbial pathogen-associated molecular patterns (PAMPs), such as peptidoglycans and lipopolysaccharides, by a group of receptors from the peptidoglycan recognition protein (PGRP) family, the IMD adaptor protein is recruiting, thus triggering a signalling cascade (Choe *et al.*, 2005). Ensuing this signalling, formation of the IKK complex occurs, responsible for the phosphorylation of the Relish transcription factor. This transcription factor is then activated through a further cleavage by the caspase Dredd. Once activated, Relish is translocated to the nucleus, where it will promote the transcription of a variety of anti-microbial peptides (Leulier *et al.*, 2000; Stöven *et al.*, 2003).

Although primarily involved in the response against bacterial infection, this pathway has also been demonstrated to be mildly involved in antiviral immunity. It has indeed been shown to be active against cricket paralysis virus (CrPV) and Drosophila C virus (DCV), although the entire pathway is not involved in the response, rather only several of its components are (Costa *et al.*, 2009; Goto *et al.*, 2018). In mosquitoes, an up-regulation of specific effectors of this pathway have also been observed, after infection by DENV and CHIKV, although the expression of its main components was not particularly affected by the infection (Xi *et al.*, 2008; Luplertlop *et al.*, 2011; Sim *et al.*, 2013).

3. Toll Pathway

Firstly characterized in *Drosophila melanogaster* through its role in embryonic development, the Toll pathway was later shown to be substantially involved in the antibacterial and antifungal response in the fly, and to also contribute to the antiviral defence (Rutschmann *et al.*, 2002; Zambon *et al.*, 2005).

As observed for the IMD pathway, the Toll response is initially triggered by the recognition of microbial PAMPs by receptors of the PGRP family, triggering a proteolytic cascade resulting in the cleavage of Späetzle (Michel *et al.*, 2001). This cytokine then binds to the Toll receptor and activates it, leading to signalling through adaptor proteins, and resulting in the phosphorylation and subsequent degradation of Cactus, a negative regulator of the pathway (Nicolas *et al.*, 1998). Degradation of this factor allows for the release of previously sequestered transcription factor Dorsal, and its further translocation to the nucleus. This transcription factor will then promote the transcription of antimicrobial peptides (AMPs) (Hoffmann, 2003).

The Toll pathway, conserved in insects, has also been shown to play an important role in the antiviral response in mosquitoes. Activation of the transcription of components of this pathway, such as Späetzle, Toll and Rel1 (orthologue of *D.*

melanogaster Dorsal), as well as several antimicrobial peptides, has indeed been observed following DENV infection in multiple tissues of *Ae. aegypti* (Xi *et al.*, 2008).

4. JAK-STAT pathway

Similar to the Toll pathway, the Janus activated kinase-Signal transducers and activators of transcription (JAK-STAT) pathway was firstly identified in *Drosophila* through its important role throughout the development. Its function in the immune response was demonstrated later, by the discovery of the implication of the *An. gambiae* STAT homolog in antibacterial defence in the mosquito (Barillas-Mury *et al.*, 1999).

This pathway is initially triggered through a stress response, following cell damage caused by infection or injury (Agaisse *et al.*, 2003). This response leads to the secretion of Unpaired (Upd) peptide ligand, which binds to the transmembrane receptor Domeless (Harrison *et al.*, 1998; Brown *et al.*, 2001). The recognition of this ligand in turn leads to the dimerization of the receptor, and the subsequent phosphorylation of its associated Janus activated kinases (JAK), Hopscotch (Brown *et al.*, 2001, 2003). These kinases then allow for the recruitment of STAT92E, which are activated by further phosphorylation, leading to dimerization of these proteins. The STAT92E dimers are then translocated to the nucleus, where they will induce the production of various effectors (Arbouzova and Zeidler, 2006).

In *D. melanogaster*, in addition to its role in antimicrobial immunity, the JAK-STAT pathway has been shown to also contribute to the immune response against viruses. Interestingly, a role for this pathway was not observed for various types of viruses, but rather only for a subset of those, such as DCV and CrPV, suggesting that the antiviral response of this pathway is virus-specific (Dostert *et al.*, 2005; Kemp *et al.*, 2013). This antiviral role has also been demonstrated in *Ae. aegypti*, when depletion of key components of this pathway led to an significant increase of DENV replication in the midguts of mosquitoes (Souza-Neto *et al.*, 2009). In addition, as for the Toll pathway, the up-regulation of several components of the JAK-STAT has also been observed following mosquito infection by DENV (Xi *et al.*, 2008).

5. RNA interference response

Other large contributors to the immune response in insects are RNA interference (RNAi) pathways. It encompasses three pathways, each of which play a specific role: the micro-RNA (miRNA) pathway, the PIWI-interacting RNA pathway (piRNA) pathway and the the small-interfering RNA (siRNA) pathway.

5.1. miRNA pathway

Small RNAs produced through the miRNA pathway are mainly involved in the regulation of gene expression. These miRNAs are encoded in the genome, and following their transcription by the RNA polymerase II into primary transcripts (referred to as pri-miRNAs), will be processed in the nucleus. This step, accomplished by the Microprocessor complex comprising the two double-stranded RNA-binding proteins

(dsRBPs) Drosha and Pasha, allows for the production of a pre-miRNA, which is then exported from the nucleus to the cytoplasm (Denli *et al.*, 2004). There, pre-miRNAs are further processed by Dicer 1 (Dcr-1), with the help of its co-factor Loquacious (Loqs), to finally generate mature miRNAs. After being loaded into a protein complex containing the Argonaute 1 (Ago1) endonuclease, miRNAs can then regulate gene expression, through several mechanisms (Ha and Kim, 2014).

Although they do not seem to harbour an antiviral function at first, considering that they are encoded by the host genome, and thus are supposed to target endogenous genes, it has been shown that some miRNAs can affect the response to viral infection (Ramos-Nino *et al.*, 2022). It has recently been shown that a specific miRNA was associated with a better replication of WNV in *Ae. aegypti*. Interestingly, the expression of this miRNA is actually down-regulated following infection by the virus, in turn decreasing the expression of a pro-viral metalloprotease, and thus restricting the replication of WNV (Slonchak *et al.*, 2014). It has also been shown that the expression of a few miRNAs can be up-regulated following a blood-meal in mosquitoes, and in turn affect the expression of immune genes, such as *cactus* and *REL1*, of the Toll pathway (Hussain *et al.*, 2013). Although the effect of miRNAs on the outcome of viral infections seems mostly related to their ability to differentially regulate the expression of pro- or antiviral genes, it has been shown that some miRNAs can effectively bind to viruses. Binding sites for those miRNAs are indeed present at the 3' UTR of CHIKV, for example, however the binding of miRNAs does not seem to be responsible for the modulation of infection (Dubey *et al.*, 2019). Finally, an analysis of the saliva of CHIKV-infected *Ae. aegypti* and *Ae. albopictus* mosquitoes also revealed the presence of circulating miRNAs that were previously undetected in the genome, whose expression seemed to have been triggered by the infection itself, and which were also involved in the modulation of the infection (Maharaj *et al.*, 2015). Although the mechanisms by which these types of small RNAs influence and regulate viral infection are not entirely deciphered to this day, it is undeniable that they have an important role to play in the shadow of the other immune pathways.

5.2. piRNA pathway

Another pathway of the RNAi allows for the production of piRNAs, small RNAs whose primary role is to maintain the integrity of the germline, by controlling the expression of TEs.

So-called piRNA clusters, comprising transposon and specific genomic loci, allow for the production of these piRNAs, following transcription by RNA polymerase II. The antisense primary transcript produced at this stage can then be processed in two ways. The first mechanism, involves the processing of the 5' end of these transcripts by a ribonuclease called Zucchini, which allows them to be loaded into PIWI proteins and to be transported to the nucleus (Ramos-Nino *et al.*, 2022). On the other hand, these transcripts can enter the second mechanism referred to as the "ping-pong" cycle, that allows for the amplification of the piRNAs pool. In this process, the antisense primary piRNAs are loaded into the Aubergine (Aub) protein, and then hybridize to a complementary sense strand transcript, which is in turn cleaved into secondary piRNAs.

These secondary piRNAs are further loaded into Ago3, and similarly hybridize with their complementary anti-sense strand transcripts, leading to their cleavage and the ensuing generation of primary-type secondary piRNAs. These piRNAs can then bind to Aub again, and keep this cycle going (Siomi *et al.*, 2010). It is considered that piRNAs produced through the first mechanism, leading to the translocation of PIWI proteins to the nucleus, are involved in transcriptional repression of TEs, while piRNAs produced through the ping-pong cycle are involved in post-transcriptional control (Ku and Lin, 2014; Wang *et al.*, 2015).

Interestingly, this PIWI protein family extended considerably in mosquitoes, and in particular, in *Aedes* and *Culex*. While the *Drosophila* genome encodes three proteins from this family, Piwi, Ago3 and Aub, *Aedes* mosquitoes encode for seven of those proteins, named Piwi1 to Piwi7 (Campbell *et al.*, 2008). Considering the main role of the piRNA pathway in the control of TEs, it makes sense that this family expanded in an organism in which half of the genome is comprised of these elements (Nene *et al.*, 2007; Matthews *et al.*, 2018). Additionally, this increase in proteins of the PIWI family could also be explained by the extension of the piRNA pathway function to other cell types. In *Drosophila*, the action of the piRNA pathway is mostly restricted to the germline, whereas it has been shown in mosquitoes, to also function in somatic cells (Morazzani *et al.*, 2012).

In these past few years, the comprehension of the biological function of this pathway has considerably increased, and with it, new roles for these piRNAs have come to light. A deep sequencing of several *Drosophila* cell lines revealed the presence of multiple virus-derived piRNAs (vpiRNAs), thus suggesting a potential role for this pathway in antiviral immunity (Wu *et al.*, 2010). Soon after this discovery, vpiRNAs displaying a ping-pong signature were also identified in several *Ae. aegypti* and *Ae. albopictus* mosquito cell lines, following infection with CHIKV, LACV and SINV (Morazzani *et al.*, 2012; Vodovar *et al.*, 2012). It is currently believed that Piwi4 could be a key component of this antiviral response in mosquitoes, considering the impact of its silencing on virus replication (Schnettler *et al.*, 2013; Varjak *et al.*, 2017). However, the precise mechanisms by which piRNAs act as factors of the antiviral response is yet to be elucidated.

5.3. siRNA pathway

The last pathway of the RNAi response allows for the production of siRNAs, in response to the detection of dsRNAs, and is currently considered to represent the main antiviral response in insects.

This pathway relies on the recognition of long dsRNA transcripts by the ribonuclease Dcr-2 and its co-factor Loqs, which will then be processed into smaller 21-nt long siRNA duplexes (Marques *et al.*, 2010). With the help of another important co-factor of the pathway, R2d2, these siRNAs will be loaded into the RNA-induced silencing complex (RISC), containing the main effector protein, Ago2. In this complex, one strand of the siRNA duplex will be cleaved and the remaining strand will serve to guide Ago2 to its target, where it will specifically hybridize with its complementary sequence. Once

bound, the endonuclease activity of Ago2 will allow for the cleaving and subsequent degradation of the target mRNA (Olson and Blair, 2015).

Long dsRNA transcripts that are targeted by this pathway can originate from the genome itself, thus leading to the production of endogenous-siRNAs (endo-siRNAs). In this capacity, the siRNA pathway can have a complementary role to the piRNA pathway, as these endo-siRNAs can be involved in the silencing of TE as well (Kawamura *et al.*, 2008). On the other hand, dsRNAs from exogenous sources can also be recognized by the proteins of the siRNA pathway, and be processed into so-called exogenous-siRNAs (exo-siRNA). It was initially considered that this exo-siRNA branch of the pathway was responsible for the antiviral response, using dsRNA intermediates of the viral replication as templates. However, it has recently been shown that there is a discrimination between the origin of exogenous dsRNAs, involving different co-factors, and leading to the production of either exo-RNAs or virus-derived siRNAs (vsiRNAs) following infection (Marques *et al.*, 2013).

As described earlier, the initial function of Dcr-2 is aided by the presence of its co-factor Loqs. In *Drosophila*, four isoforms of this protein have been identified, Loqs-PA, Loqs-PB, Loqs-PC (only identified in cells) and Loqs-PD, each of them presenting a specific role (Förstemann *et al.*, 2005; Zhou *et al.*, 2009). Interestingly, it would seem that one of these isoforms only, Loqs-PD, is required for the processing of exogenous and endogenous dsRNAs, but is not involved in the processing of dsRNA from viral sources, thus suggesting that this protein is the main determinant in the discrimination between origins of the dsRNAs (Marques *et al.*, 2013).

In mosquitoes, only three of those isoforms are described, none of which seem to have an equivalent function to Loqs-PD. In contrast to *Drosophila*, where both Loqs-PA and Loqs-PB isoforms seem to be able to interact and play a role in the miRNA pathway, it has recently been shown that in mosquitoes, Loqs-PA plays an important role in the siRNA pathway, while Loqs-PB is mostly involved in the miRNA pathway (Haac *et al.*, 2015). It is however not known how the siRNA pathway in mosquitoes is able to discriminate between origin of the dsRNA transcript in the absence of an orthologue to Loqs-PD.

All pathways described here represent the main mechanisms of immune defence in insects, however various other pathways have been reported to contribute to the regulation of infections, that were not presented here. Among this variety of immune responses, it is considered that the RNAi pathway is the main actor of antiviral immunity, mounting a strong specific response against these pathogens. Considering the high efficiency of this pathway and others, in combatting viral infections in insects, it raises the question as to why those mosquitoes are able to host and transmit such a vast number of viruses.

V. Discovery of a new antiviral dsRBP, specific to Aedes *mosquitoes*

As previously mentioned, the ingestion of a blood meal from an infected host leads to the entry of viruses in the midgut, where they must first establish an infection

in order to further spread to other tissues. Being the first cells that viruses encounter upon entering the midgut, enterocytes are instrumental in determining the outcome of the establishment of the infection. The antiviral response mounted by these cells is thus decisive to prevent dissemination of the virus.

The study of the siRNA response in this particular cell type, in *Ae. aegypti,* revealed that this pathway was not able to control viral replication. Indeed, the silencing of the main effector of the siRNA pathway, Ago2, did not result in an increase in DENV and ZIKV viral loads, thus demonstrating that this pathway was not involved in the antiviral response in this tissue. However, the efficiency of silencing through this pathway remained intact when dsRNA from other sources were used. This suggests that, as it is observed in *Drosophila*, the siRNA pathway also has the ability to discriminate between dsRNAs of different origins (Olmo *et al.*, 2018).

In *Drosophila*, this discrimination between the exo-/endo-siRNA pathway and the vsiRNA pathway relies on the presence of a specific isoform of Loqs, Loqs-PD, which is absent from *Aedes* mosquitoes (Marques *et al.*, 2013). An analysis of the *Ae. aegypti* genome was performed in order to identify genes encoding dsRBPs in this mosquito, which led to the identification of an uncharacterized gene, paralog of *loqs* and *r2d2*. This gene, which was named *loqs2*, is specific to *Aedes* mosquitoes, and is not expressed in the midgut, but shows the highest expression in reproductive tissues, such as ovaries and testis, as well as in early embryos (Olmo *et al.*, 2018).

This peculiarity was thought to be a clue to explain the difference in the siRNA response in the midgut, compared to the rest of the mosquito, and it was hypothesized that this protein could be a co-factor of the pathway, possibly responsible for the discrimination of dsRNA from viral origin. To further understand the role of this protein, its antiviral properties were assessed, first through a silencing of its expression, which led to an increase in DENV viral load in the carcass of the mosquitoes. Transgenic mosquitoes were also generated, ectopically expressing Loqs2 in the midgut, and showed a reduced DENV and ZIKV viral load in this tissue, as well as a decreased dissemination in the carcass, confirming its role in the control of viral replication (Olmo *et al.*, 2018).

To further understand its function, and more particularly, its relation to the siRNA pathway, the interactome of Loqs2 was investigated. This experiment, performed in *Ae. aegypti* Aag2 cells, allowed for the identification of 79 potential partners of Loqs2, of which Loqs and R2d2 were the most significant hits (Olmo *et al.*, 2018). Although not enough to demonstrate the implication of Loqs2 in the siRNA pathway, these results show an intricate relationship between this protein and co-factors from this pathway, and allow us to speculate on this possibility.

The identification of this novel dsRBP, that has been demonstrated to have antiviral properties similar to multiple components of the RNAi pathway, highlights the importance of this class of proteins in the defence against viruses, and encourages to further study and characterize them.

VI. Diversity of dsRBP roles highlights their biological importance

It is estimated that there are approximately a dozen dsRBPs in *Aedes*, almost half of which are components of the RNAi pathway (Estevez-Castro *et al.*, 2021). These proteins are characterized by the presence of a consensus domain that was firstly characterized in *Xenopus* 30 years ago, allowing them to bind dsRNA (Johnston *et al.*, 1991). This consensus domain is the most abundant RNA-binding domain (dsRBD) among proteins, however it is not the only domain that can bind to dsRNA. Indeed, multiple proteins have been shown to interact with and bind to dsRNA, that do not carry this domain (Saunders and Barber, 2003). In regards to this, a previously uncharacterized domain, PWI, has recently been shown to have the capacity to bind all kind of nucleic acids, including dsRNAs, that do not have the consensus structure of classical dsRBDs (Szymczyna *et al.*, 2003). The identification of new domains capable of binding dsRNA in a variety of proteins further emphasizes the versatility of dsRBPs and their involvement in diverse biological processes.

As mentioned previously, a significant part of these identified dsRBPs are involved in the RNAi response, although their function largely differs in this pathway. Some of those dsRBPs, such as Dcr-1, Dcr-2 and Drosha, possess a catalytic activity, which allows them to process their template RNA into the appropriate small RNAs (Olson and Blair, 2015). Other proteins containing dsRBDs, such as Pasha, R2d2 and Loqs are co-factors of these pathways, and help direct the appropriate response, as it is the case for Loqs, which has been shown to be determinant in the discrimination of the origin of the dsRNA transcript processed in its pathway (Marques *et al.*, 2013). The involvement of most of these proteins in immunity, as described previously, highlights the importance of dsRBPs in the defence against viral infection.

It has also been shown that the immune response can be modulated by another dsRBP, that is not involved in the RNAi pathway. This protein, Adenosine deaminase acting on RNA (ADAR) is responsible for adenosine-to-inosine (A-to-I) editing, following its binding to a dsRNA template. Generally, inosines are read by the cellular machinery as guanosines, thus leading to changes in the encoded protein sequence, as well as alteration of RNA structure and splicing. This capacity to edit RNA has been demonstrated to antagonize the RNAi pathway, rendering the RNAs unrecognizable by Dcr-2, and also to affect the production of AMPs in *Drosophila* (Deng *et al.*, 2020). Interestingly, ADAR has also been shown to bind to Drosha and Dicer proteins, and thus to affect the processing of pri-miRNA. In addition to its interference with the RNAi pathway, it has been shown that ADAR can also affect development, as null mutants in *Drosophila* showed severe locomotion defects, sleep defects, and age-dependant neurodegeneration, among other effects (Deng *et al.*, 2020). This shows the diversity of the roles of dsRBPs, adding a new layer of complexity to their function.

Development can also be affected by dsRBPs, through their ability to regulate gene expression as observed for a protein identified in *Drosophila*, called Disconnect-interacting protein 1 (DIP1). This protein has been shown to be involved in the regulation of gene expression during development, in *D. melanogaster*, and to be involved in the differentiation of multiple cell types during embryogenesis (DeSousa *et al.*, 2003).

Another protein containing dsRBDs has been described in *Drosophila*, as being involved in the regulation of gene expression. This ATP-dependent DEXH-box RNA/DNA helicase, maleless (mle), has been shown to participate in the regulation of the levels of X chromosome-linked gene in males as part of a bigger protein complex (Kuroda *et al.*, 1991).

The versatility of this class of proteins is also further demonstrated by their involvement in other important biological processes, such as oogenesis. Staufen, one of the firstly identified dsRBPs in *Drosophila*, is a protein involved in establishing the polarity of the embryo, by transporting specific mRNAs from the anterior to the posterior pole of the oocyte, thus establishing concentration gradients of the proteins encoded by these mRNAs (Johnston *et al.*, 1991). Interestingly, it seems that one of the dsRBD of this protein plays a role in translational control, through protein-protein interactions (Tian *et al.*, 2004). In addition to Staufen, other dsRBPs play an important role in reproduction, by affecting the fertility of either males or females. Blanks is a protein that has been identified in *Drosophila* a few years ago, as a male specific protein, exclusively expressed in testes of adult flies, that is critical for sperm maturation in the seminal vesicles (Gerbasi *et al.*, 2011; Sanders and Smith, 2011). The fertility defects observed in male mutants for this gene could possibly be attributed to its function in RNAi, as it was shown to bind specific siRNAs, and to be necessary for efficient an RNAi response (Gerbasi *et al.*, 2011; Nitschko *et al.*, 2020). The DExD/H-box helicase Belle (Bel), orthologue of the dsRBP DDX3 in humans, has also been shown to be involved in fertility in *Drosophila*, as has been DDX3 (Johnstone *et al.*, 2005; Poynter *et al.*, 2019; De Colibus *et al.*, 2022). In addition to being essential for the viability and growth of larvae in *Drosophila*, Bel is required for female fertility, and has been shown to be involved in spermatogenesis (Johnstone *et al.*, 2005). Interestingly, Bel has also been shown to interact with multiple components of the RNAi pathway (Zhou *et al.*, 2008). Also, in addition to their role in antiviral immunity, a role for some RNAi proteins has been shown in fertility as well. Notably, the loss of Loqs function in ovaries renders mutant females sterile, due to its important role in the miRNA pathway, resulting in a disruption of normal pre-miRNA processing and further defects in germ-line stem cell maintenance (Förstemann *et al.*, 2005). Additionally, it has also been discovered that some *Drosophila* species, such as *Drosophila pseudoobscura* and *Drosophila persimilis*, encode multiple paralogues of Ago2, while most other animal species only possess one (Hain *et al.*, 2010). Most of the paralogs of Ago2 identified in these species are exclusively expressed in males, and seemed to have evolved towards a testis-specific role, diverging from their original antiviral function, but further studies are required to decipher the specificities of their function (Lewis *et al.*, 2016). Interestingly, only two other dipteran species have been shown to encode multiple Ago2 paralogs, one of those species being *C. quinquefasciatus*, however the function of the Ago2 paralog in these species has not yet been investigated (Campbell *et al.*, 2008).

A potential role for Loqs2 in the immune response has already been shown upon ectopic expression, however, current understanding of the functions of dsRBPs in multiple organism demonstrate great diversity and versatility, suggesting the potential for the involvement of Loqs2 in other biological processes. Considering the crucial function of this type of proteins in organisms, ranging from fertility and gene regulation

to antiviral defence, and the fact that the function of numerous of those dsRBPs has not yet been elucidated, it is critical to work towards characterizing them, if we aim to better understand the mechanisms governing these organisms.

VII. Aim of this work

The battle against arboviruses remains an ongoing challenge, and the urgency to develop effective measures to control their spread keeps increasing. The study of the interactions between viruses and their vector, is a promising approach, as it could allow the identification of key targets. The recent identification of the dsRBP Loqs2 represents an important step forward in this direction. However, a thorough characterization of this protein and its function is crucial, given that dsRBPs have been shown to play multiple roles in different biological functions. This could provide valuable insights into the mechanisms underlying the interaction between mosquito and virus, and how to effectively regulate this relationship to prevent the spread of arboviruses.

The aim of the present work is to comprehensively characterize Loqs2, focusing on three key aspects that will help to better comprehend its role in mosquito biology. Firstly, we will delve into its origin and evolution, in order to gain a deeper understanding of its development to this day. Secondly, we will explore the antiviral properties of Loqs2 in the midgut, an important aspect considering its potential as a target for the control of arboviruses. Finally, we will investigate its natural function, which could provide valuable insights into its potential roles in the biology of mosquitoes, beyond its antiviral function.

Through this work, we hope to gain a more complete understanding of the diverse roles of Loqs2, and ultimately contribute to the development of effective strategies for the control of arboviruses.

Part 1

Study of the origin and evolution of logs2

We demonstrated a potential antiviral function for this newly discovered Loqs2 protein, however little is known about it at the moment. Its specificity to *Aedes* mosquitoes is of particular interest in our research, as it could prove to be one of the factors that participate in making these mosquitoes such efficient vectors. This prompted us to further characterize this protein. First, we investigated its origin and evolution until the present day, through multiple phylogenetic analyses.

I. loqs2 *originated in the ancestor of the* Stegomyia *subgenus*

As mentioned previously, one striking feature of *loqs2* is the presence of two dsRBDs. In order to ascertain the origin of this gene, we retrieved genome sequences of multiple mosquito and fly species and screened these sequences for proteins containing at least one dsRBD. We then examined the phylogenetic relationships between them and *loqs2* (**Figure 1.A**).We observed that Loqs2 proteins locate in a sister clade of Loqs, which confirms that these two genes are indeed paralogs.

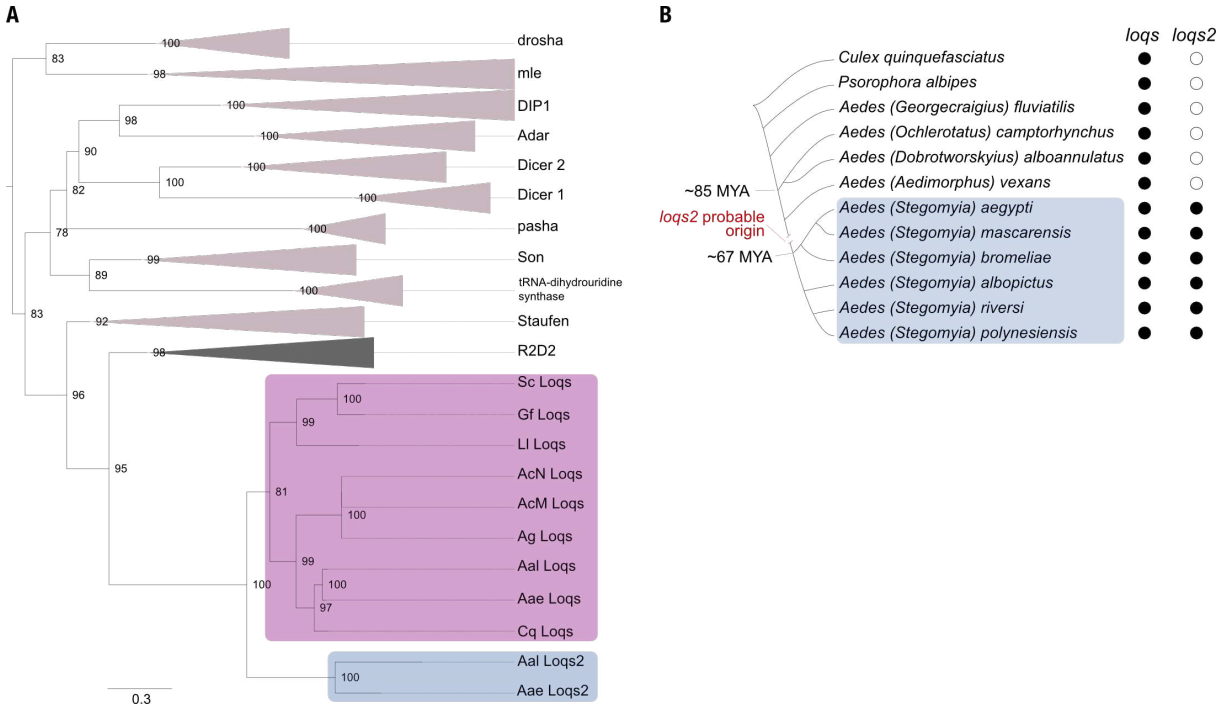


***Figure 1.* loqs2 *is a paralog of* loqs *that originated in the* Stegomyia *subgenus*.**
[A] Maximum likelihood tree constructed with the amino acid sequences of the dsRNA-binding proteins of Ae. aegypti (Aae), Ae. albopictus (Aal), C. quinquefasciatus (Cq), An. gambiae (Ag), Anopheles coluzii Mali-NIH (AcM), Anopheles coluzii Ngousso (AcN), Lutzomyia longipalpis Jacobina (Ll), Stomoxys calcitrans USDA (Sc) *and* Glossina fuscipes IAEA (Gf). *Collapsed branches are indicated by the pink and grey triangles to facilitate visualization. For the same reason, the tree is rooted at the midpoint. Node values correspond to 1,000 ultra-fast bootstrap iterations. [B] Schematic cladogram indicating the possible origin of* loqs2.

Loqs2 is exclusively detected in *Aedes* mosquitoes, and does not have orthologues in other species. We thus attempted to trace back the origin of the *loqs2* gene in *Aedes* mosquitoes. Most mosquito genomes are not publicly available or not entirely assembled to this day, which makes it difficult to precisely pinpoint when the gene

originated. We were, however, able to retrieve publicly available RNA-Seq or whole genome sequencing data from mosquitoes belonging to different subgenera, namely *Georgecraigius* (Caragata *et al.*, 2017), *Ochlerotatus* and *Dobrotworskyius* (Shi *et al.*, 2017), *Aedimorphus* (Chung *et al.*, 2020), as well as four species from the *Stegomyia* subgenus, to which *Ae. aegypti* and *Ae. albopictus* belong (Crawford *et al.*, 2017; Redmond *et al.*, 2020). We chose to also include a species from a different genus, *Psorophora albipes*, as it is still closely related to the *Aedes* genus (Chagas *et al.*, 2013). Sequences of *loqs* and *loqs2* from *Ae. aegypti* and *Ae. albopictus* were used as a reference to fish potential orthologs in the aforementioned species. Sequences aligning with *loqs* were found in all species tested here, however this was not the case for *loqs2* (**Figure 1.B**). Sequences or fragments of sequences matching *loqs2* were only found in the four mosquitoes from the *Stegomyia* subgenus. We thus considered that the radiation of this subgenus was the point of origin of *loqs2*, which is estimated to have taken place between 85.57 to 67.07 MYA (Wilkerson *et al.*, 2015). The absence of genomic data for most other mosquito species prevented us from narrowing down this time period.

As mentioned above, *loqs2* is closely related to *loqs*, and is also expected to produce a protein with dsRBDs. There is however a difference between both, in that the two dsRBDs of Loqs2 are located closer to each other than the first two domains of Loqs, which also contains an additional dsRBD (**Figure 2.A**). We thus investigated the phylogenetic relationships between these two domains of Loqs, and the different domains of the other dsRBPs studied previously, at the amino acid sequence level. Interestingly, we observed that both dsRBD from Loqs2 (Loqs2-D1 and Loqs2-D2) clustered with the first domain of Loqs (Loqs-D1)(**Figure 2.B**). We further explored this strong similarity between Loqs-D1 and both Loqs2 dsRBD by performing a global alignment of these sequences from both *Ae. aegypti* and *Ae. albopictus*. This allowed us to confirm that Loqs2-D1 and Loqs2-D2 were the most similar to Loqs-D1 (**Figure 2.C**). Together, these results suggest that *loqs2* originated from two independent duplication events of the first dsRBD of *loqs* (**Figure 2.D**).

II. loqs2 evolved under a positive selective pressure

Outcomes of a gene duplication are variable, and will mostly depend on the selective pressure that the gene is evolving under. The most common outcome is the conservation of the original gene's function through purifying selective constraints, while the duplicated copy becomes silenced (pseudogenization). In rarer occurrences, the duplicated gene can accumulate mutations, leading to a subdivision of the original function, and rendering both genes interdependent to perform this function. Finally, a duplicated gene can also evolve to accumulate mutations that will lead to the acquisition of a new function (neo-functionalization), that will fixate in the population if advantageous (Lynch and Conery, 2000; Prince and Pickett, 2002). Considering that Loqs retained its function independently of Loqs2, we hypothesized that the last scenario applied to the evolution of this gene, which likely accumulated mutations and possibly developed alternative functions (**Figure 3**).

Phylogenetic analyses were thus performed to evaluate the selective pressure under which each of *loqs2* dsRBDs were evolving, when compared to the first dsRBD of

loqs. We observed a significant relaxation of the selective pressure for both *loqs2* domains, thus corroborating our hypothesis that the accumulation of mutations in *loqs2* was unhindered, which lead to a diversification of the protein's function further on (Estevez-Castro *et al.*, 2021). A further investigation of the evolution of this gene, allowed us to determine that this gene is diversifying faster than *loqs* and *dicer* genes in *Aedes* mosquitoes, reinforcing the idea that it is evolving under a different selective pressure than *loqs*, and showing that it is still actively diversifying.

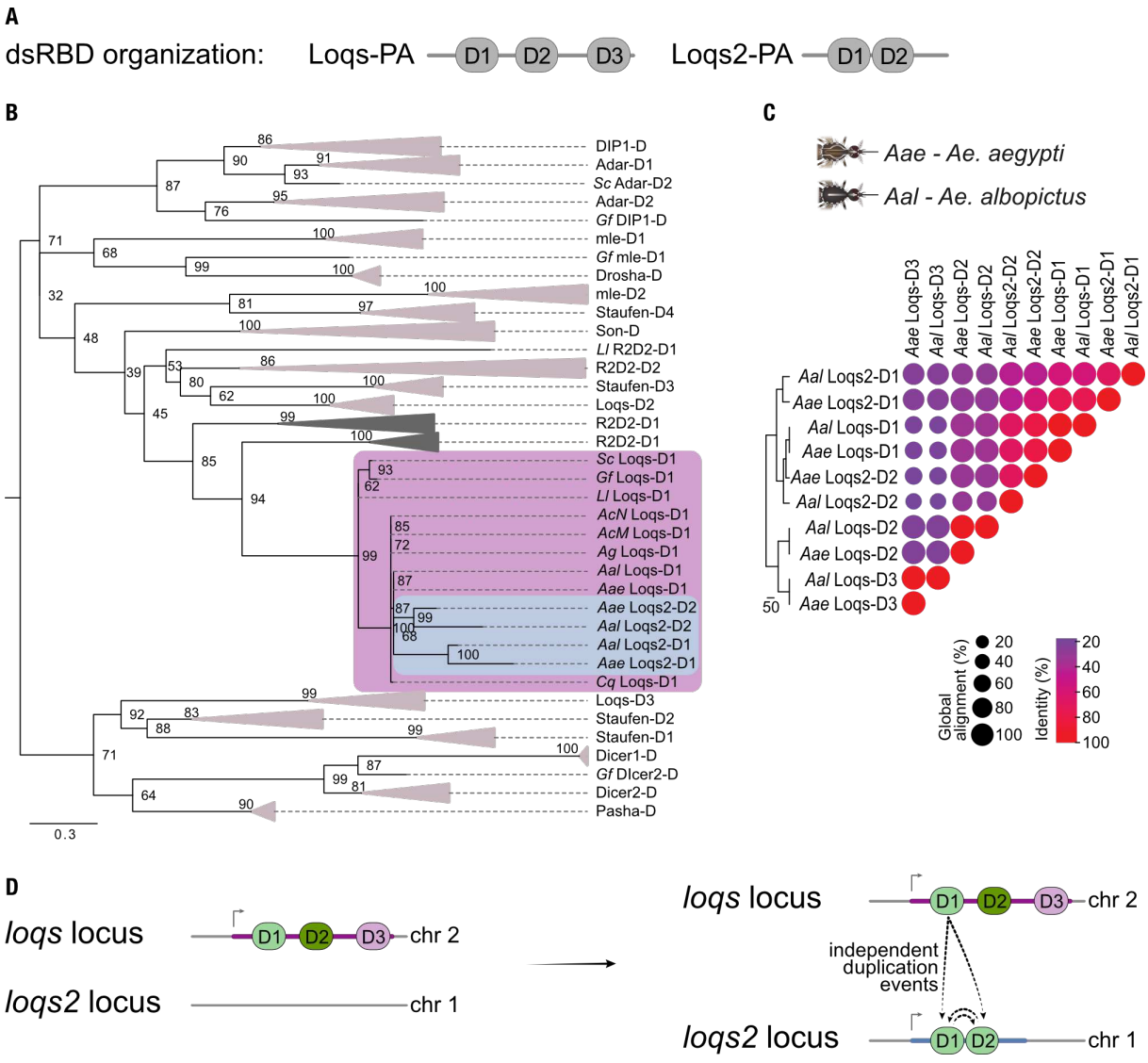


Figure 2.* loqs2 *originated from two independent duplication events of the first dsRBD of* loqs.** *[A] Domain organization of* loqs *and* loqs2. *[B] Phylogenetic relationships among the dsRBDs of the dsRBPs from* ***Figure 1.A*. Phylogeny was inferred by maximum likelihood and the tree is rooted at the midpoint for visualization purposes. For the same reason, collapsed branches are indicated by the pink and grey triangles. Node values correspond to the results of 1,000 ultra-fast bootstrap iterations. Loqs2 monophyletic clade and Loqs-D1 paraphyletic clade are highlighted in blue and pink, respectively. [C] Correlation matrix showing global alignment identity and alignment percentage from the comparison between Loqs and Loqs2 dsRBD amino-acid sequences. [D] Putative model for* loqs2 *origin.*

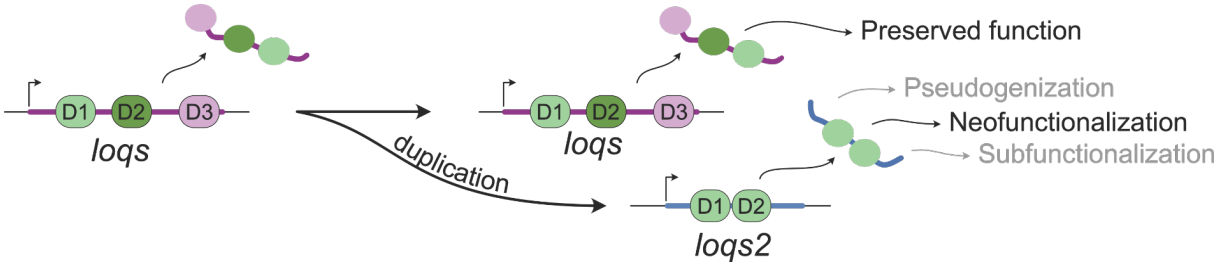


***Figure 3. Relaxed positive selection shaped the evolution of* loqs2.**
Proposed model of the evolutionary path followed by loqs *and* loqs2 *after the duplication events. Written in grey are the other common outcomes of gene duplication.*

Overall, our results indicate that *loqs2* is a gene that originated from a co-factor of the RNAi pathway, *loqs*, and that evolved under a relaxed selective pressure which allowed for an accumulation of mutation and possibly the gain of a new function, through an active diversification.

Part 2

Characterization of Loqs2 antiviral function

Phylogenetic analyses of the origin and the evolution of *loqs2* concluded that this gene evolved from duplications of the first domain of *loqs*, and was maintained through a positive selective pressure, likely driving the acquisition of new functions (Estevez-Castro *et al.*, 2021). We hypothesized that these new functions could be complementary to those of Loqs, which is well known to play roles in different RNAi pathways and in the antiviral response.

Olmo and colleagues recently demonstrated the involvement of Loqs2 in the control of virus dissemination, as well as the regulation of viral replication in the midgut of *Ae. aegypti* mosquitoes, when ectopically expressed in this tissue (Olmo *et al.*, 2018).

Considering these results, we chose to initially focus on the investigation of this specific feature of the protein, in an attempt to decipher its contribution to the antiviral response in *Aedes* mosquitoes. To this aim, we focused on exploring the protein interactome of Loqs2, and characterizing the partners identified through these studies.

I. Loqs2 interacts with multiple antiviral proteins in the midgut

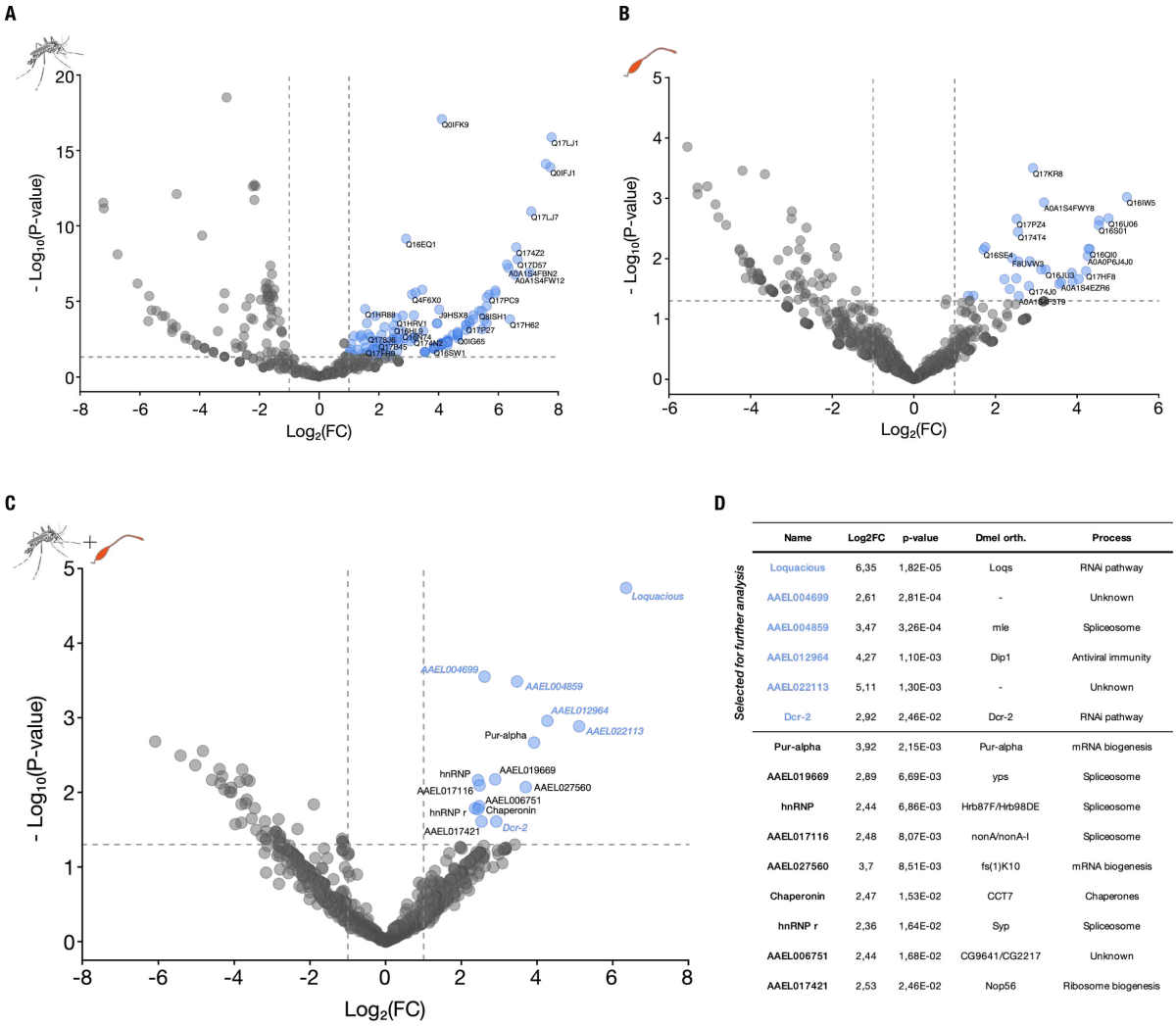
As mentioned above, preliminary studies were performed on Loqs2 at the time of its discovery by (Olmo *et al.*, 2018). One of these experiments focused on the identification of Loqs2 protein partners in *Ae. aegypti* Aag2 cells, overexpressing the tagged protein. From this analysis, 79 proteins were detected, of which 4 were highly significant. Among these highly significant hits were the two main co-factors of the siRNA pathway, Loqs and R2D2, which happen to be the closest related proteins to Loqs2 (Olmo *et al.*, 2018).

Of note, *Ae. aegypti* Aag2 cells were generated originally from a pool of embryos (Peleg, 1968) and are thus composed of a variety of cell types. In laboratory conditions, it was not possible to detect endogenous *loqs2* mRNA in these cells (Shivaprasad *et al.*, 2022). The conditions used in this initial experiment might thus not have been optimal for the identification of Loqs2 protein partners, since the proteome would differ greatly in these cells, compared to the midgut of mosquitoes.

In order to better comprehend the behaviour of Loqs2 in the antiviral response in mosquitoes, we repeated this experiment *in vivo*, using the transgenic mosquito line generated by (Olmo *et al.*, 2018). This transgenic line overexpresses a tagged Loqs2 protein specifically in the midgut, under the control of the blood-induced carboxypeptidase (CP) promoter. Non-transgenic siblings of the aforementioned line were used as controls in all the following experiments.

The feasibility of this experiment was tested first, by using protein extracts from whole 3 to 5 days old female mosquitoes, to retrieve a higher yield. These females were offered a bloodmeal and collected after 24 h, when the CP promoter-induced expression is the highest. Proteins detected by mass spectrometry were considered significant hits if enriched with a fold-change (FC) > 2 and a p-value < 0.05. In this analysis, 156 proteins met the aforementioned criteria, of which 7 were highly significant, with a

p-value < 1 x 10 $^{-10}$ (**Figure 4.A**). Although the co-immunoprecipitation conditions were rather stringent, the possibility remains that interactions were artificially formed with proteins from other tissues after cell lysis.



	Name	Log2FC	p-value	Dmel orth.	Process
Selected for further analysis	Loquacious	6,35	1,82E-05	Loqs	RNAi pathway
	AAEL004699	2,61	2,81E-04	-	Unknown
	AAEL004859	3,47	3,26E-04	mle	Spliceosome
	AAEL012964	4,27	1,10E-03	Dip1	Antiviral immunity
	AAEL022113	5,11	1,30E-03	-	Unknown
	Dcr-2	2,92	2,46E-02	Dcr-2	RNAi pathway
	Pur-alpha	3,92	2,15E-03	Pur-alpha	mRNA biogenesis
	AAEL019669	2,89	6,69E-03	yps	Spliceosome
	hnRNP	2,44	6,86E-03	Hrb87F/Hrb98DE	Spliceosome
	AAEL017116	2,48	8,07E-03	nonA/nonA-l	Spliceosome
	AAEL027560	3,7	8,51E-03	fs(1)K10	mRNA biogenesis
	Chaperonin	2,47	1,53E-02	CCT7	Chaperones
	hnRNP r	2,36	1,64E-02	Syp	Spliceosome
	AAEL006751	2,44	1,68E-02	CG9641/CG2217	Unknown
	AAEL017421	2,53	2,46E-02	Nop56	Ribosome biogenesis

Figure 4. Loqs2 interacts with a diverse range of proteins when ectopically expressed in the midgut. *Proteins enriched in immunoprecipitates of 3xFLAG-Loqs2 in [A] whole mosquitoes and [B] midguts of transgenic* Ae. aegypti *overexpressing the protein ectopically in the midgut (right side of the graph), in comparison to wild-type mosquitoes (left side of the graph). [C] Proteins enriched in the combination of both previous analyses. Blue circles represent significant hits, with a p-value < 0.05 and a Log2FC > 1. Dotted lines represent cutoff values. Spectral counts were submitted to a negative-binomial test using generalized linear model regression. P-values were corrected using the Benjamini-Hochberg method. [D] List of 15 candidates identified in the combined analysis, and their corresponding enrichment and significance. When existing, the corresponding orthologues in* D. melanogaster *(Dmel) were added to the table, as well as the main biological process each protein is associated with (inferred using KEGG pathways).*

A second experiment was thus performed, on protein extracts from female midguts only, with a few differences to the previous procedure. We had previously estimated the amount of proteins found in the blood to be almost 10 times higher than the amount of proteins in the midguts of *Ae. aegypti* alone. Although this was negligible for analyses on whole mosquitoes, it was not the case for midguts, as we observed a high degradation of all proteins in bloodfed midgut extracts. Therefore, midguts were cut open and washed to remove as much of the blood as possible, before preparing the

protein extracts. Proteins detected by mass spectrometry were considered significant under the same criteria as the previous analysis, which resulted in the detection of 33 proteins, of which only one, Loqs, stood out as highly significant (**Figure 4.B**).

Comparing these analyses, we were able to identify several proteins detected in both datasets, and thus decided to perform a complementary analysis, combining these results. This resulted in an increased stringency, as we only considered to be significant hits, the proteins that had been identified in both datasets, according to the same criteria as in the original analyses, with an FC > 2 and p-value < 0,05. This analysis allowed us to generate a list of 15 strong possible interactants of Loqs2 (**Figures 4.C** and **4.D**).

Delving into the study of the interactome of Loqs2, we investigated the 15 candidates identified through the previous analysis. Since *Ae. aegypti* is not a model species, it was sometimes not possible to find a description for the proteins of interests. For this reason, we also looked for orthologues of the genes in *D. melanogaster* when possible (**Figure 4.D**). Based on the function or process in which the proteins or their orthologues were involved in, we considered a few candidates to further study and characterize: two core RNAi pathway proteins, Loqs and Dcr-2, two dsRNA binding proteins with orthologues in *D. melanogaster*, AAEL004859 and AAEL012964, and finally, two mosquito-specific proteins, AAEL04699 and AAEL022113.

1. Core RNAi pathway proteins

As described previously, the RNAi, and most specifically the siRNA pathway relies on the sequential action of multiple proteins, among which the main actors are the nucleases Dcr-2 and Ago2. The action of these proteins is facilitated by the help of several co-factors, including the two proteins mentioned in this study, Loqs and R2d2.

We previously described the intertwinement of Loqs2 in this antiviral pathway, which is why we chose to continue deciphering the implications of this protein in this already well-established pathway.

Of note, the two main isoforms of Loqs, Loqs-PA and Loqs-PB, were detected in the mass spectrometry analyses. The majority of peptides identified in the analysis being common for both isoforms, it was not possible for us to assign them to either, and to discriminate which of them was represented at higher levels, or if they were both equally interacting with Loqs2. Since both isoforms have been described to associate with two different parts of this pathway, the first one being involved mainly in siRNA-based silencing, and the second one in miRNA biogenesis, it could also indicate a wider implication of Loqs2 in the RNAi pathway and not solely in the antiviral response (Haac *et al.*, 2015).

2. Proteins with orthologues in D. melanogaster

Two of the selected candidates, AAEL01294 and AAEL004859, were not described in *Ae. aegypti* at the time of the analysis, but had orthologues in *D. melanogaster*, Disconnected interacting protein 1 (Dip1) and maleless (mle), respectively.

Dip1 has first been identified as a regulator of gene expression during development in *D. melanogaster*, as it is widely expressed during embryogenesis, and is involved in the differentiation of specific cell types (DeSousa *et al.*, 2003). Since then, the protein has been shown to be involved in a multitude of biological pathways, ranging from transcription regulation, to tRNA processing and maturation, as well as chromosomal silencing (Bondos *et al.*, 2004; Catanese and Matthews, 2011), and an antiviral function has also been demonstrated against DCV in *D. melanogaster* (Zhang *et al.*, 2015). Very recently, a link with the antiviral response has also been established in *Ae. aegypti*, when Dip1 was detected in the interactome of DENV 3' UTR and in the interactome of the NS1 protein of DENV in cells (Besson *et al.*, 2022; Yeh *et al.*, 2022).

In *D. melanogaster*, mle has been identified as one of the four main components of the male-specific lethal (MSL) complex, responsible for dosage compensation, a mechanism involved in the regulation of the levels of X chromosome-linked gene products between sexes (Kuroda *et al.*, 1991). mle is an ATP-dependent DEXH-box RNA/DNA helicase, which human orthologue RNA helicase A (RHA/DHX9) has been shown to act in numerous biological processes from translation initiation to RISC assembly. Similarly, mle has been shown to have a wide and diverse interactome, with partners involved in splicing, translation and RNAi (Cugusi *et al.*, 2015). More recently, the protein has also been identified in the interaction of DENV 3' UTR in *Ae. aegypti,* suggesting a role in antiviral immunity as well (Yeh *et al.*, 2022).

These two proteins, AAEL012964 and AAEL004859, which will from now on be referred to as their *D. melanogaster* orthologues Dip1 and mle, have both been shown to be able to participate in antiviral immunity, in processes that are likely independent of the RNAi pathways. This makes them interesting candidates to study and characterize with regards to their interaction with Loqs2.

3. Uncharacterized, mosquito-specific proteins

Finally, we selected two additional candidates, that were uncharacterized at the time of the analysis, AAEL004699 and AAEL022113. We were unable to infer a possible function for these proteins, due to the absence of orthologues in *D. melanogaster*, and their specificity to *Culicinae*.

When identified during our analyses, AAEL004699 was still uncharacterized, however, the presence of orthologues in *Aedes* mosquitoes only and its multiple RNA binding domains reminded us of the main characteristics of Loqs2, which made this protein of particular interest for our study. Since then, the protein, renamed aedine broadly active antiviral protein (aBravo), has been identified as an interactant of Dcr-2 in *Ae. aegypti* Aag2 cells. It has also been shown to be antiviral against ZIKV, CHIKV and Semliki Forest virus (SFV), independently of Dcr-2 (Varjak *et al.*, 2020).

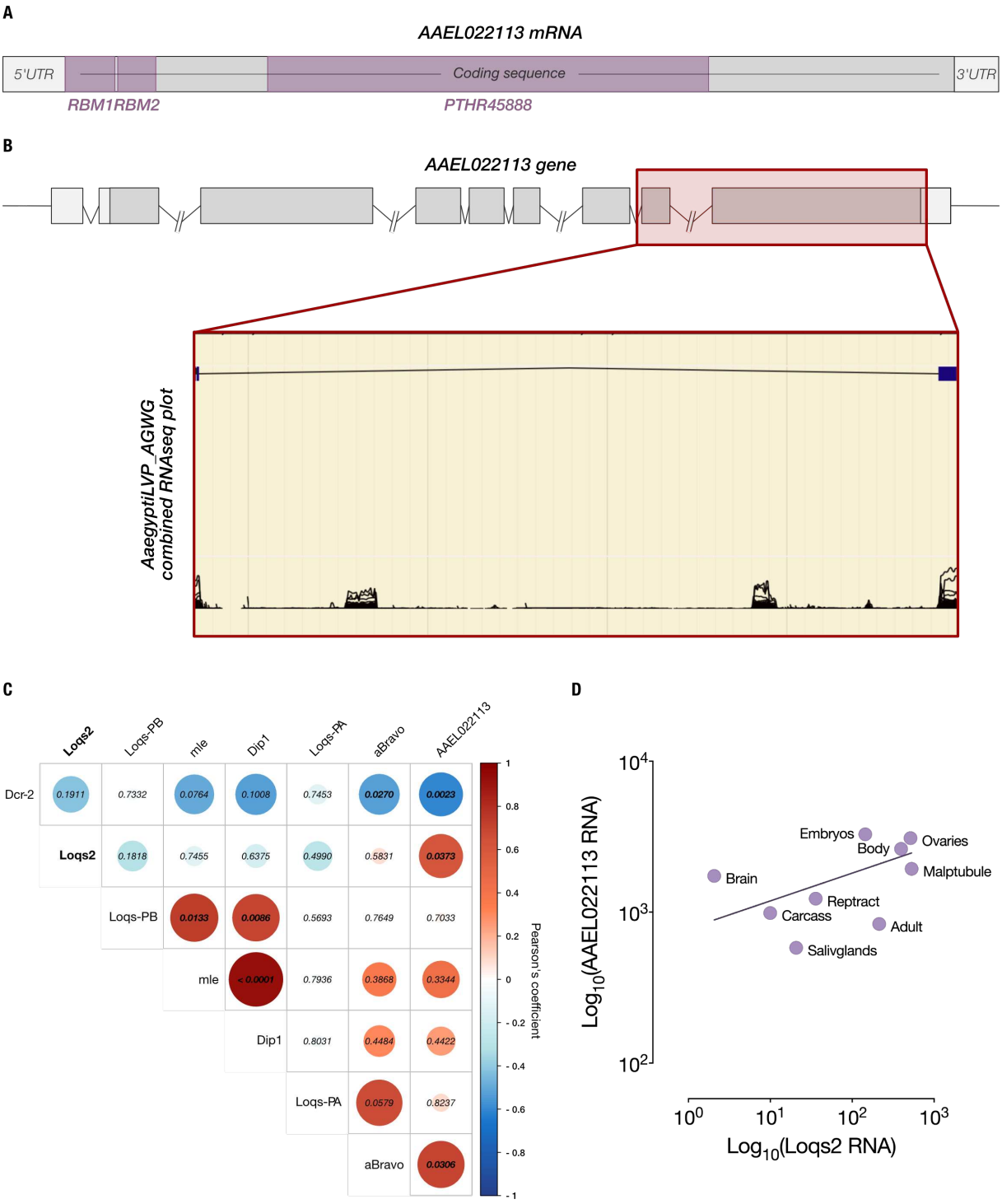


Figure 5. AAEL022113 is a* Culicinae*-specific uncharacterized gene.
[A] Schematic of the predicted InterPRO and PANTHER domains of AAEL022113. [B] Gene organization of AAEL022113 and extract of the combined RNASeq plot from Ae. aegypti *LVP strain, as displayed on Vectorbase Release 62. [C] Pearson's correlation matrix of tissue expression patterns from all selected candidates. Correlation is shown by a darkening red color, and anti-correlation is shown by a darkening blue color. Significance is shown by the size of the circle and p-values are indicated inside each circle. Significant p-values (< 0.05) are highlighted in bold. [D] XY correlation plot of AAEL022113 and Loqs2 mRNA expression in individual tissues (Malptubule = Malpihian tubules; Reptract = Reproductive tract; Salivglands = Salivary glands).*

Similarly, AAEL022113 has orthologues only in *Aedes* and *Culex* mosquitoes, and contains multiple RNA-binding domains (**Figure 5.A**). However, the current annotation of the gene on Vectorbase release 62 seems to be inaccurate since RNA-Seq data show the presence of reads in the last intron of the gene, suggesting the presence of two additional exons (**Figure 5.B**). These exons, if established, could contain conserved domain sequences that would help us better fathom the possible function of this protein. In an attempt to gather additional information on this protein, we studied the expression levels of our candidate genes in different mosquito tissues, using publicly available RNA-Seq data. Our aim was to identify a potential peculiar expression pattern, similar to what we observed for Loqs2, through the calculation of a Pearson's correlation coefficient.

Interestingly, we observed a high correlation between Loqs2 and AAEL022113 expression patterns in the tissues of mosquitoes (**Figure 5.C**). Indeed, similarly to Loqs2, AAEL022113 is also highly expressed in ovaries and in Malpighian tubules, and displays lower expression levels in salivary glands and in the brain (**Figure 5.D**). The similarities between the expression patterns of these two proteins could hint towards a similar function, possibly an involvement in the same pathways or biological processes.

These last two candidates that were selected from our analyses also have peculiar features, that resemble those of Loqs2, and makes them promising for further characterization.

II. Silencing the expression of the candidate genes

To allow further functional studies, we first tested the efficiency of silencing that we could achieve for all of the candidate genes. Considering that the further characterization of the candidates would be done *in vivo*, the silencing was performed by micro-injections of dsRNAs in 3 to 5 days old *Ae. aegypti* adult females, in a wild-type background (Bangkok strain), followed, 48 h later, by a non-infectious bloodmeal. The expression of each gene was measured in the midgut of injected mosquitoes after 4 days, and compared individually to their expression in the midguts of dsGFP-injected mosquitoes (**Figure 6.A**).

Silencing was successfully achieved, in the midgut, for all candidates tested, with an efficiency ranging from 60 to 70 % for most of the candidates (**Figures 6.B to 6.E**), with the exception of *aBravo*, for which a higher silencing efficiency was attained, showing an 85 % decrease in expression (**Figure 6.F**). Using a dsRNA designed to target all isoforms of *loqs*, silencing of the expression of this gene was more difficult than the other candidates, however we were still successful in reducing its expression by 50 % (**Figure 6.G**).

The efficient silencing observed for all of the candidates allowed us to proceed with their characterization, and in particular the investigation of their possible antiviral activity.

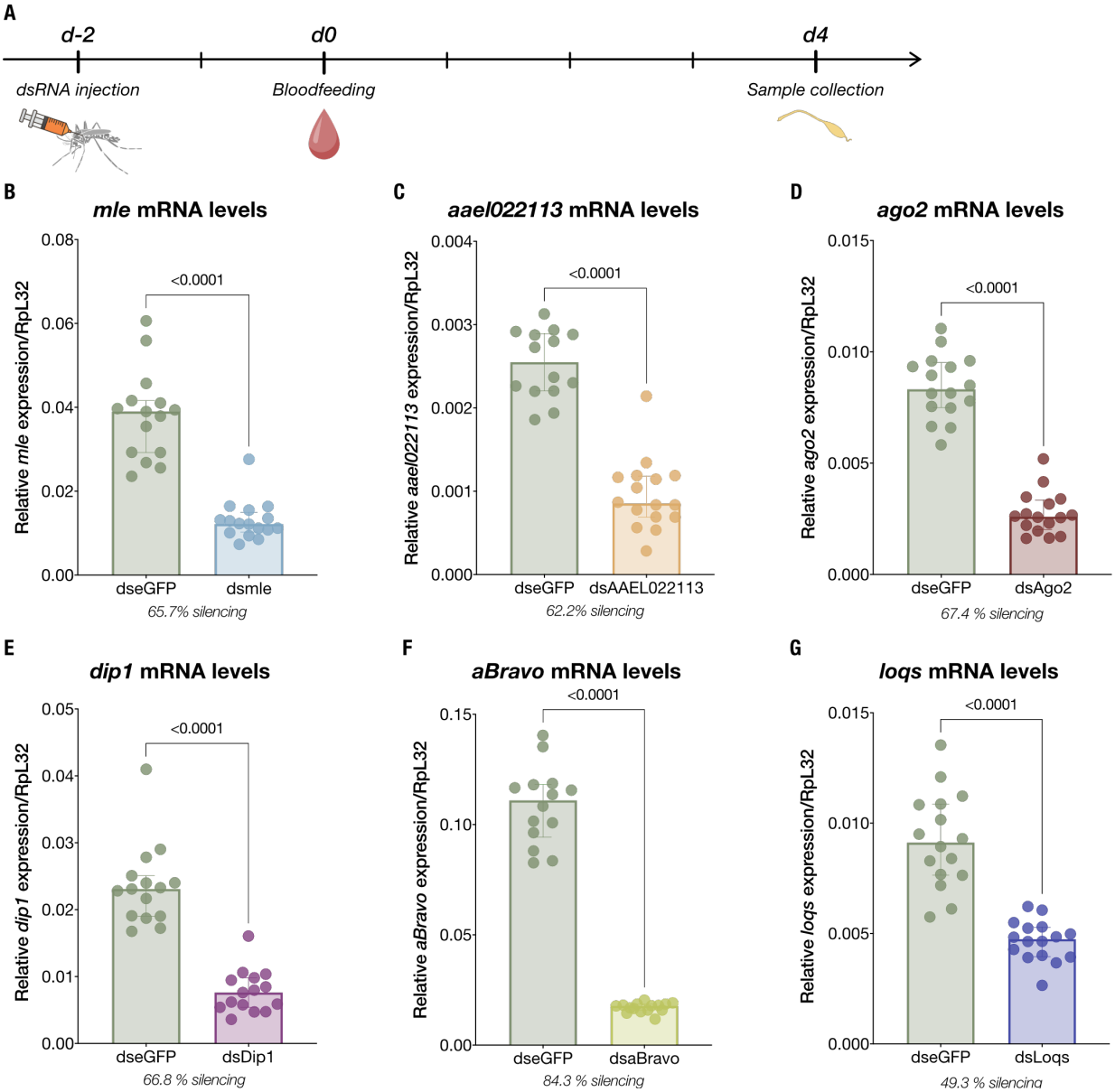


Figure 6. All potential partners of Loqs2 can be efficiently silenced in the midgut of mosquitoes. *[A] Timeline of the silencing experiment showing key steps. Relative expression of [B] mle, [C] AAEL022113, [D] Ago2, [E] Dip1, [F] aBravo and [G] Loqs to RpL32, in mosquitoes micro-injected with the corresponding dsRNAs, compared to dsGFP-injected mosquitoes. Each dot represents an individual mosquito midgut. Median is shown with interquartile range, and p-value was calculated using two-tailed Mann-Whitney test. Efficiency of silencing for each gene is indicated below the corresponding graph.*

III. Characterization of the antiviral properties of Loqs2 interactants

Most candidates selected for further characterization, or their orthologues, had been shown to have an antiviral function, in *Ae. aegypti* or in *D. melanogaster*, respectively. For two of these candidates, however, the function was either not described (AAEL022113) or the association to the antiviral response was inferred from interactomes only (mle). We thus chose to investigate these two candidates first, and to assess their role in the antiviral immune response *in vivo*.

1. mle controls ZIKV replication in the midgut

Study of the antiviral role of mle in mosquitoes was performed by silencing the gene in 3 to 5 days old adult *Ae. aegypti* females. dsRNA directed against *mle* was micro-injected in mosquitoes, which, after 48 h, were then infected with ZIKV through a bloodmeal. Simultaneously, control mosquitoes were injected with dsGFP (negative control) and dsAgo2 (positive control), and all mosquitoes were collected at 4 and 8 days post-infection (dpi).

The expression of *mle* was efficiently silenced in the midgut of mosquitoes, with a 64 % and 45 % decrease, at 4 and 8 dpi, respectively (**Figure 7.A**). This reduced expression of the gene affected viral replication in the midgut, as we observed a 3-fold increase in ZIKV viral load in the midguts of infected mosquitoes, which was consistent over time (**Figure 8.A**). In the control, *ago2* was successfully silenced as well, with a 66 % and 71 % decrease in expression, at 4 and 8 dpi, respectively (**Figure 7.B**), which also led to an increase in the viral load in the midgut, by around 6-fold (**Figure 8.B**).

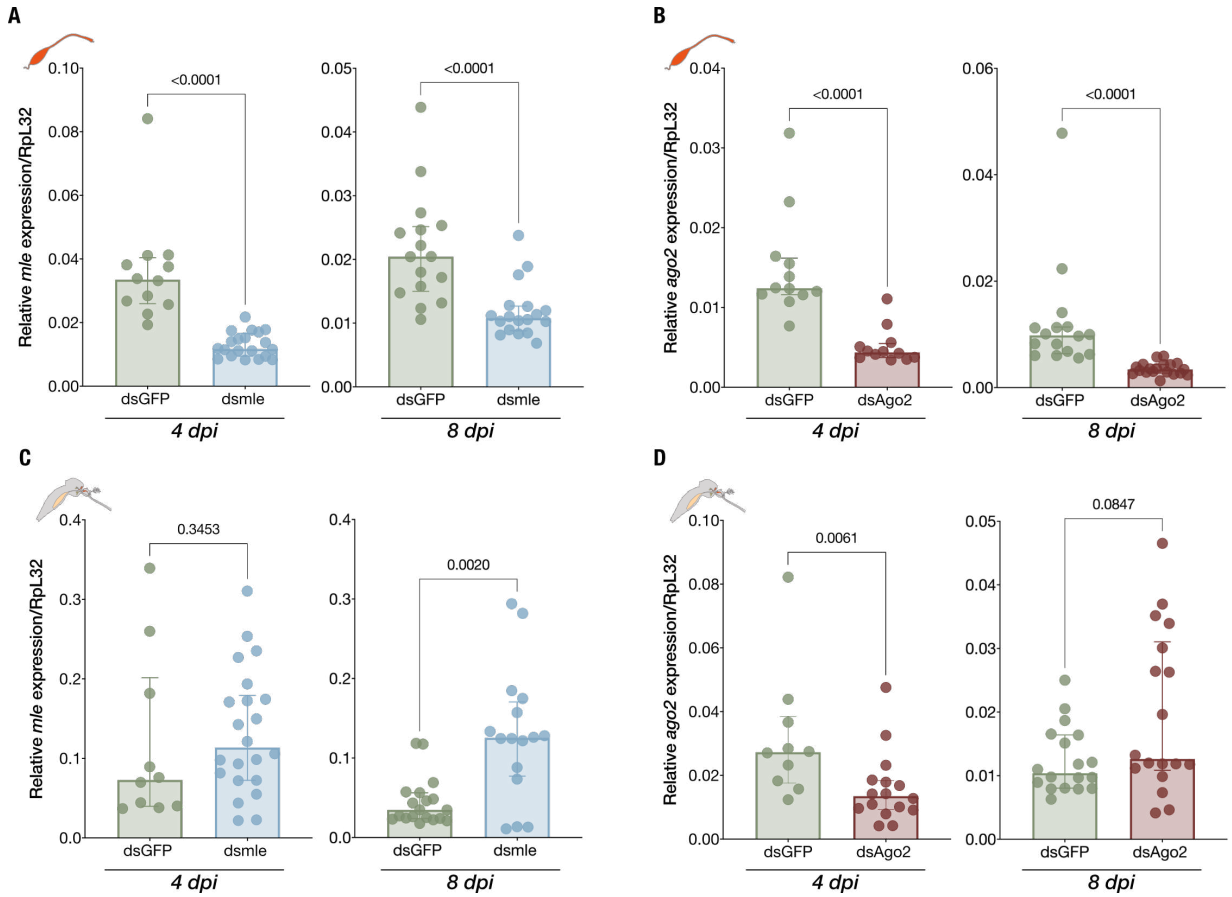


Figure 7. Expression of* mle *and* Ago2 *is efficiently silenced in ZIKV infected mosquitoes.
Relative expression at 4 and 8 dpi of [A] mle *and [B]* Ago2 *in midguts of mosquitoes micro-injected with the corresponding dsRNAs, compared to dsGFP-injected mosquitoes. Relative expression at 4 and 8 dpi of [C]* mle *and [D]* Ago2 *in carcasses of mosquitoes micro-injected with the corresponding dsRNAs, compared to dsGFP-injected mosquitoes. Each dot represents an individual mosquito. Median is shown with interquartile range and p-value was calculated using two-tailed Mann-Whitney test.*

In the carcass, silencing of both *mle* and *ago2* genes was inefficient, with increases in target gene expression at some time points (**Figures 7.C** and **7.D**). Regarding the effect on ZIKV infection, the number of infected carcasses at 4 dpi was too low to observe any effect of the silencing, and at 8 dpi, we did not observe significant differences in viral loads although there was a tendency of increased prevalence of infection when *mle* or *ago2* were silenced (**Figures 8.C** and **8.D**).

Overall, our preliminary studies suggest that mle, similarly to the antiviral protein Ago2, is able to regulate viral replication in the midgut of mosquitoes. Our results, however, do not allow for a conclusion on the role of mle in the control of systemic infection.

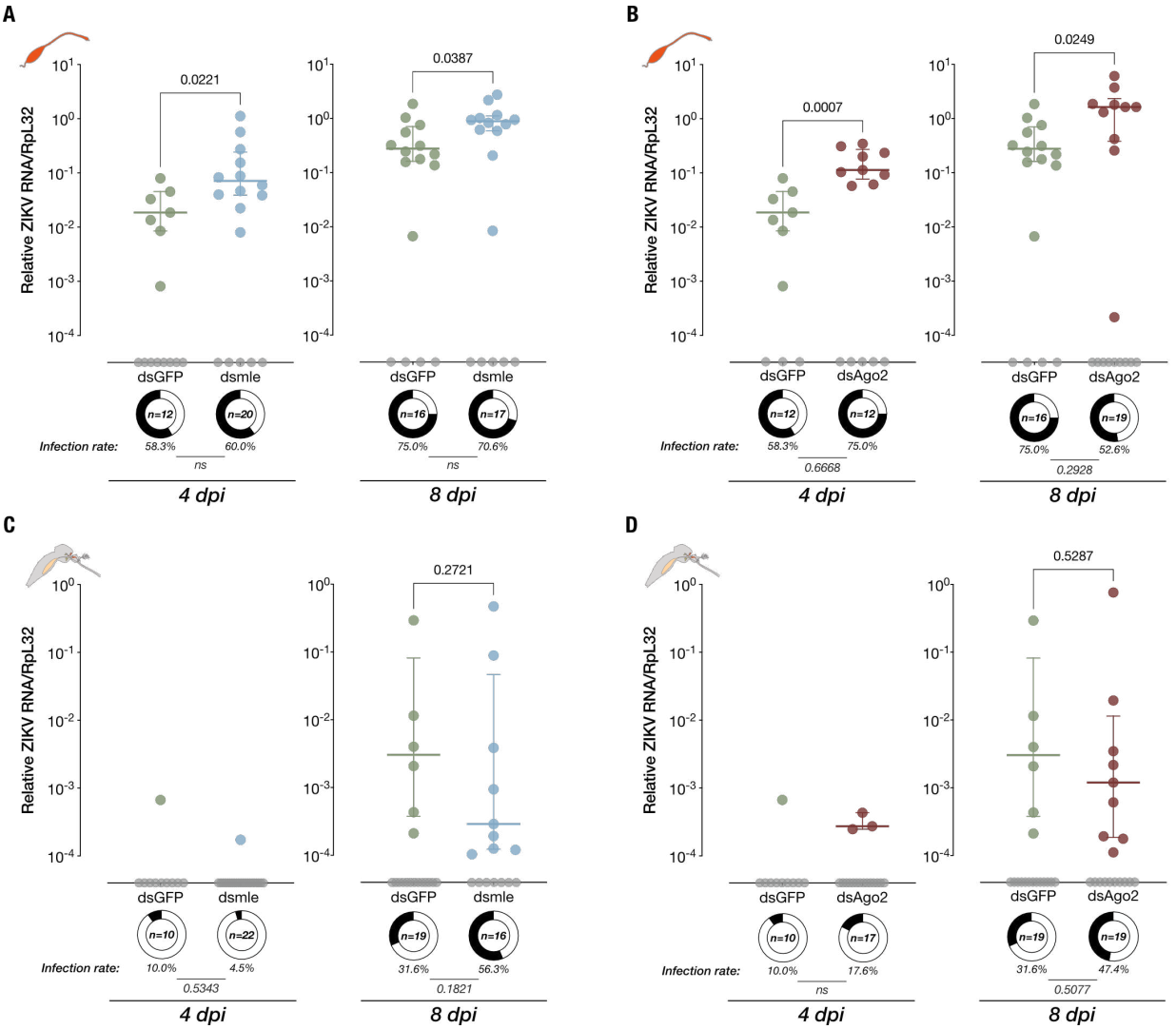


Figure 8. mle controls viral replication in the midgut of ZIKV-infected mosquitoes.
ZIKV viral load and prevalence at 4 and 8 dpi in the midguts of ***[A]*** mle *and* ***[B]*** Ago2 *dsRNA-injected mosquitoes, compared to dsGFP-injected mosquitoes. ZIKV viral load and prevalence at 4 and 8 dpi in the carcasses of* ***[C]*** mle *and* ***[D]*** Ago2 *dsRNA-injected mosquitoes, compared to dsGFP-injected mosquitoes. Each dot represents an individual mosquito. Grey dots represent non-infected mosquito. Total number of mosquitoes analyzed in this experiment is indicated in the prevalence pie-chart. Pie-charts represent the percentage of infected (black) and non-infected (white) samples. Median is shown with interquartile range, and p-value was calculated using two-tailed Mann-Whitney test for the viral load, and two-tailed Fischer's exact test for the prevalence. ns = not significant (p-value > 0.9999).*

2. AAEL022113 controls ZIKV replication in the midgut

As done previously, investigation of the possible antiviral role of AAEL022113 was performed through the silencing of this gene in 3 to 5 days old *Ae. aegypti* females. Micro-injection of dsRNA directed against *AAEL022113* in mosquitoes was followed after 48 h by an infection with ZIKV, through a bloodmeal. Simultaneously, mosquitoes were micro-injected with dsGFP as a control, and all mosquitoes were collected at 4 and 8 dpi.

Surprisingly, the efficiency of *AAEL022113* silencing in the midgut was lower than in the initial experiments, with a 20 % and a 55 % reduction in gene expression at 4 and 8 dpi, respectively (**Figure 9.A**). Nonetheless, this decrease in expression, led to a 3-fold increase in viral load at 4 dpi but to no significant difference at a later timepoint (**Figure 10.A**).

Silencing of the gene in carcasses was similar to what was observed in the midguts, with a 15 % and 41 % decrease in expression at 4 and 8 days, respectively (**Figure 9.B**). In this case however, no significant difference in viral load was observed, at any timepoint when compared to the control (**Figure 10.B**).

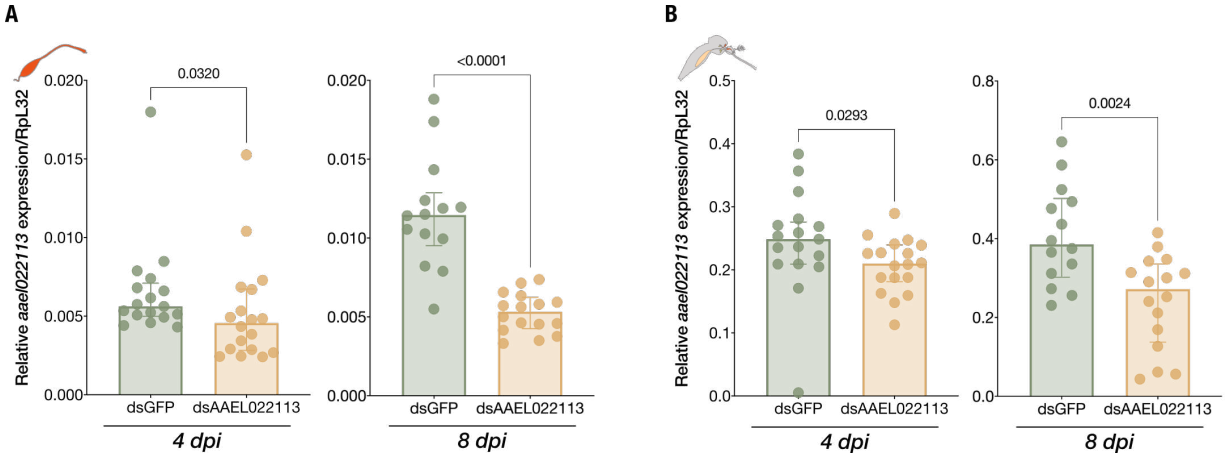


Figure 9. Expression of* AAEL022113 *is efficiently silenced in ZIKV infected mosquitoes.
Relative expression at 4 and 8 dpi of AAEL022113 *in [A] midguts and [B] carcasses of mosquitoes micro-injected with the corresponding dsRNA, compared to dsGFP-injected mosquitoes. Each dot represents an individual mosquito midgut or carcass. Median is shown with interquartile range, and p-value was calculated using two-tailed Mann-Whitney test.*

These preliminary results suggest that AAEL022113 is involved in the regulation of viral replication in the midgut of mosquitoes, but likely not in the control of systemic infection.

Overall these data support our hypothesis that Loqs2 has antiviral properties, and that it is likely that several antiviral processes are involved in this response, as there are multiple interactions between the proteins identified as antiviral, and the proteins of the RNAi pathway.

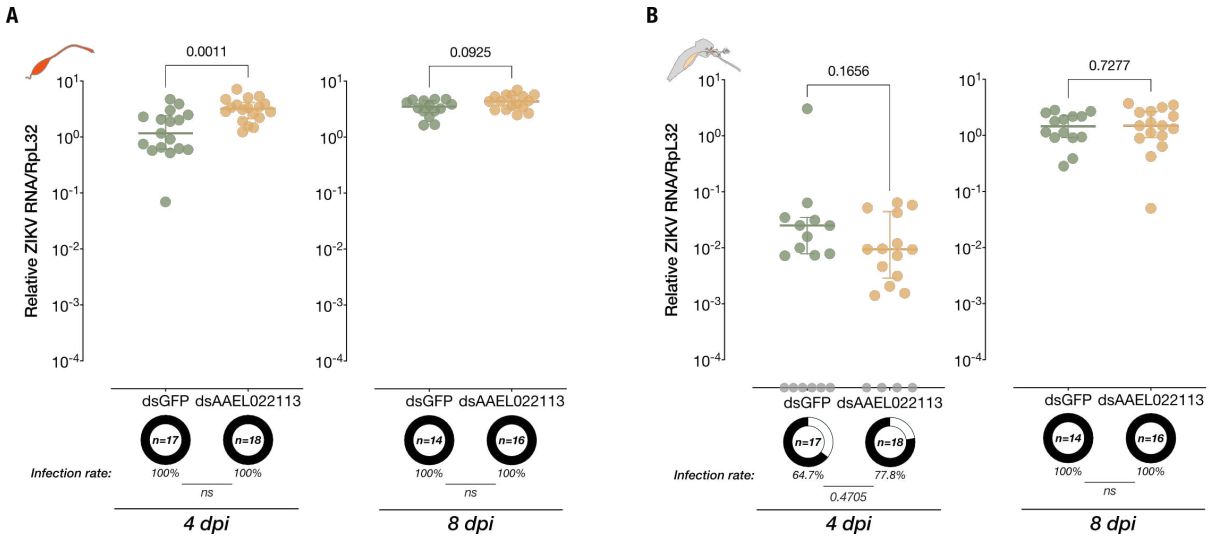


Figure 10. AAEL022113 controls viral replication in ZIKV infected mosquitoes.
*ZIKV viral load and prevalence at 4 and 8 dpi in the [**A**] midguts and [**B**] carcasses of* AAEL022113 *dsRNA-injected mosquitoes, compared to dsGFP-injected mosquitoes. Each dot represents an individual mosquito midgut or carcass. Grey dots represent non-infected mosquito. Total number of mosquitoes analyzed in this experiment is indicated in the prevalence pie-chart. Pie-charts represent the percentage of infected (black) and non-infected (white) samples. Median is shown with interquartile range, and p-value was calculated using two-tailed Mann-Whitney test for the viral load, and two-tailed Fischer's exact test for the prevalence.*

Part 3

Investigation of Logs2 natural function

We were able to demonstrate that Loqs2 is able to control viral infection in the midgut, and can reduce virus dissemination in the mosquito (Olmo *et al.*, 2018). Furthermore, our previous analyses emphasized that this protein interacts with numerous antiviral factors, reinforcing our postulate that Loqs2 can act as an antiviral protein. However, we currently have no knowledge of the possible functions of Loqs2 in the tissues where it is naturally expressed.

I. Generation of a Loqs2 loss-of-function mutant

The mosquito line over-expressing Loqs2 in the midgut of mosquitoes already allowed us to gain an insight into the function of the protein, however, this tissue does not naturally express the protein. At this stage, it was necessary for us to develop new tools to allow us to study the protein in a more natural context.

It had already been demonstrated that it was possible to successfully implement the CRISPR-Cas9 system in *Ae. aegypti*, and to generate knock-out (KO) mosquito lines using this technique (Kistler *et al.*, 2015; Li *et al.*, 2017). These methods require the injection of single-guide RNAs (sgRNAs) either in a Cas9-expressing mosquito line, or simultaneously with the Cas9 protein. Doing so, a limited amount of sgRNA is injected, that can be targeted by nucleases, and degraded before it can be loaded into the Cas9 protein. We aimed to overcome this issue by allowing a continuous production of those sgRNAs in the mosquitoes, expressing them under the control of U6 promoters, simultaneously with the Cas9 protein, expressed itself under the control of Exuperentia (Exu), a germline-specific promoter (**Figure 11.A**).We designed three sgRNAs, using the online tool CRISPOR, to target specifically the first dsRBD of *loqs2* (**Figure 11.B**). This strategy would allow us to disrupt the first domain, so that even if the deletion is in frame and still allows for the translation of a protein, it would likely not be functional.

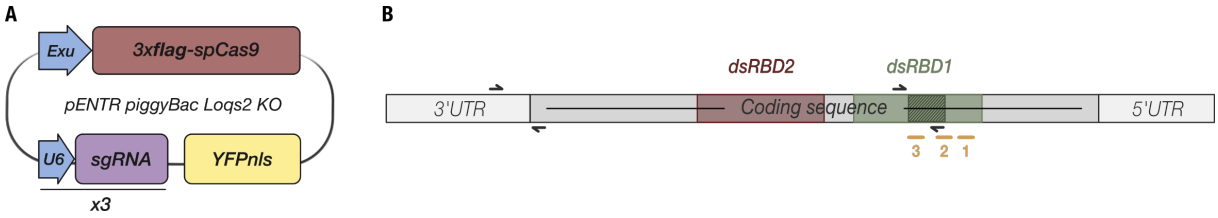


Figure 11. The CRISPR-Cas9 method was used to target the first dsRBD of Loqs2.
[A] Schematic representation of the construct injected in fertilized mosquito eggs to generate the KO line. [B] Schematic representation of loqs2 mRNA, showing the two dsRBDs (green and red, respectively). Target sites for the 3 sgRNAs are indicated with orange lines and numbered accordingly. Black arrows represent primer annealing sites for qPCR, and striated region indicate the deletion.

Following the transgenesis strategy established in our laboratory (Olmo *et al.*, 2018), we micro-injected the aforementioned construct into freshly laid eggs of a wild-type *Ae. aegypti* strain, and further selected the larvae displaying transient expression of our fluorescent marker, (yellow fluorescent protein; YFPnls) to be intercrossed (**Figure 12**). In the following generation, we isolated the transgenic mosquitoes and genotyped females to find CRISPR-Cas9 induced deletions. We were able to identify several individuals presenting various deletion patterns, two of which presented the same

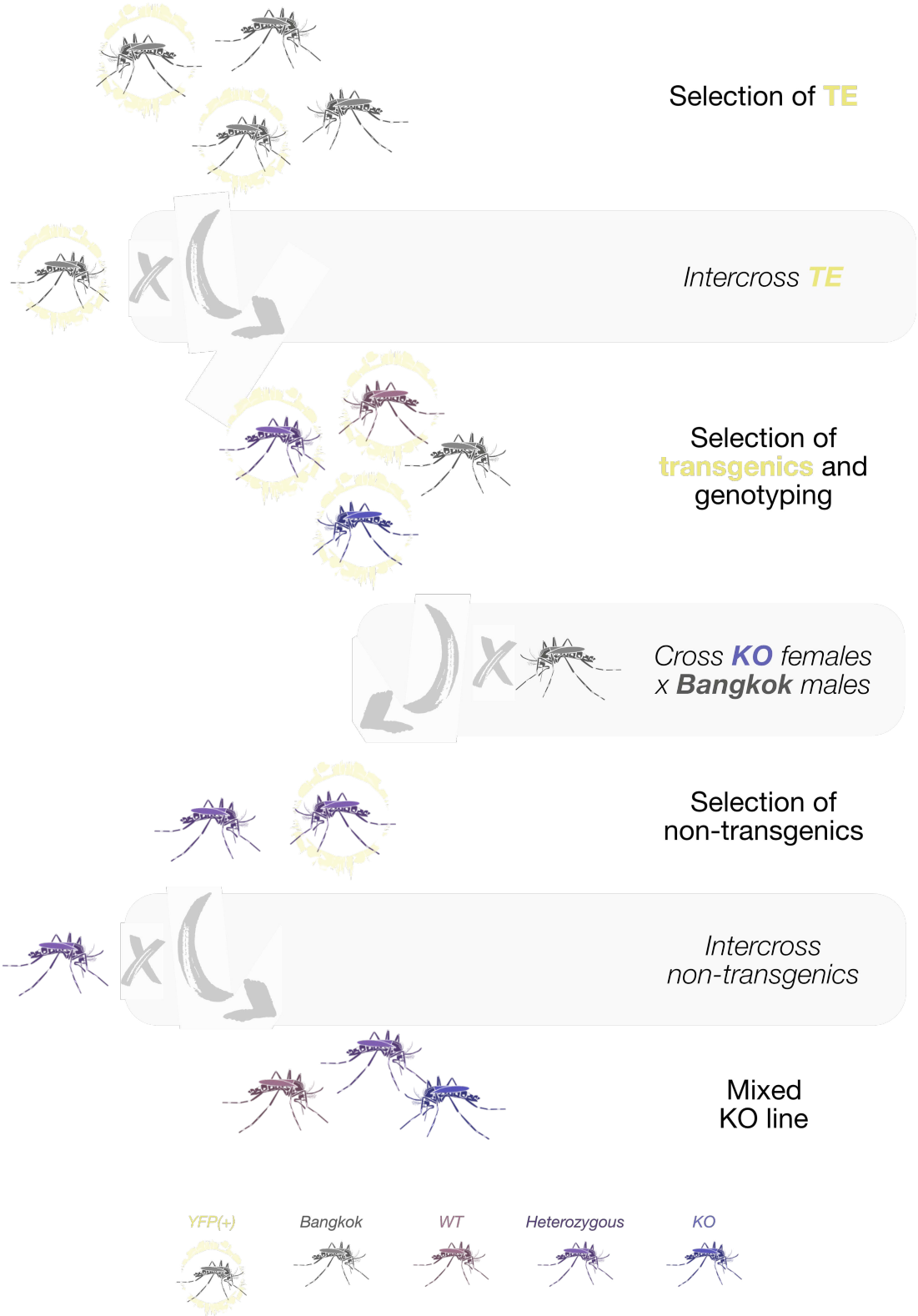


Figure 12. Strategy for the selection of a Loqs2 KO line.
Following micro-injections in the eggs, mosquitoes were hatched and larvae with transient expression of YFPnls (TE) that were subsequently intercrossed. In the first generation, transgenic mosquitoes expressing YFPnls constitutively were selected and genotyped. From those, females presenting a KO genotype were crossed to Bangkok (wild-type strain) males. In the following generation, non-transgenic heterozygous mosquitoes were intercrossed and the following generation presented all 3 genotypes (WT, Heterozygous and KO), and was considered the first generation of this new line. Bangkok mosquitoes are represented in grey, and mosquitoes showing after selection of the transgenics are represented by their genotype: WT (pink), Heterozygous (purple) or KO (blue). Yellow circles around the mosquitoes represent YFPnls expression.

deletion pattern, that we chose to further outcross to our wild-type Bangkok strain, in order to keep the most genetic diversity. In the following generation, where all mosquitoes were heterozygous for the deletion, we selected the non-transgenic individuals and intercrossed them. The next generation then contained mosquitoes with either two wild-type alleles, two alleles with a deletion, or one of each, referred to in the rest of this manuscript as WT, KO and Heterozygous (or Het.), respectively. This was considered the first generation of Loqs2 KO mosquitoes, and mosquitoes were kept as a pool of these three genotypes for all subsequent generations. When no specific genotype is described, this line will be referred to as "mixed line" in the rest of this manuscript.

Sequencing of the KO mosquitoes from this line showed a 46 bp deletion in the first dsRBD of *loqs2* (**Figure 13.A**), causing a disruption of the reading frame, and the apparition of an early stop codon at the end of the first dsRBD. This deletion was easily detectable by PCR, which was the main genotyping method used throughout the rest of this work (**Figure 13.B**). Measure of *loqs2* mRNA level in these mosquitoes was performed, by using a primer pair spanning the deleted region and a second pair annealing at the 3' UTR (**Figure 11.B**). The detection of *loqs2* mRNA using the first primer pair confirmed the deletion in the KO mosquitoes, since the levels were below our detection threshold of 10^{-4}, while the expression of *loqs2* was stable in both heterozygous and WT mosquitoes (**Figure 13.C**). *Loqs2* 3' UTR, however, was detected in all mosquitoes, independently of their genotype, at a very similar level (**Figure 13.D**). Although *loqs2* mRNA levels seem to be slightly higher in the KO mosquitoes than in the WT, we hypothesized that this is an artefact due to the comparatively low number of WT mosquitoes in this experiment, and that this difference would probably become negligible when studying a larger sample group.

The presence of *loqs2* mRNA in the KO mosquitoes indicate that despite the deletion, the mRNA was not degraded and translation of a protein remained possible. Indeed, it is predicted that instead of the usual 31.9 kDa protein, this truncated mRNA could be translated into two smaller proteins of 11 kDa and 18.1 kDa, containing the truncated first dsRBD, and the second dsRBD, respectively (**Figure 13.E**). We had previously ordered custom-made polyclonal antibodies, produced against the whole Loqs2 protein, that we used here to assess the presence of the proteins (either whole or truncated) in our KO individuals. Using midguts from mosquitoes over-expressing Loqs2, and midguts from wild-type mosquitoes as positive and negative controls, respectively, we were able to show that no protein is detectable in the ovaries of our KO mosquitoes, and that there is also no sign of the predicted truncated proteins in these individuals (**Figure 13.F**).

To assess the transmissibility of the KO allele, we followed the genotype of mosquitoes in the mixed line over several generations, and we observed a mendelian inheritability of the allele in the initial generations in females, with around 20% WT, 50% Het and 30% KO individuals (**Figure 14.A**). It seems however that the female WT population might be decreasing over time, while the KO individuals are slightly increasing. This inheritability rate is very different in male mosquitoes. Indeed, in the first generation genotyped, the number of KO individual was extremely low, around 2%, while we observed almost 50% of both WT and Het individuals (**Figure 14.B**).

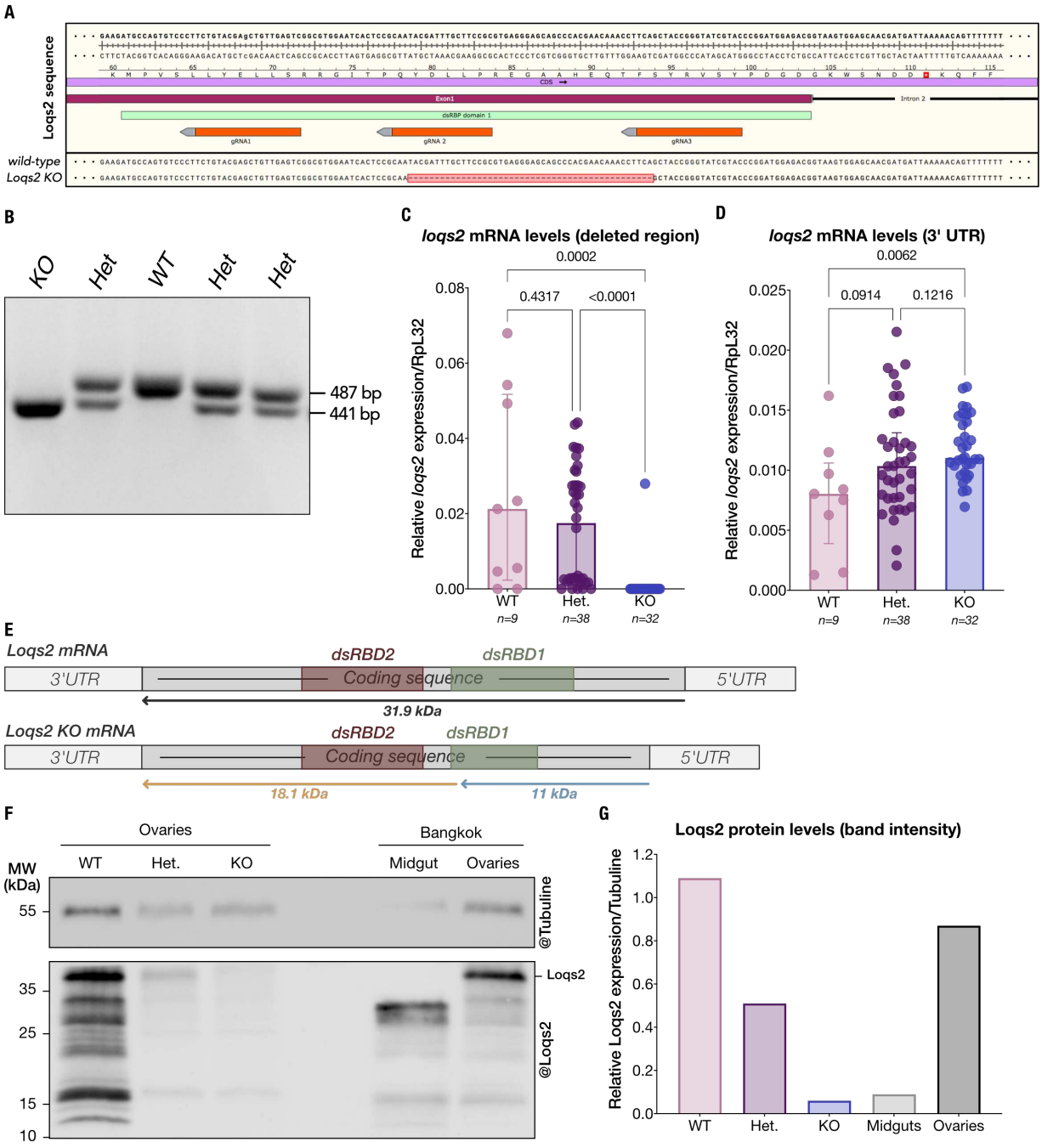


Figure 13. Loqs2 KO line was validated by multiple molecular biology techniques.
*[**A**] Sequence alignment of Loqs2 KO mosquitoes compared to wild-type (Bangkok), showing a 46 bp deletion in the middle of the first dsRBD. [**B**] Genotyping of 5 randomly selected females from the Loqs2 KO pool, annotated with the corresponding genotype. Detection of loqs2 mRNA using primer annealing in the [**C**] deleted region or in the [**D**] 3'UTR, as shown on the schematic representation in **Figure 11.B**. Number of individuals analyzed is indicated below each bar. Median with interquartile range is displayed and pairwise comparisons were performed using two-tailed Mann-Whitney test. [**E**] Schematic representation of* loqs2 *mRNA and* loqs2 *KO mRNA. Both dsRBDs are represented in green and red, respectively. Loqs2 full-length predicted protein is represented by a black arrow, and the two predicted truncated proteins in the Loqs2 KO line are represented by a blue and an orange arrow, respectively. [**F**] Loqs2 protein expression in ovaries of WT, heterozygous and KO mosquitoes from the Loqs2 KO pool. Midguts and ovaries from the Bangkok wild-type strain were used as negative and positive controls, respectively. Tubuline expression was also measured as a loading control. [**G**] Bar-plot representation of the relative Loqs2 expression over tubuline, measured from the western blot using ImageJ.*

Interestingly, the population of KO males seems to increase in the following generation, to 8%, while the percentage of WT individuals is decreasing.

This sex-bias in the inheritability of the gene can be explained by the genomic localization of *loqs2*. Indeed, this gene is located in close proximity to the m/M sex-determining locus, on the first chromosome (**Figure 14.C**). This 63-Mbp region containing several sex-specific genes such as *myo-sex* or *Nix* is known for showing extremely low to no recombination rates (Fontaine *et al.*, 2017). It is likely that recombination rates of genes located in the proximity of this region are also disturbed, compared to the rest of the genome. Considering that our mixed line was generated from KO females, crossed to wild-type males (**Figure 12**), it could explain why we observe less males carrying this mutation.

These results confirm that we were able to successfully generate a mosquito line in which the *loqs2* gene was partially deleted, and in which the function of Loqs2 is successfully abolished due to the absence of the protein.

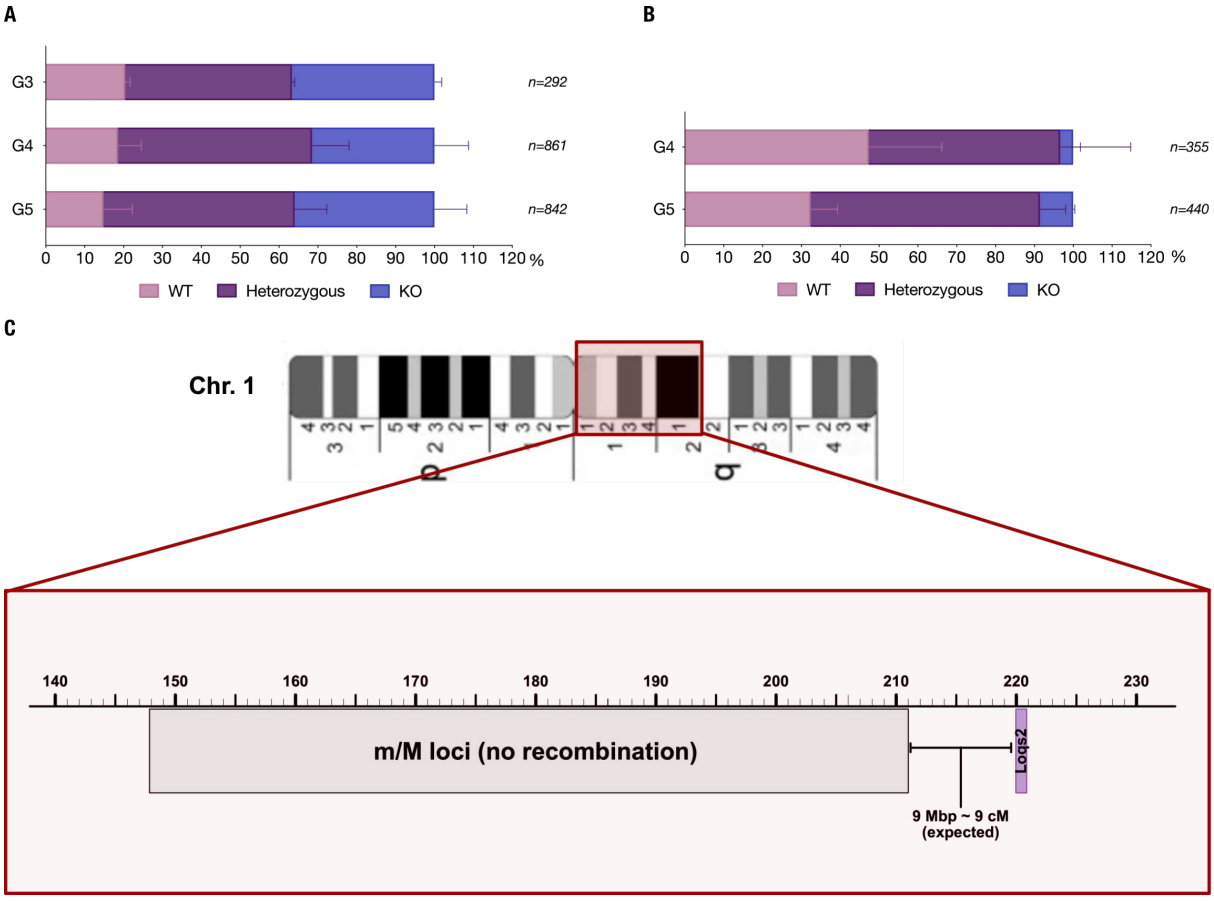


Figure 14. Loqs2 KO is not inherited equitably between males and females.
[A] Bar plot representing the repartition of each genotype in females from 3 consecutive generations of the Loqs2 KO pool. Number of genotyped individuals for each generation is indicated at the end of the bar. [B] Repartition of each genotype in males from 2 consecutive generations of the Loqs2 KO pool. Number of genotyped individuals for each generation is indicated at the end of the bar. Mean with standard deviation is displayed. [C] Schematic representation of chromosome 1 of Ae. aegypti*, and localization of loqs2 in comparison to the region of the m/M loci that show low to no recombination.*

II. Loqs2 does not show antiviral activity in natural conditions

We had previously demonstrated that when ectopically expressed in the midgut of mosquitoes, Loqs2 shows antiviral properties, and we aimed to assess if this was also observed in other tissues, where Loqs2 is naturally expressed. In this context, we focused our investigation on the tissue showing the highest expression of this protein, ovaries.

Multiple infection experiments were thus performed on our newly generated KO mosquitoes, in order to investigate this hypothesis. To allow for maximum reproducibility between KO and WT individuals, we infected mixed line, and separated mosquitoes by their genotype during the analyses. All genotypes are not present at the same ratios in the mixed line, which explains why the WT sample size is much lower than the other two. For each experiment, the total sample size was calculated according to the previously observed ratios of each genotype in the pool, to have at least 10 WT individuals. Most experiments described here were performed once, unless stated otherwise, and need to be repeated.

Following the natural route of infection, we first offered a ZIKV infectious bloodmeal to the mosquitoes, and monitored viral load and prevalence of infection in midguts, carcasses and ovaries, every other fourth day, for 12 days. The collection of mosquitoes at 8 days after infection was performed twice, in two independent experiments. For the first one (circles), mosquitoes were infected through a bloodmeal using sheep blood, and for the second one (diamonds), the infectious bloodmeal was prepared with human blood. A third independent experiment (hexagons) was performed to collect the remaining two timepoints, with a bloodmeal prepared using sheep blood. At 4 days, viral replication is mostly limited to the midgut, which explains the absence or extremely low prevalence of infection observed in both carcasses and ovaries (**Figure 15.A**). After a few more days, the virus starts to disseminate to the different tissues of the mosquito, and is thus detectable in all three tissues tested in this experiment. Even after early dissemination of the virus at 8 days, no significant difference in neither viral load nor prevalence is observed in any of the tissues (**Figure 15.B**). Similarly, both viral load and prevalence do not significantly differ between the three genotypes in all tissues tested at 12 days (**Figure 15.C**). Although it would seem that in the midgut, we detect a viral load in less KO mosquitoes than WT, it may be due to the low number of individuals in the WT group, and this reiterates the need to repeat the experiment and increase the sample size. In the meantime, our results clearly indicate that Loqs2 is not involved in the control of virus replication or dissemination in the context of an infection by bloodmeal.

Figure 15. Loqs2 is not antiviral against ZIKV in natural infection conditions.
*ZIKV viral load and prevalence in midguts, carcass and ovaries of WT, heterozygous and KO mosquitoes, at [**A**] 4 dpi, [**B**] 8 dpi and [**C**] 12 dpi. Each dot represents an individual tissue, and the total number of individuals analyzed is indicated in the prevalence pie-charts. Pie-charts represent the percentage of infected (black) and non-infected (white) samples. Each replicate is indicated by a different type of dot (circle, diamond, hexagon). Median with interquartile range is displayed and pairwise comparisons were performed using two-tailed Mann-Whitney tests for the measures of viral load, and two-tailed Fischer's exact test for the prevalence. ns = not significant (p-value > 0.9999).*

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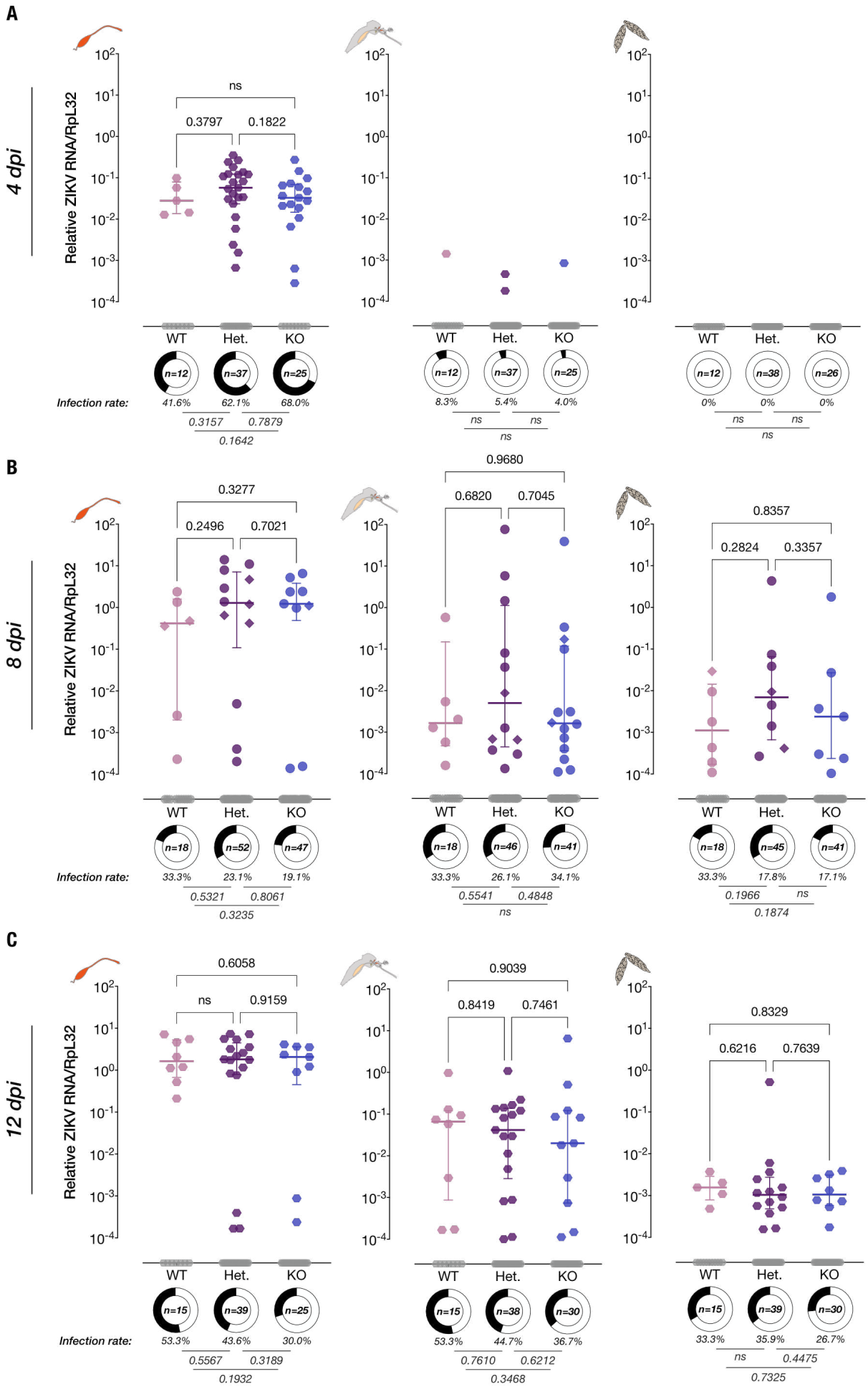

A
4 dpi
Relative ZIKV RNA/RpL32
ns
0.3797
0.1822
WT
Het.
KO
n=12
n=37
n=25
Infection rate:
41.6%
62.1%
68.0%
0.3157
0.7879
0.1642
8.3%
5.4%
4.0%
n=38
n=26
0%
B
8 dpi
0.3277
0.2496
0.7021
0.9680
0.6820
0.7045
0.8357
0.2824
0.3357
n=18
n=52
n=47
n=46
n=41
n=45
33.3%
23.1%
19.1%
26.1%
34.1%
17.8%
17.1%
0.5321
0.8061
0.3235
0.5541
0.4848
0.1966
0.1874
C
12 dpi
0.6058
ns
0.9159
0.9039
0.8419
0.7461
0.8329
0.6216
0.7639
n=15
n=39
n=25
n=38
n=30
53.3%
43.6%
30.0%
44.7%
36.7%
35.9%
26.7%
0.5567
0.3189
0.1932
0.7610
0.6212
0.3468
0.4475
0.7325

We had previously shown that when exposed to an infectious bloodmeal, not all mosquitoes were productively infected, and some mosquitoes could eliminate the virus after a few days (Olmo *et al.*, 2018). In this context, it is difficult to assess if the absence of difference in virus dissemination in our KO line is related to the loss of Loqs2, or if it is linked to this clearance of the virus, or even a combination of both. In order to overcome this limitation, we bypassed the infection of the midgut, by injecting the virus in the hemocoel of mosquitoes and investigating the response to direct systemic infection. Confirming the results obtained previously, no difference was observed between the viral loads of all three genotypes in both carcasses and ovaries, neither at 4 nor at 8 days (**Figure 16.A**). However, after 12 days, a significant decrease in ZIKV viral load was observed in the carcasses of KO individuals, as compared to the WT mosquitoes, that is statistically significant, while there seems to be an opposite trend in the ovaries, although not significant (**Figure 16.B**). Surprisingly, our results suggest here that Loqs2 might have a slightly pro-viral activity in the carcass of mosquitoes, in the context of direct systemic infection. Once again, increasing the sample sizes by repeating the experiments would allow us to have a better insight and to strengthen our conclusions.

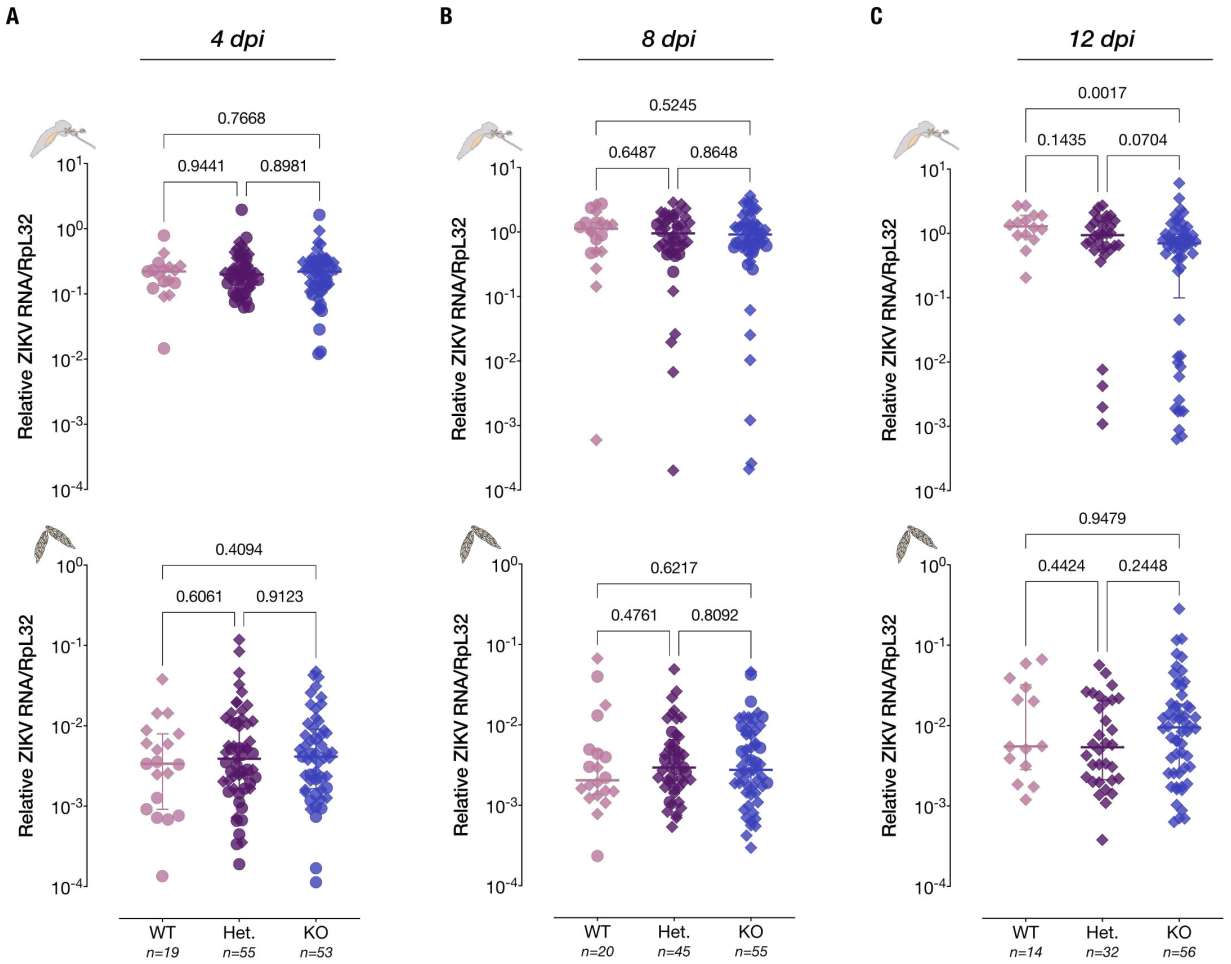


Figure 16. Loqs2 possibly shows pro-viral activity during direct systemic infection.
*ZIKV viral load in carcass and ovaries of WT, heterozygous and KO mosquitoes of the Loqs2 KO line, at **[A]** 4 dpi, **[B]** 8 dpi and **[C]** 12 dpi. Each dot represents an individual tissue, and the total number of individuals analyzed is indicated below each group. Each replicate is indicated by a different type of dot (circle, diamond). Median with interquartile range is displayed and pairwise comparisons were performed using two-tailed Mann-Whitney tests.*

Considering that viruses from different families have different infection strategies, replicating and disseminating at different rates, there is a possibility that different proteins are involved in the control of these infections (Robison *et al.*, 2020). Loqs2 could thus have very limited interactions with virus of the *Flaviviridae* family, but could be involved in the antiviral response against viruses from other families. We tested this hypothesis by again injecting our KO individuals, this time with SINV, a virus from the *Togaviridae* family. The rapid replication and dissemination of viruses from this family prompted us to monitor viral load at earlier timepoints than in our previous experiments, 2 and 4 dpi. We also considerably decreased the amount of virus injected in our mosquitoes, in an attempt to delay the reach of the infectious plateau as much as possible, and thus injected the equivalent of 1 pfu per mosquito. This very low amount of virus did not allow the infection of all injected mosquitoes, contrary to the ZIKV injection experiment described before. Although displayed on each graph for the sake of transparency, the prevalence observed in this experiment is not related to the presence or absence of Loqs2, but solely to the amount of virus injected that was too low to induce a productive infection in several individuals. At the very early stages of infection, no significant difference was observed in the viral load in the carcass, and although it would seem that the viral load is higher in the ovaries of Het and KO mosquitoes, the number of infected WT individuals is too low to show a significance in this trend (**Figure 17.A**). After 4 days, it seems that the infection already reached a plateau in the carcass, thus showing no difference in the viral load between the genotypes, and although not reaching as high of a viral load as in the carcass, the differences in viral load observed at 2 days in ovaries disappeared at this later timepoint (**Figure 17.B**). Overall, our results indicate that Loqs2 does not seem to be involved in the antiviral response against SINV either, although this experiment needs to be repeated to ensure a homogeneous infection of all individuals tested, and to increase the sample size.

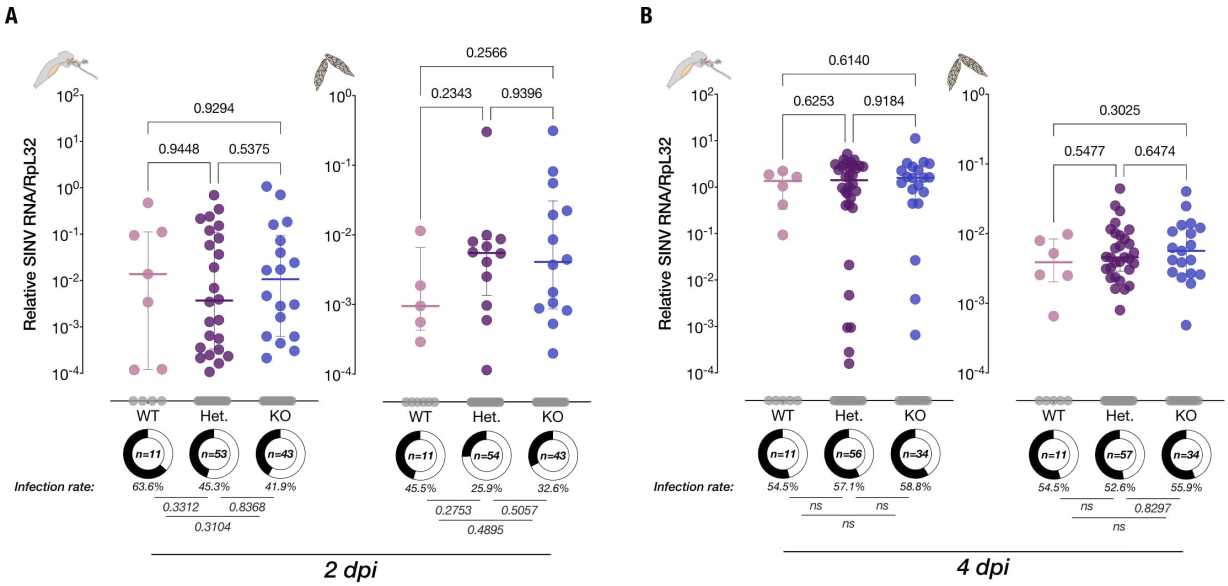


Figure 17. Loqs2 is not antiviral against SINV.
*SINV viral load in carcasses and ovaries of WT, heterozygous and KO mosquitoes of the Loqs2 KO line, at **[A]** 2dpi and **[B]** 4dpi. Each dot represents an individual tissue, and the total number of individuals analyzed is indicated in the prevalence pie-chart below each group. Pie-charts represent the percentage of infected (black) and non-infected (white) samples. Median with interquartile range is displayed and pairwise comparisons were performed using two-tailed Mann-Whitney tests for the measures of viral load and two-tailed Fischer's exact test. ns = not significant (p-value > 0.9999).*

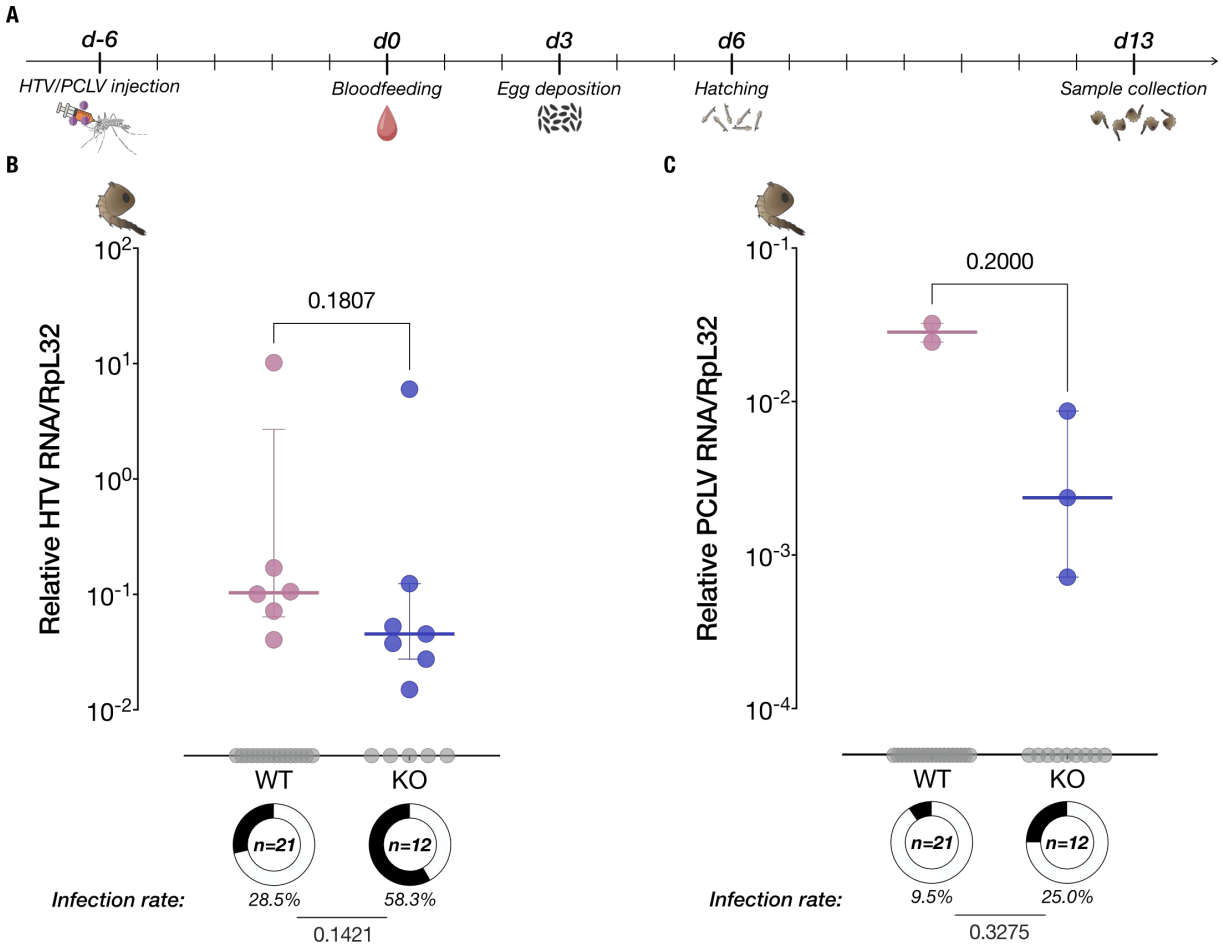


Figure 18. Loqs2 may be involved in the control of vertical transmission of viruses.
*[**A**] Timeline of the experiment showing key steps.Viral load and prevalence of [**B**] HTV and [**C**] PCLV in groups of 5 pupae from WT and KO mosquitoes of the Loqs2 KO line. Each dot represents a group of 5 pupae, progeny of an individual female. Total number of individuals analyzed is indicated below each group, in the prevalence pie-chart. Pie-charts represent the percentage of infected (black) and non-infected (white) samples. Median with interquartile range is displayed and pairwise comparisons were performed using two-tailed Mann-Whitney tests for the measures of viral load and two-tailed Fischer's exact test for the prevalence.*

In all experiments performed here, we were able to detect similar virus levels in ovaries from KO and WT mosquitoes, suggesting that Loqs2 is not involved in the control of infection in this tissue. However, considering its high expression in the germline, we hypothesized that Loqs2 may be involved in the control of vertical transmission of viruses. Here, we chose to test the insect-specific viruses, Humaita-Tubiacanga virus (HTV) and Phasi Charoen-like phasivirus (PCLV), to viruses that are naturally detected in the virome of most *Ae. aegypti* mosquitoes, and are vertically transmitted in the wild (Olmo *et al.*, 2023). However, vertical transmission was difficult to observe in laboratory conditions, following intra-thoracic injections, making these viruses good candidates to test our hypothesis. Mosquitoes were thus injected intra-thoracically with a mix of HTV and PCLV, and allowed to recover for 5 days, which is the observed time for PCLV to reach ovaries (Olmo *et al.*, 2023). Following a non-infectious bloodmeal, mosquitoes were allowed to lay eggs, and HTV and PCLV viral loads were measured in this progeny, at the pupal stage. The progeny of 21 WT females and 12 KO females were tested, in groups of 5 pupae each (**Figure 18.A**). Interestingly,

although the viral load was similar for both genotypes, we could observe a difference in the prevalence of infection, with almost twice as many mosquitoes infected in by HTV for the KO as compared to the WT individuals (**Figure 18.B**). Likewise, very low levels of PCLV were detected in both genotypes, but again, the prevalence of infection was twice as high in KO, compared to WT mosquitoes (**Figure 18.C**). These differences in the prevalence, however, were not statistically significant, likely due to the low sample size.

Overall, our results suggest that Loqs2 is not actively involved in the antiviral response, however, it is possible that the protein plays a role in vertical transmission of viruses, although this hypothesis needs to be further confirmed.

III. Loqs2 affects fitness and fertility in mosquitoes

Our original hypothesis stated that Loqs2 was evolving towards neofunctionalization. Considering our recent results, it would seem that this new function is not explicitly related to antiviral immunity as we initially thought. We thus explored the involvement of Loqs2 in other biological processes. Furthermore, most of the partners of this protein identified in the midgut of mosquitoes are also involved in various processes outside of immunity, ranging from splicing to translation, to gene regulation during development. Also, we attempted to generate a mosquito line expressing Loqs2 ubiquitously, and this resulted in developmental arrest at the 4th larval stage, thus suggesting an involvement of Loqs2 in the development of mosquitoes (Estevez-Castro *et al.*, 2021).

Using the Loqs2 KO mosquitoes previously generated, we investigated this hypothesis by following the life cycle of these individuals, from oviposition to death of adult mosquitoes. To this aim, we isolated the WT and KO mosquitoes from the mixed line and tried to homozygotize them into two individual lines. We were successful in producing homozygous WT mosquitoes, but unfortunately, due to the very low amount of male KO (**Figure 14.B**), it was difficult to generate a homozygous KO line, as no mosquitoes would hatch from these crossings. Considering that the inheritance of the KO allele seemed to be sex-linked, with low levels of recombination (**Figure 14.C**), we decided to cross heterozygous males with KO females, so that the females that we would use for our experiments would remain KO. Females from both of these lines were offered a non-infectious bloodmeal, and were allowed to lay eggs. Starting at this point, the number of eggs was assessed, as well as the number of individuals at each developmental stage after that. Each dot on the following graphs represents the measures for the progeny of one female, and the number indicated below each group represents the total number of female parents that were studied. These experiments were repeated three times, on three consecutive generations (circles, diamonds and hexagons, respectively).

The total amount of eggs deposited per female, and the consecutive number of larvae that came out of them were counted, in order to calculate the hatching rate, and measure the fertility. Although females from both groups laid a similar number of eggs (**Figure 19.A**) the amount of neonate larvae measured at the day of hatching was considerably lower in the KO group, resulting in a significant decrease in the hatching

rate in these mosquitoes (**Figure 19.B**). We also observed a significant decrease in the survival of mosquitoes until the adult stage, with numerous individuals dying during development (**Figure 19.C**). However, despite the higher mortality in the KO group, no delay in development was observed for these individuals, in comparison to the WT individuals (**Figures 19.E** and **19.F**). Considering the sex-bias that we described previously, we also assessed the female-to-male ratio in adult mosquitoes and observed no significant difference between the two groups (**Figure 19.D**).

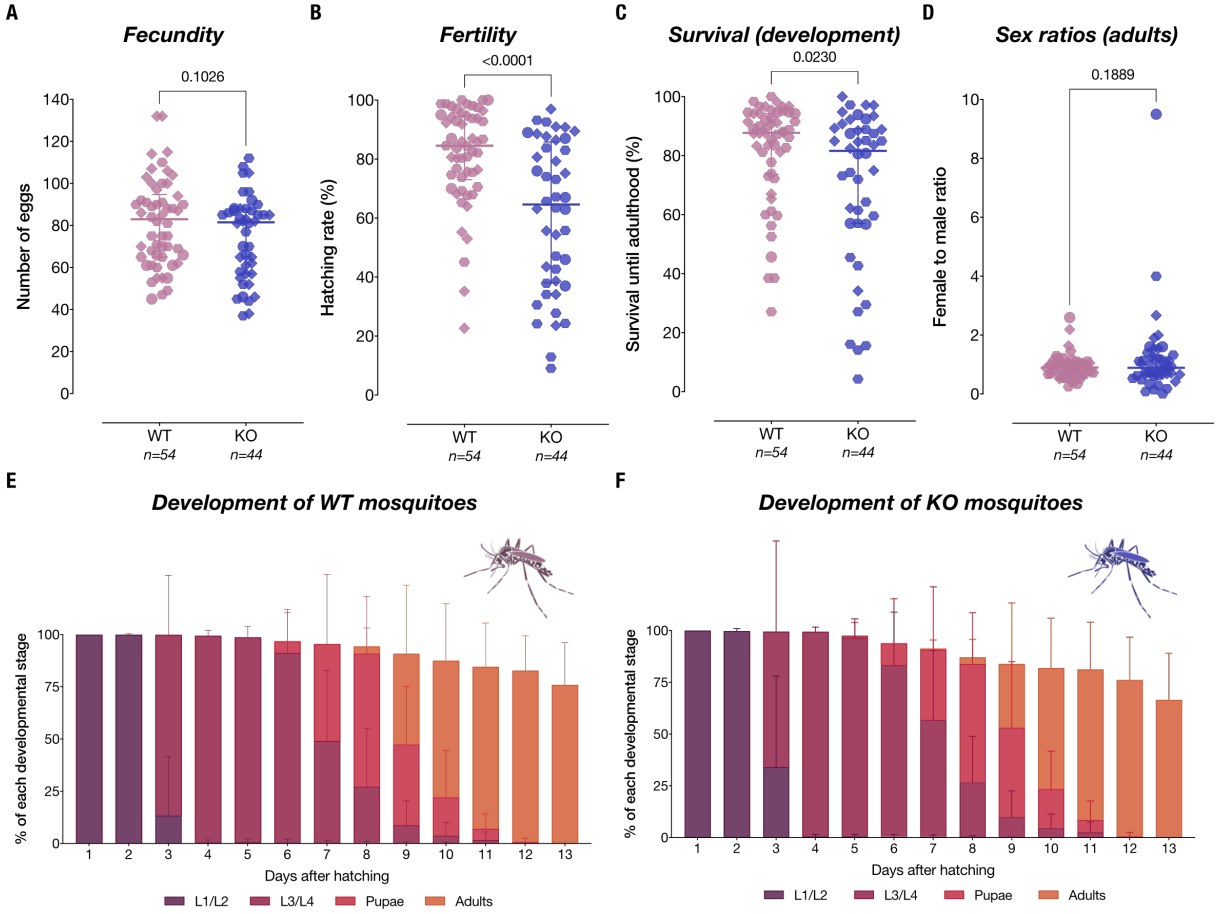


Figure 19. Loqs2 impacts fertility and survival during development.
*[**A**] Number of eggs deposited by individual females of the WT and Loqs2 KO mosquito lines. [**B**] Hatching rate for the progeny each individual female. [**C**] Survival of the progenies until adulthood. Each individual dot represents the progeny of one individual parent. Total number of parents (females) are indicated below each group. Median with interquartile range is displayed and pairwise comparisons were performed using two-tailed Mann-Whitney tests. [**D**] Female-to-male ratio of adult mosquitoes in each group. [**E**] Development rate of progenies from the [**E**] WT line and [**F**] KO line. For each day, the percentage of progenies in each developmental stage, from each individual female parent was calculated, and the mean is displayed on this graph, with standard deviation.*

As adults, we continued investigating the phenotype of these progenies, and monitored their survival over time, in regular rearing conditions. These mosquitoes were grouped as individual progenies from one female, and the survival of each group was monitored separately. In total, there were around 20 groups per mosquito line tested. As it would be unreadable to have all 40 survival curves on one plot, we chose to pool the data of each group, day by day. Considering that all mosquitoes from each group were

reared in the same conditions, with the same experimental conditions, this method allows us to render a representative survival curve for each mosquito line. Over the course of almost 60 days, we were able to observe a significant difference in mosquito survival between the two groups, with KO mosquitoes dying at slower rates than WT mosquitoes (**Figure 20.A**). To be certain that the difference that we observed was not due to a Simpson's paradox (Simpson, 1951), we generated a plot with all curves (approximately 40), and were able to observe the same trend, with a consistent better survival of KO mosquitoes (**Figure 20.B**).

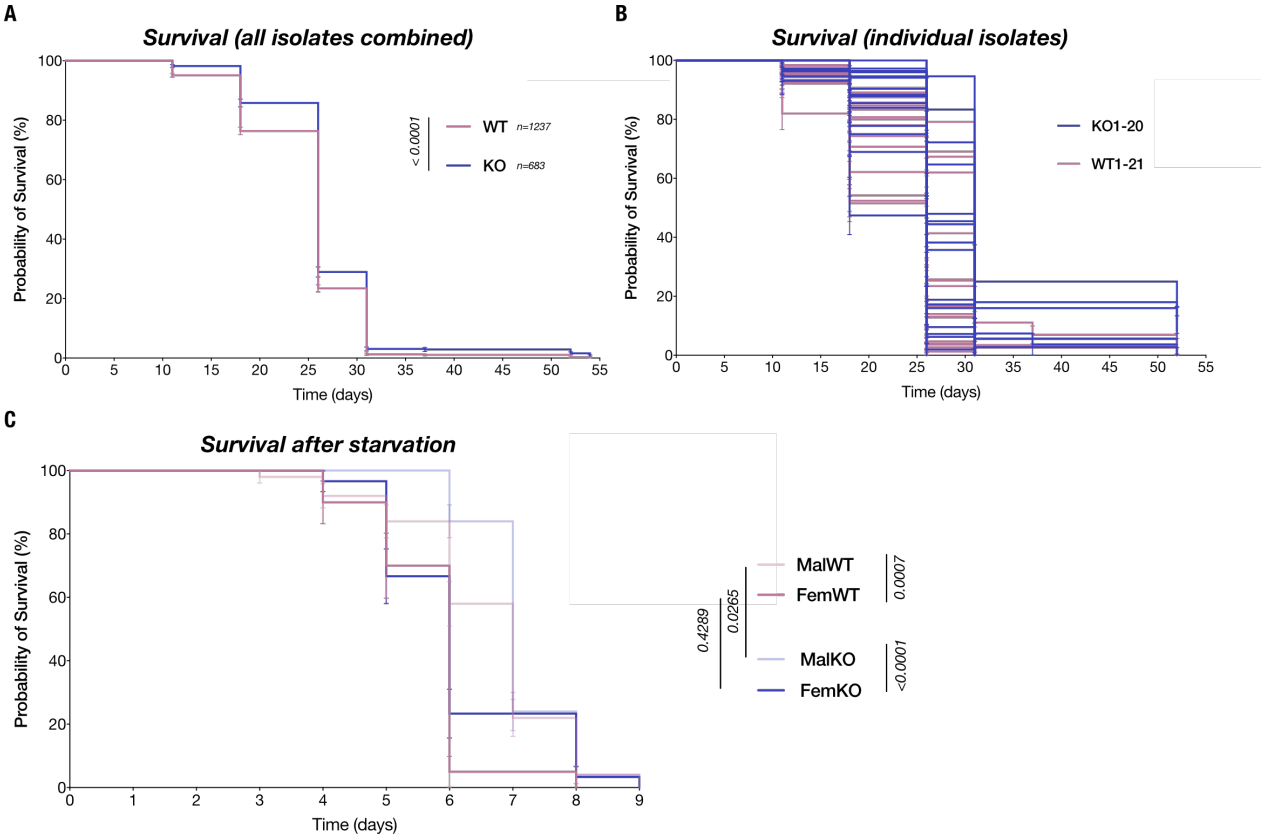


Figure 20. Loqs2 affects survival of mosquitoes, especially when placed under stress.
*[**A**] Kaplan-Meier survival curves of pooled WT mosquitoes in comparison to KO mosquitoes in regular rearing conditions. Total number of mosquitoes in the pool are indicated beside in the legend. [**B**] Kaplan-Meier survival curves of each KO and WT isolates, prior to pooling. [**C**] Kaplan-Meier survival curves of females and males of the WT and KO mosquito lines following starvation. Pairwise comparisons were performed using Gehan-Breslow-Wilcoxon test.*

Finally, we investigated the resilience of mosquitoes from our KO group, by monitoring their survival under stress. We chose to test their resistance to starvation, by depriving them of the sucrose solution they usually feed on. Mosquitoes were sorted by sex and isolated in groups of 5 individuals as soon as they emerged, and offered a water-soaked cotton pad for the duration of the experiment, to avoid dehydration. Mosquitoes survived up to 9 days, during which females from both mosquito lines died earlier than males (**Figure 20.C**). Interestingly, no difference was observed between females of each group, however, males from the KO group showed higher survival rates than those from the WT group. This result supports the data obtained previously, showing a better overall survival of the KO mosquitoes than the WT.

Overall, the phenotypic study of our Loqs2 KO mosquitoes confirmed that Loqs2 likely plays a role outside of immunity, affecting both fertility and survival of the mosquitoes at different stages in their life.

IV. Loqs2 is likely involved in multiple biological processes

We have now demonstrated that although in specific contexts, Loqs2 can present antiviral properties, it would seem that its main function lies elsewhere, and is most likely mainly related to reproduction. In order to gain new insights on this function, and attempt to dissect the mechanisms Loqs2 is involved in, we were interested in investigating the effects of the loss of function of this protein on the transcriptome of ovaries

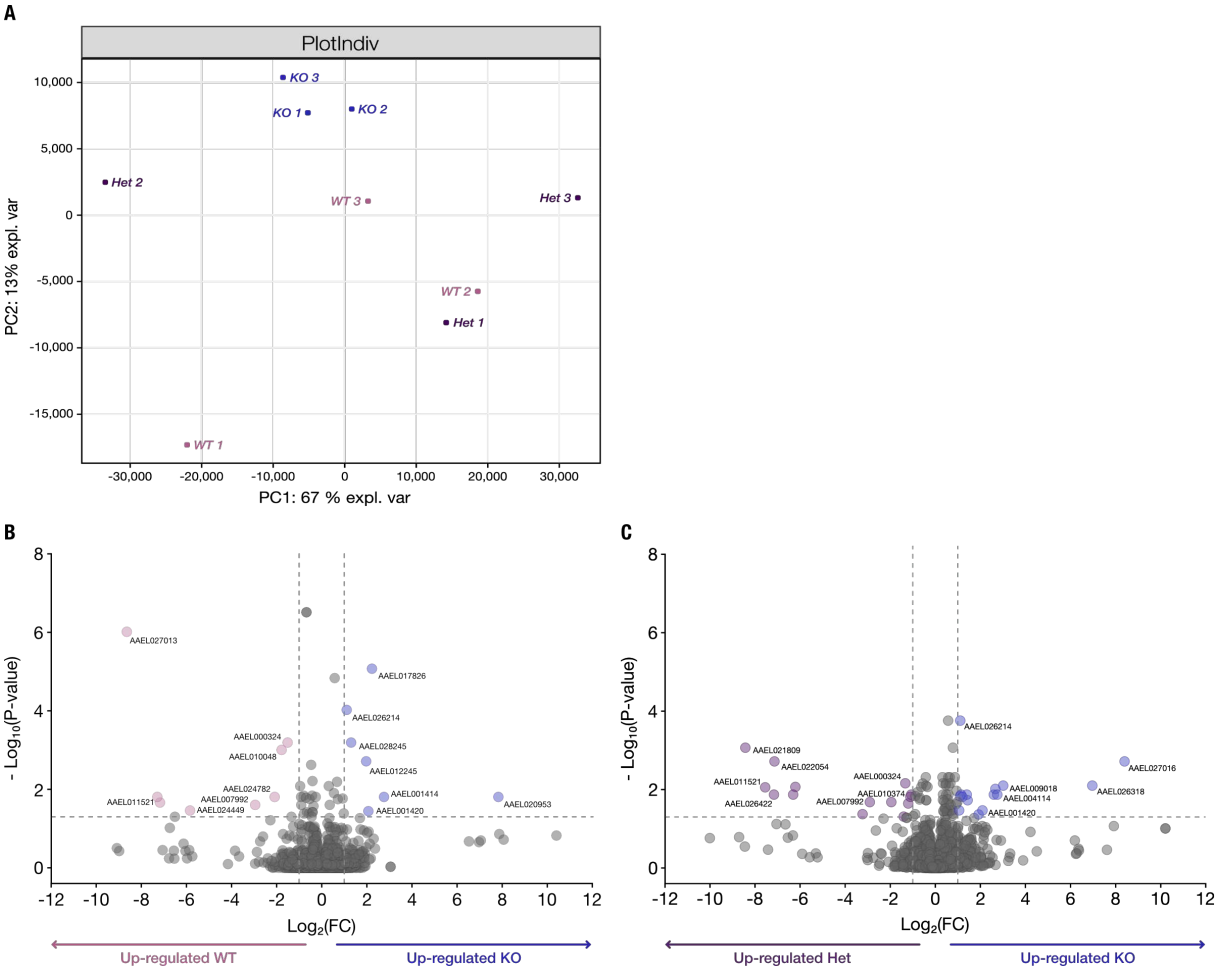


Figure 21. Defects in loqs2 expression do not significantly affect gene expression in ovaries. *[A] Principal component analysis of the triplicates of each library [B] Differentially expressed genes in ovaries from WT (pink) and Loqs2 KO (blue) mosquitoes. [C] Differentially expressed genes in ovaries from Het (purple) and Loqs2 KO (blue) mosquitoes. Colored circles represent significant hits, with a p-value < 0.05 and a Log2FC > 1. Dotted lines represent cutoff values. P-values were corrected using the Benjamini-Hochberg method*

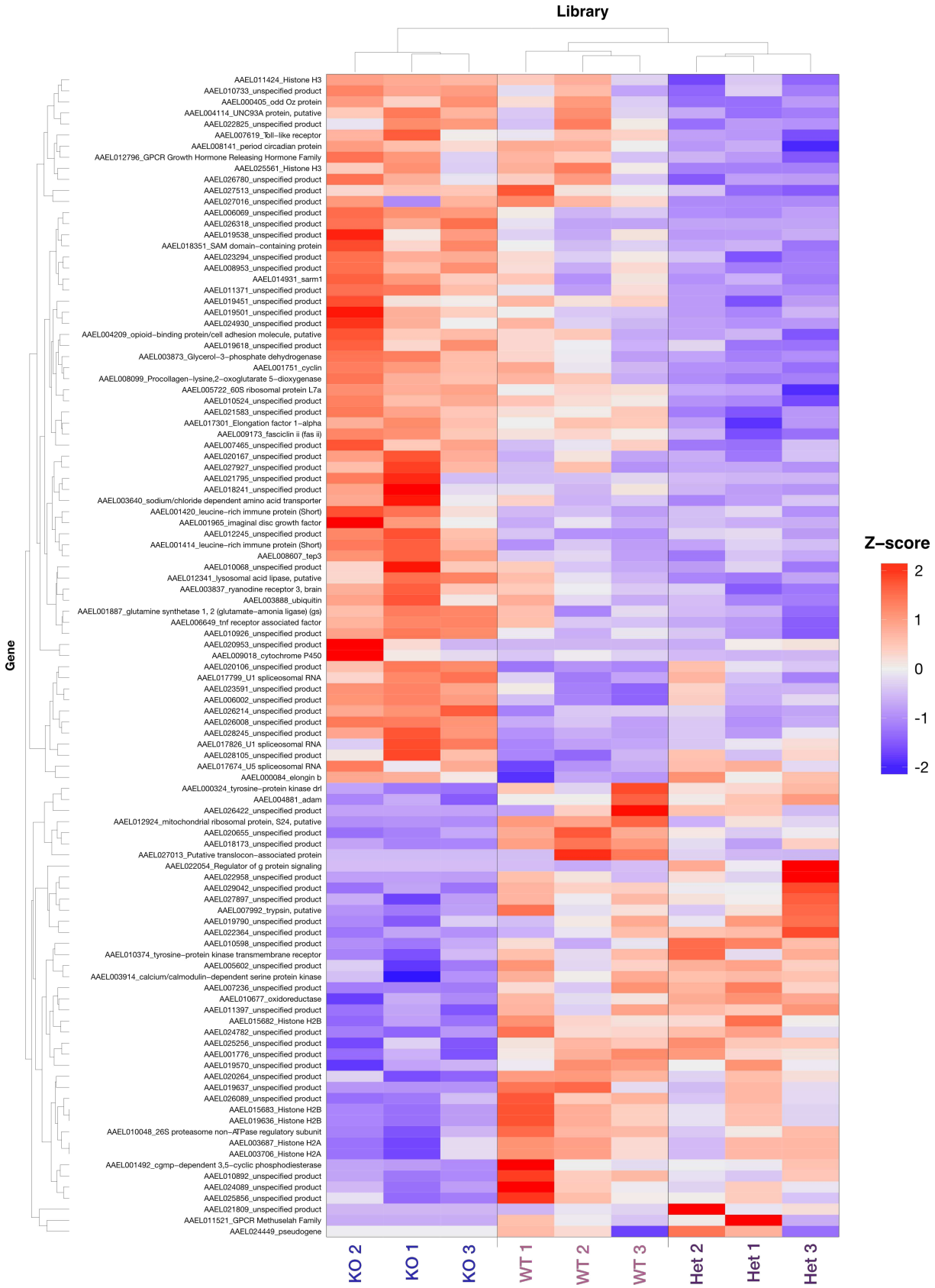


Figure 22. Loss of Loqs2 affects expression of histone genes and spliceosomal RNAs.
Heatmap of the differentially expressed genes between all three conditions. Genes represented here were identified with an adj. p-value < 0.05, in any of the pairwise comparisons between samples. Z-score normalizations indicate up-regulation (darkening red) and down-regulation (darkening blue) of each gene.

To this aim, we generated ribosomal RNA (rRNA) depleted long RNA libraries from pools of ovaries from 3 to 5 days old virgin females of WT, Het and KO genotypes. Genes were considered significantly up- or down-regulated with a FC > 2 and an adj. p-value < 0.05. Overall, our analyses did not show a striking difference between all of the samples (**Figure 21.A**). It seems that only a few genes were affected by the loss of Loqs2, with only 7 and 8 genes being up- and down-regulated, respectively, when compared to WT (**Figure 21.B**), and around double that amount when compared to the transcriptome of Het mosquitoes (**Figure 21.C**). Comparing the difference in expression between the significantly enriched genes in all of these samples, we observed a similar effect on most genes up- and down-regulated in the WT and Het samples, implying that the loss of Loqs2 in heterozygous mosquitoes is compensated by the remaining copy (**Figure 22**). Numerous genes that are differentially expressed in the Loqs2 loss-of-function mutants are uncharacterized, and little is known about them except for a possible nucleic acid binding activity and nuclear localization for most of them. Interestingly, the expression of several genes encoding spliceosomal RNAs seems to be up-regulated in the absence of Loqs2, while expression of several histone genes seems to be decreased when comparing KO and WT genotypes (**Figure 22**). Overall, very few pathways seemed to have been affected by the loss of Loqs2, however among those that have been, several have been shown to have also been disrupted following constitutive overexpression in larvae (**Figure 23**) (Estevez-Castro *et al.*, 2021).

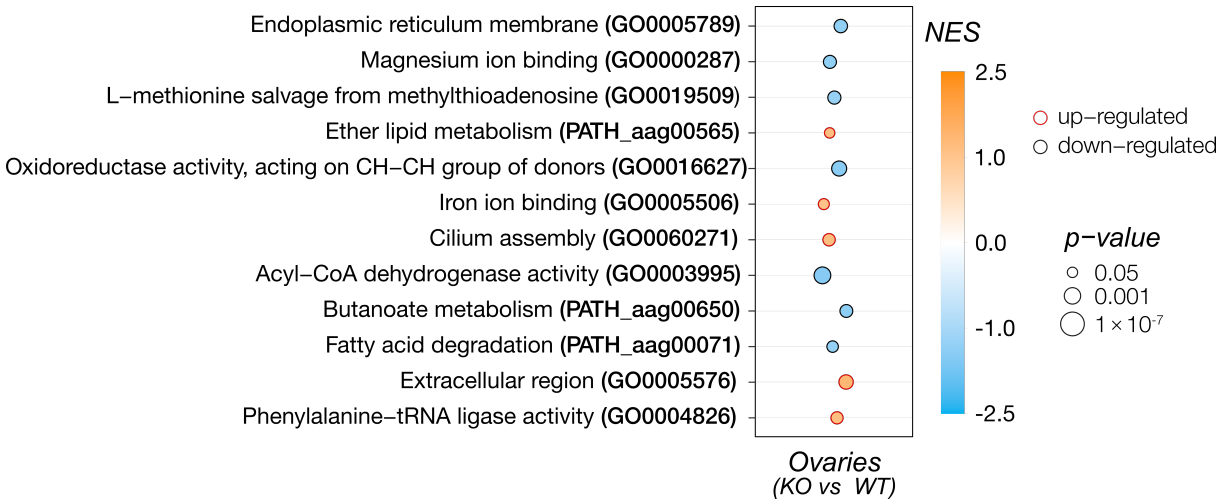
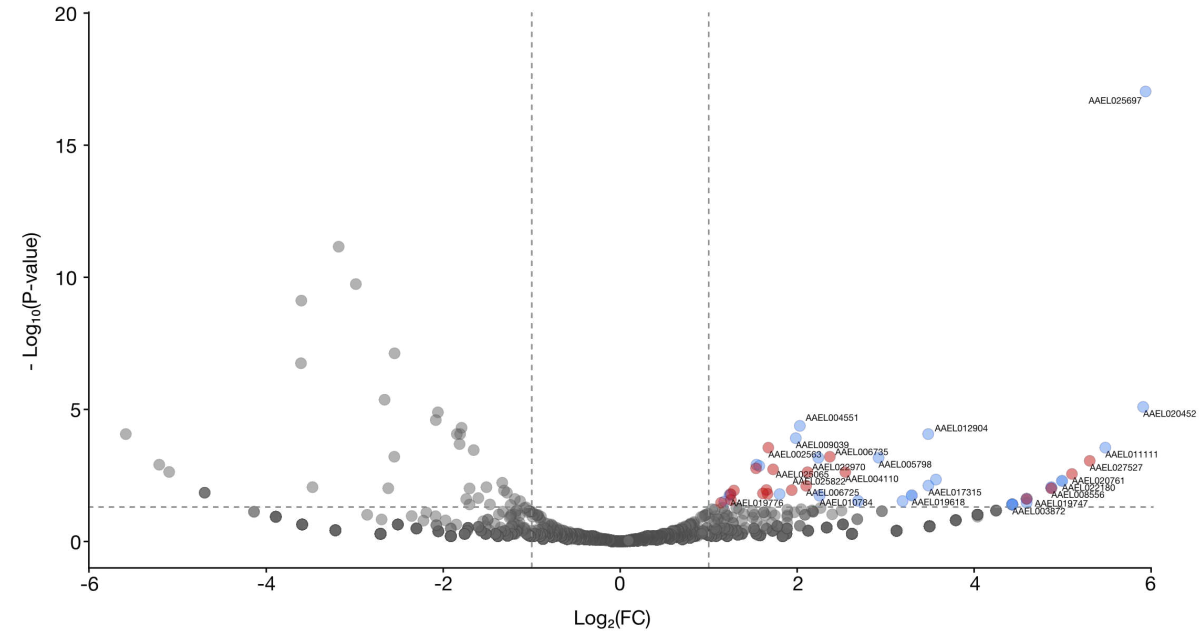


Figure 23. Loss of Loqs2 mildly affects several biological pathways.
Biological processes significantly up- or down-regulated in ovaries of Loqs2 KO mutants, in comparison to WT mosquitoes.

This transcriptome of the ovaries raised questions on the difference between the biological processed in which Loqs2 is involved when ectopically expressed in the midgut, compared to a tissue where it is naturally expressed. Considering these results, and in order to deepen our understanding of the mechanisms by which Loqs2 functions, we further investigated the interactome of this protein in the ovaries.

A



B

Vectorbase ID	Log2FC	adj. p-value	Dmel orthologue
AAEL025762	6,6	7,62E-10	Plp
AAEL025697	5,9	9,29E-18	Hsc70-5
AAEL020452	5,9	8,05E-06	-
AAEL011111	5,5	2,81E-04	Nab2
AAEL027527	5,3	8,85E-04	Adar
AAEL020761	5,1	2,78E-03	CG6686
AAEL022180	5,0	5,08E-03	Cip4
AAEL001612	5,0	5,08E-03	Dcr-1

C

Vectorbase ID	Log2FC	adj. p-value	Dmel orthologue
AAEL027527	5,3	8,85E-04	Adar
AAEL020761	5,1	2,78E-03	CG6686
AAEL008556	4,9	9,68E-03	CG7028
AAEL019747	4,6	2,43E-02	CG31211
AAEL004110	2,5	2,37E-03	Pitslre
AAEL006735	2,4	6,19E-04	Gem2
AAEL022970	2,1	2,38E-03	-
AAEL006725	2,1	7,82E-03	-
AAEL025822	1,8	1,61E-02	scaf6
AAEL025065	1,7	1,86E-03	-
AAEL002563	1,7	2,81E-04	Hpr1
AAEL011744	1,7	1,57E-02	abs
AAEL023617	1,7	1,12E-02	Slu7
AAEL025258	1,6	1,52E-02	barc
AAEL008700	1,6	1,38E-03	Smn
AAEL013985	1,3	1,17E-02	-
AAEL009282	1,3	2,68E-02	CG10907
AAEL003605	1,1	3,40E-02	Sf3b1

Figure 24. Loqs2 interacts with numerous proteins in the ovaries.
*[**A**] Proteins enriched in immunoprecipitates of Loqs2 in ovaries of wild-type mosquitoes. Polyclonal antibodies against FHV RNA-dependent RNA polymerase, prepared and purified by the same company as Loqs2 antibodies, were used as a control on the same protein samples. Blue circles represent significant hits with an adjusted p-value < 0.05 and a Log2FC > 1, and dotted lines represent cutoff values. Red circles represent significant hits under the same criteria as blue circles, that are associated with the splicing mechanisms. [**B**] List of the most highly enriched proteins in this analysis, with a Log2 FC > 5, and their D. melanogaster (Dmel) orthologues. [**C**] List of the 18 proteins involved in splicing among the candidates identified in this analysis (corresponding to the red circles on the Volcano-plot), and their D. melanogaster (Dmel) orthologues (biological processes were inferred using KEGG pathways).*

To this aim, we co-immunoprecipitated Loqs2, from non-vitellogenic ovaries of 3 to 5 days old mated females, using the antibody directed against Loqs2. The control immunoprecipitation was performed on these same protein extracts using antibodies produced by the same company and in the same species as the Loqs2 antibodies,

directed against the viral RNA-dependent RNA polymerase from Flock House virus (FHV). Proteins detected by mass spectrometry were considered significant hits when enriched with a FC > 2 and an adj. p-value < 0.05. Using these criteria, we were able to identify 54 significantly enriched proteins (**Figure 24.A**). Interestingly, from the previous interactome of Loqs2 in the midguts, only Loqs was detected as a significant interactant in this analysis, even though not as strongly. Of note, however, among the protein with the highest enrichment (FC > 10), we were able to identify both Dcr-1 and Adar, two other dsRNA-binding proteins, one of which is also involved in the RNAi pathway (**Figure 24.B**). This interactome of Loqs2 also shows the presence of a large number of proteins involved in the splicing, representing around one third of the total number of candidates (**Figure 24.C**). Interestingly, although we did not initially select them for further characterization at the time, we also detected proteins involved in the splicing process in the list of potential partners of Loqs2 in the gut (**Figure 4.D**).

Among the main interactants and differentially expressed genes discovered in this analysis, and also the protein partners identified in the midguts, we noticed a divergence in the subcellular localization of multiple of those proteins. Being it in these two analyses in ovaries, or in the previous analysis in midguts, several protein interactants of Loqs2 are known to be cytoplasmic, in particular the proteins of the RNAi pathway, while other localize in the nucleus, mainly the proteins involved in the splicing process. Also, our transcriptomic analyses revealed that Loqs2 may be involved in regulating the expression of histones, which would also imply a nuclear localization. Interestingly, although they were not among the most highly enriched proteins, we also identified components of different complexes from the oxidative phosphorylation pathway, localizing in the mitochondria. These observations prompted us to investigate the localization of Loqs2 in cells, and particularly in cells from ovaries, considering its naturally high expression in this tissue.

To this aim, we performed an immunostaining of Loqs2, using the specific antibodies directed against the protein. Ovaries were collected from 3 to 5 days old virgin females, that did not receive a bloodmeal. The oogenesis was thus arrested at the pre-vitellogenic stage, with primary egg chambers containing only 7 nurse cells and an oocyte, surrounded by around 300 follicle cells (**Figure 25.A**) (Valzania *et al.*, 2019). No fluorescence was detected in the ovaries of our KO individuals, while it was highly detected in the wild-type cells, confirming again the absence of Loqs2 protein in the KO line (**Figure 25.B**). In the wild-type cells, Loqs2 seems to be expressed in all cell types in the ovaries, and to be diffused in the cytoplasm with a possible concentration at the nuclear periphery. It does not seem to localize to granules, as shown for multiple proteins of the RNAi pathway (Nishida *et al.*, 2013), and does not seem to enter the nucleus. Nevertheless, these are preliminary results that should be further analyzed.

Overall, these results confirm that in natural conditions, Loqs2 does not seem to interact with proteins related to immunity, and that the antiviral role of Loqs2 that we described previously is likely restricted to the midgut, when the protein is ectopically expressed in this tissue. Our findings also suggest that Loqs2 is a versatile protein, capable of interacting with partners involved in various biological processes, in multiple

organelles, and that it likely plays an important role in the regulation of gene expression, adding yet another layer of complexity to this already peculiar protein.

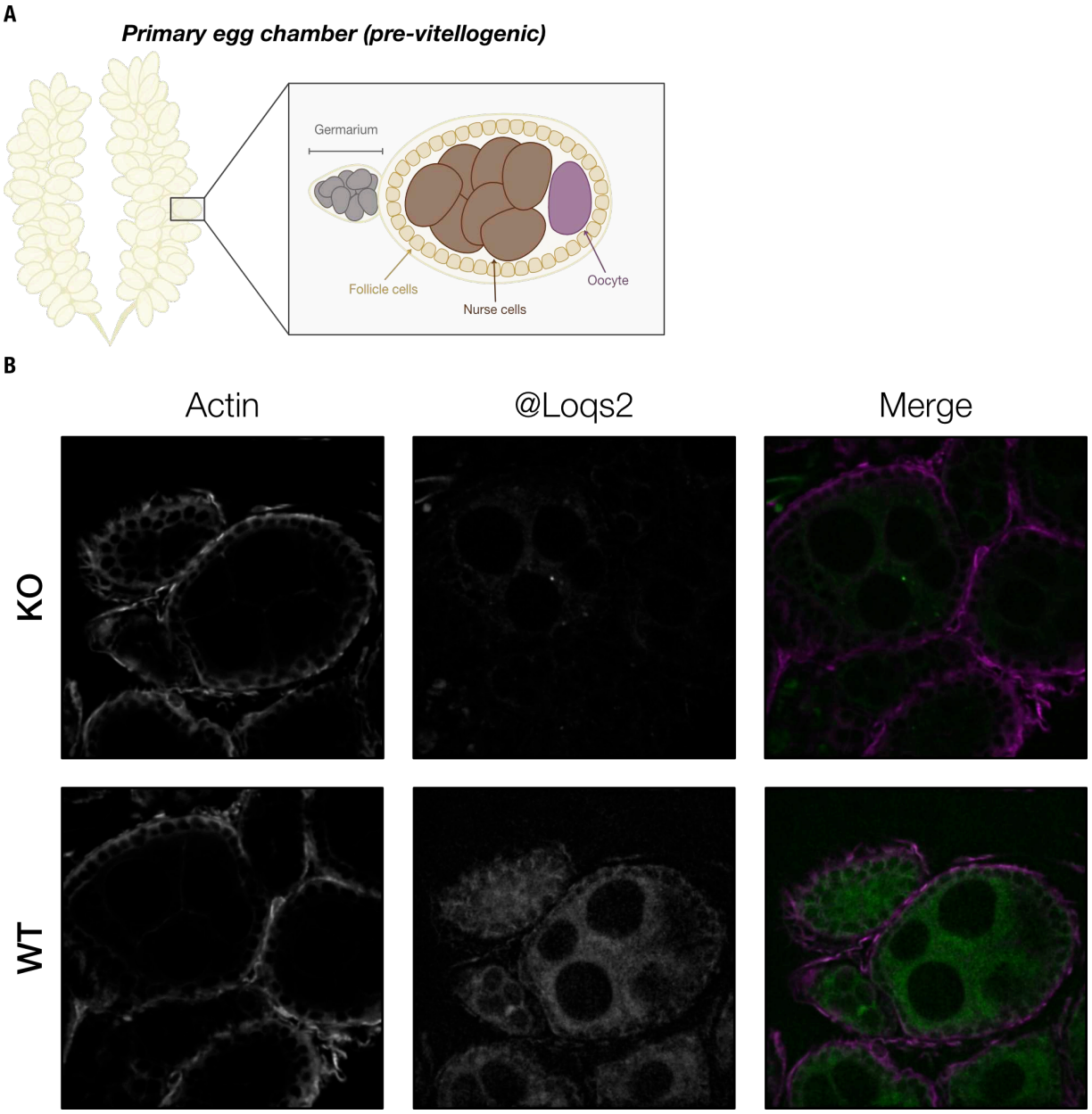


Figure 21. Loqs2 is expressed in the cytoplasm of all somatic and germ-cells in ovaries.
*[**A**] Schematic representation of a primary egg chamber in the pre-vitellogenic stage. The oocyte and 7 nurse cells are surrounded by around 300 follicle cells. At the top of the egg chamber is the germarium, containing stem cells (adapted from Valzania et al., 2019). [**B**] Immunostaining of the ovaries of WT mosquitoes. Ovaries of Loqs2 KO mosquitoes were used as a negative control. Phalloïdin was used to stain actin (pink), and Loqs2 (green) was detected using antibodies directed against the protein. Oocytes are not visible on these pictures, only nurse cells, follicle cells and stem-cells from the germarium are visible.*

Discussion

I. Evolution and origin of Loqs2

The siRNA pathway in *Drosophila* relies on the ability of a specific isoform of the Loquacious co-factor of Dcr-2, Loqs-PD, to discriminate between dsRNA of exogenous origin, and dsRNA of viral origin (Marques *et al.*, 2013). Despite the absence of this isoform in mosquitoes, it was recently shown that the siRNA pathway is still able to distinguish dsRNAs of viral origin from other types of dsRNAs, although the factors involved in this process are not identified yet (Olmo *et al.*, 2018). The discovery of *loqs2*, a new *Aedes*-specific dsRBP, paralog of *loqs*, that is not expressed in the tissue where the processing of virus-derived dsRNAs is compromised was a first step in shedding light on this process (Olmo *et al.*, 2018).

Trying to uncover the processes underlying the appearance and stabilization of *loqs2* in the genome of *Aedes* mosquitoes, we were able to date its origin around 67 MYA, coinciding with the branching of the *Stegomyia* subgenus (Estevez-Castro *et al.*, 2021). This event would be considered to have occurred recently, on the mosquito evolutionary scale, being that the radiation with *Drosophila* took place during the Permian period, around 273 MYA. However, the radiation between the two genera *Aedes* and *Psosophora* dates back to the Creataceous period, only 102 MYA, implying that *loqs2* appeared relatively fast in the *Aedes* genus (Reidenbach *et al.*, 2009; da Silva *et al.*, 2020; Estevez-Castro *et al.*, 2021). The absence of publicly available, or quality genome sequences for numerous other non-medically important mosquitoes of this genus hindered a further specification of the origin of *loqs2*. Indeed, we were able to show that *loqs2* originated from two independent duplication events of the first dsRBD of *loqs*, on the first chromosome. Having access to quality genomic data of other mosquitoes, closely related to the *Stegomyia* subgenus, may allow us to determine how these events took place.

Interestingly, as mentioned above, our analyses led to the demonstration that *loqs2* originated from two independent duplications of a domain of *loqs*, and not a complete gene duplication. This is quite unusual considering that dsRBPs are mostly products of gene duplication events, followed by a speciation of the gene, and motif duplication events are expected to be relatively rare (Tian and Mathews, 2003; Dias *et al.*, 2017). Also, all dsRBDs do not have the same function, they are subdivided in two groups, with type A dsRBDs, able to bind dsRNA with a great affinity, and type B dsRBDs, showing a very decreased affinity for dsRNA, but which are rather involved in protein-protein interactions (Krovat and Jantsch, 1996; Hitti *et al.*, 2004; Chang and Ramos, 2005). Numerous dsRBPs contain multiple of these domains, and it has been demonstrated that proteins containing multiple type A dsRBDs, such as the Protein kinase R (PKR) in humans, can bind to dsRNA more effectively (Krovat and Jantsch, 1996). Interestingly, in the case of *loqs*, the first domain is of type A, while the remaining two domains are associated to the binding to proteins (Miyoshi *et al.*, 2010). Considering that the two domains of *loqs2* derive from the first domain of *loqs*, this would suggest that the protein may display a high affinity for dsRNA, and that its function may rely on the strength of its binding to this type of template, rather than its ability to interact with other proteins. This difference in the constitution of the gene, with two domains of similar nature rather than two different type of dsRBDs, as well as the little conservation

observed between *loqs2* and *loqs* outside of these domains suggests that the function of Loqs2 now diverges from that of its predecessor.

Our further analyses supported this hypothesis of a diversification of the function of Loqs2. We were able to show that this gene was evolving under a relaxed positive selective pressure, thus letting mutations accumulate freely, and getting fixed in the population when advantageous (Lynch and Conery, 2000; Prince and Pickett, 2002). In this case, the fate of the duplicated gene is usually the acquisition of a new function, that could complement that of the original gene or be vastly different. We were also able to demonstrate that *loqs2* is still evolving fast, suggesting that the gene is still actively diversifying and likely finetuning its function, or that it needs to be able to rapidly adapt to external pressures. In *Drosophila*, several components of the RNAi pathway, namely *dcr-2*, *ago2* and *r2d2*, are among the top 3% fastest evolving genes, at rates similar to what we observed for *loqs2,* and it has also been observed in *Ae. aegypti* (Obbard *et al.*, 2006; Bernhardt *et al.*, 2012). This rapid evolution of RNAi genes is likely forced by their constant interaction with viruses, and the evolutionary arms race that results from this. Numerous viruses encode viral suppressors of silencing (VSR), whose role are to interfere with the RNAi response and to escape the antiviral defence. In the face of that, there is a strong need for the components of this response to adapt and counter the action of these VSRs (Marques and Imler, 2016). Although the possibility remains that the fast evolution of *loqs2* could also be driven by this arms race with viruses, it is more likely a result of its potential involvement in the other biological processes that Loqs2 seems to affect.

In conclusion, our results demonstrate the unusual origin of *loqs2*, from two independent domain duplication events, rather than a single complete gene duplication. We also demonstrated that this gene was evolving under a relaxed selective pressure, likely allowing it to diversify its function. Finally, we showed that *loqs2* was still currently evolving rapidly, suggesting that it is still actively diversifying, and which could also imply a necessity for rapid adaptation. Overall these results emphasize the importance of further characterizing the function of Loqs2.

II. Characterization of Loqs2 antiviral activity

Altogether, our results suggest that Loqs2 plays a role in antiviral defense. It has indeed been shown that silencing of the expression of this gene followed by an infection with DENV led to an increased viral load in the carcass, and that mosquitoes ectopically expressing Loqs2 in the midgut showed decreased viral load in this tissue, as well as less dissemination than wild-type mosquitoes (Olmo *et al.*, 2018). These experiments were the basis of our hypothesis that Loqs2 is an antiviral protein, and we aimed at further characterizing this protein, and understand the mechanisms by which it can be involved in this process.

Olmo and colleagues initiated this in-depth study of the function of Loqs2, by identifying 79 potential partners of Loqs2, in *Ae. aegypti* Aag2 cells overexpressing the protein, among which the highest hits were the two main co-factors of the siRNA pathway, Loqs and R2d2 (Olmo *et al.*, 2018). This further corroborated our assumptions

that Loqs2 was strongly related to the antiviral defence, and in particular to the RNAi pathway. However, it is often challenging to interpret results from experiments performed in this particular cell line, considering that it was originally generated from a pool of embryos, in which gene expression is vastly different from that of individual tissues of adult mosquitoes (Peleg, 1968). Therefore, potential partners identified through this experiment might not represent the actual range of partners interacting with Loqs2 in the context of its antiviral function.

This work allowed us to gain a significant insight into the answer to this question, by focusing on the study of the protein *in vivo*. Our experiments were conducted using the same mosquitoes as those used to demonstrate the antiviral properties of Loqs2. These mosquitoes overexpressed a tagged version of the Loqs2 protein, under the control of the blood meal-induced CP promoter in the midgut. Combining the analyses of Loqs2 interactome from the co-immunoprecipitation of the protein in washed midguts, and in whole mosquitoes, we were able to generate a short list of highly likely interactants of Loqs2. As expected from the previous analysis, Loqs was identified as the main interactant, but although we did not detect R2d2 in this analysis, we were still able to detect Dcr-2. Among the 15 candidates identified in this experiment, we chose to focus on the characterization of only a few of them to begin with, and later on, to continue with the rest of the list. The proteins that were selected as candidates were both proteins of the RNAi pathway, Loqs and Dcr-2, two dsRBPs with orthologues in *Drosophila*, AAEL004859 (orthologue of mle) and AAEL012964 (orthologue of Dip1), and finally two unknown proteins, unique to *Aedes* mosquitoes, or to *Culicinae*, AAEL004699 and AAEL022113, respectively. In the time between these analyses and the writing of this manuscript, AAEL004699 was shown to be antiviral in *Ae. aegypti* cells, and was renamed aBravo (Varjak *et al.*, 2020).

Of note, this analysis was performed 24 h following a bloodmeal, this timepoint showing the highest expression of Loqs2 in our mosquitoes. Interestingly, as observed through the data of the *Aedes aegypt*i atlas, developed by Nicolas Buchon's lab, the expression of numerous immunity-related genes is lowered at that time, only to increase again to higher levels after 48 h (Hixson *et al.*, 2022; http://aegyptiatlas.buchonlab.com). In more details, for example, these data show an almost 2-fold decrease of the expression of *loqs* in the midgut, 24 h after a bloodmeal, followed by an increase in the expression, twice as high as the original expression level, after 48 h. Looking at the other candidates that we selected, namely Dip1, mle, AAEL022113 and aBravo, the same trend can be observed, with an important decrease in expression at 24 h and a large increase at 48 h. The high enrichment observed for these proteins in our analyses, despite their lowered expression at the timepoint we selected emphasizes the strength of their interaction with Loqs2. It would be interesting to repeat this experiment at a later timepoint, 48 h after the bloodmeal, as an example, to monitor the effects of the increase in expression of these candidates on their interactions with Loqs2.

In addition to the key components of the siRNA pathway, Loqs and Dcr-2, whose antiviral function has already been established, the other candidates were also selected based on their potential as antiviral factors. Dip1, whose primary role has been described as the regulation of gene expression during development, has also been shown to display

antiviral properties against DCV in *D. melanogaster* (DeSousa *et al.*, 2003; Bondos *et al.*, 2004; Catanese and Matthews, 2011; Zhang *et al.*, 2015). In mosquitoes, the protein has recently been detected in the interactome of DENV 3' UTR, and NS1 protein in cells, suggesting a role in antiviral immunity as well (Besson *et al.*, 2022; Yeh *et al.*, 2022). Similarly, mle has also been detected as a potential interactant of DENV 3' UTR in mosquito cells, and has been shown to potentially interact with Ago2, also suggesting an involvement in the immune response, as it is the case for its human orthologue RHA/DHX9 (Cugusi *et al.*, 2015; Baldaccini and Pfeffer, 2021; Yeh *et al.*, 2022). *Aedes*-specific protein aBravo has also recently been identified as an interactant of Dcr-2, and displayed antiviral properties against SFV, CHIKV and ZIKV in *Ae. aegypti* cells (Varjak *et al.*, 2020). Finally, the last selected candidate, AAEL022113, was not described in the literature, however, its peculiar features, such as the presence of RNA-binding motifs, and its specific expression pattern in mosquito tissues reminded of those observed for Loqs2, and prompted us to further characterize this protein as well.

We were able to confirm the antiviral properties of mle in *Ae. aegypti*, and its involvement in the control of viral replication in the midgut, however, its role in the control of systemic infection remains to be determined. We also characterized a first role for the previously unknown AAEL022113 protein, by demonstrating its ability to regulate viral replication in the midgut of *Ae. aegypti* mosquitoes. These experiments allowed us to demonstrate their individual antiviral function, and it would now be needed to decipher their potential involvement in the antiviral function of Loqs2, by silencing the expression of these genes in mosquitoes ectopically overexpressing Loqs2 in the midgut. Also, our analyses on the interactome of Loqs2 was performed on non-infected mosquitoes. Considering all the changes that precede or follow the infection of cells by a virus, it is likely that Loqs2 partners vary in this context, and identifying the changes in its interactome when facing the threat of an infection could help us better understand the mechanisms by which Loqs2 acts in the antiviral response. Investigating the RNA targets of Loqs2 could also give us valuable insight to work towards elucidating its role and the biological processes it is involved in.

The aim of this work was also to determine if this antiviral function that we observed extended to the tissues where Loqs2 is naturally expressed. To this aim, we generated loss-of-function mosquitoes, in which the expression of the *loqs2* gene is disrupted. Using this new tool, we were able to investigate the involvement of Loqs2 in the antiviral response in other tissues. We showed that in mosquitoes infected with ZIKV through a bloodmeal, following the natural route of infection, Loqs2 does not seem to play a role in the antiviral defence, in any of the tissues tested. Our results also indicate that even in the case of direct systemic infection, following infection of viruses in the thorax of mosquitoes, Loqs2 did not control viral replication or dissemination to other tissues at most timepoints. In this particular case, the absence of response by Loqs2 could be explained by the fact that bypassing the midguts prevents the priming of the immune response in the early stages of infection, and that the absence of interactions between the virus and the gut microbiota led to a different infectivity of the former (Moreira *et al.*, 2009; Joyce *et al.*, 2011; Oliveira *et al.*, 2011; Apte-Deshpande *et al.*, 2012; Ramirez *et al.*, 2014). This in turn could suggest that Loqs2 may depend on this priming of the immune response to itself be able to function antivirally. This hypothesis could

be tested by offering a non-infectious blood-meal to the mosquitoes, prior to injecting the virus in the thorax and monitoring the antiviral response by Loqs2.

Interestingly, at the latest timepoint tested in our experiments of direct systemic infection, 12 days after the injection of ZIKV, we were able to see a slight, but significant decrease of the viral load, in mosquitoes lacking Loqs2, which would suggest that Loqs2 also has pro-viral properties in this context. These results seem to contradict the ones we previously obtained through the silencing of Loqs2, which showed an antiviral function of Loqs2 in the carcass of mosquitoes. There are multiple explanations for this, the most likely one being the establishment of compensatory mechanisms, due to the absence of Loqs2 throughout all developmental stages, while in the silencing experiments, mosquitoes were deprived of Loqs2 only transiently, at the adult stage. It is thus possible that these compensatory mechanisms take a toll on the immune system, to place the integrity of the organism first. In addition, also seemingly contradictory, it would not be impossible for a protein to have both pro-viral and antiviral properties. It has indeed recently been observed for the RNAi co-factor Loqs, which is known to participate in the antiviral response, but has also been shown to interact with DENV genome and proteins, in ways that promote viral replication, independently of its role in the RNAi pathway (Besson *et al.*, 2022; Shivaprasad *et al.*, 2022). It would be interesting to further investigate the effects of the absence of Loqs2 on the other immune pathways involved in the antiviral defence. Indeed, it was discovered in *D. melanogaster* that Dcr-2, although mainly involved in the siRNA pathway, can independently trigger the expression of an antiviral peptide named Vago, which has in turn been shown to activate the JAK-STAT pathway in *Culex* mosquitoes, thus leading to the control of WNV infection (Deddouche *et al.*, 2008; Paradkar *et al.*, 2012). It remains possible that the absence of antiviral effect, or possible pro-viral effect, is due to a similar mechanism, in which Loqs2 is involved in the regulation of another immune pathway.

Considering that the tissues where Loqs2 shows the highest expression were the reproductive tissues, we also investigated the possibility that this protein was involved in the control of vertical transmission. It has been shown that *Ae. aegypti* mosquitoes have the ability to transmit arboviruses, such as DENV and ZIKV, to their progeny (Sánchez-Vargas *et al.*, 2018; Lai *et al.*, 2020; Manuel *et al.*, 2020). Although highly variable, depending mostly on experimental procedures, these rates were consistently quite low. We chose to work with two insect-specific viruses, HTV and PCLV, to test this hypothesis. These viruses are found in nature, infecting most of the populations of *Ae. aegypti*, and are vertically transmitted (Olmo *et al.*, 2023). We were able to show that although not affecting the overall viral load in the progeny, the absence of Loqs2 seemed to affect the prevalence of infection. For both viruses, we observed twice as many progenies carrying the viruses in the line lacking Loqs2, compared to the wild-type line. We assume that the statistical tests performed did not consider this difference significant due to the small sample size, and these experiments will have to be repeated in order to confirm our observations.

In conclusion, our work allowed us to demonstrate that Loqs2 interacts with multiple partners, around half of which have been shown to be involved in the antiviral response, when ectopically expressed in the midgut, where it itself shows antiviral

properties. This study of the interactome of Loqs2 also allowed us to identify and start characterizing a new promising antiviral protein, specific to *Culicinae*. We were able to show, however, that the antiviral function of Loqs2 does not seem to extent to most of the other tissues, in the context of arboviral infection, although it would be interesting to deepen these studies by investigating other important tissues where the protein is expressed, such as the salivary glands and the fat body. Finally, our results suggested the possibility that Loqs2 naturally functions in the preservation of the germline, being potentially involved in the control of vertical transmission. This feature is an aspect of Loqs2 function that needs to be further explored, and could be the focus of a future work.

III. Natural function of Loqs2

As we were able to demonstrate that the antiviral function we observed for Loqs2 when ectopically expressed in the midgut does not seem to extend to the tissues where Loqs2 is naturally expressed. Considering the usual variety of functions observed for dsRBPs, another aim of our work was to investigate and characterize other possible roles for this protein, in the context of its natural expression.

We observed that *loqs2* expression levels vary greatly between all tissues, with the highest expression in ovaries and fat body, followed closely by early embryos, while the expression levels drop significantly from late embryos to adulthood (Akbari *et al.*, 2013; Matthews *et al.*, 2016; Estevez-Castro *et al.*, 2021). Interestingly, it was also observed that overexpressing *loqs2* under the control of a ubiquitous promoter in mosquitoes led to developmental arrest at the larval stage, impairing the pupation (Estevez-Castro *et al.*, 2021). Analyses of the transcriptome of these larvae revealed an imbalance in the metabolism, and no major changes in the abundance of small RNAs derived from the RNAi pathway was observed (Estevez-Castro *et al.*, 2021). These results did not point towards a clearly defined role for Loqs2 in causing this developmental arrest, and the study of the abundance of small RNAs (miRNAs, siRNAs and piRNAs) did not highlight any significant changes, thus suggesting that this function would not be dependent on the RNAi pathways (Estevez-Castro *et al.*, 2021).

To further characterize the function, we developed a new method to generate KO mosquito lines, to be added to the repertoire of tools available for the study of *Aedes* mosquitoes. Two methods, using the CRISPR-Cas9 technique were previously described in the literature, relying either on the injection of sgRNA directed towards the target gene in addition to a purified Cas9 protein, or in transgenic mosquitoes already expressing the Cas9 protein in the germline (Kistler *et al.*, 2015; Li *et al.*, 2017). We worked towards developing a new approach to the generation of KO mutants, still based on the CRISPR-Cas9 method, that would bypass the limiting amounts of injected material, and risk of their degradation before they could reach their target. We designed constructs expressing the Cas9 protein under the control of the Exu germline promoter, as well as 3 sgRNAs under the control of different U6 promoters, and a fluorescence marker and inserted them into the genome of our mosquitoes by transgenesis. Having this fluorescence marker allowed us to do a first selection in the G0, by selecting only the larvae showing transient expression, and to directly intercross them. In the following

generation, we were able to use fluorescence to select mosquitoes expressing the construct, and genotype those to find mutants. These two rounds of selection before the genotyping step allowed us to considerably reduce the number of mosquitoes to genotyping before we could find one in which the gene would be knocked-out on both alleles. Further crosses allowed us to rid the mosquitoes of the construct, and we were left with a KO line, ready to be characterized. We found that this method required less resources, and was less time-consuming than the ones we previously experimented, and we were able to use this approach to generate Loqs2 KO mosquitoes.

This new tool that we generated was substantial for the characterization of the function of Loqs2. First, we were able to show that the KO was consistently transmitted to the progenies, following mendelian inheritability, in females, with around 20 % WT, 50 % Het and 30 % WT, although these rates seemed to start varying slightly in the later generations, with the rate of KO increasing lightly. In males, however, inheritability rates were radically different, and we were surprised to find only 2 % of KO. A possible hypothesis to explain this sex-bias would be the genomic localization of *loqs2*. This gene is indeed located on the first chromosome of *Ae. aegypti*, in very close proximity to the m/M sex determining locus, a 63-Mbp region containing multiple sex-specific genes such as *myo-sex* and *Nix*, and known for showing close to no recombination (Fontaine *et al.*, 2017). In order to preserve this region, it is likely that the recombination rates of genes located in its close proximity is disturbed as well, compared to the rest of the genome. The fact that our line was generated through an initial cross of KO females to wild-type males is likely to explain the decreased number of males carrying the mutations in this context. Interestingly, however, in the following generation that was genotyped, there was a 4-fold increase in the percentage of KO males in the population. The increase in the number of males carrying this mutation could in turn explain the slightly higher number of females with the same genotype. It would be necessary to continue monitoring the evolution of the inheritability of this KO over the following few generations, in order to assess the stabilization of KO mosquitoes in the population.

Corroborating this hypothesis, we were also able to show an increased survival of mosquitoes lacking Loqs2, compared to wild-type mosquitoes, suggesting that Loqs2 might have a detrimental effect on the survival of mosquitoes. Of note, in our first trials, due to the extremely low number of homozygous KO males, we were not able to generate a homozygous KO line. Considering the sex bias that we had observed, we decided to generate our KO line by crossing homozygous KO females with heterozygous males, so that we could retain homozygous KO females over the generations, and likely increase the proportion of KO males in the population over time. For the aforementioned experiment, the survival of mosquitoes was assessed as a whole, and we did not separate the individual survival of males and females. Keeping this in mind, we don't know at the moment if this increased survival is imputable to a specific sex and their corresponding genotype, but we suspect that this survival is overall a consequence of the absence of Loqs2. It would still be necessary to generate a line in which both males and females are homozygous, in order to confirm these results.

We were also able to demonstrate a better resistance to a specific type of stress in mosquitoes lacking Loqs2. For this particular experiment, we separated mosquitoes

by genotype and sex, and monitored their survival to starvation individually. We saw a striking difference between the survival of KO males and the other groups, with these mosquitoes showing an increased resistance to this particular stress, although no mosquito survived more than 10 days in this condition. These results, again suggest a detrimental action of Loqs2 on the fitness of mosquitoes, which raises the question as to how a gene with such a fitness cost could have been selected and become fixated in the population in the first place. It would be interesting to assess the resistance of those mosquitoes to other types of stress, such as drought, temperature changes and also their resistance to different types of insecticides. It is possible that although Loqs2 seems to be taking a toll on their ability to resist to the stress that we placed them under, it would be the opposite under a different type of stress.

To further characterize the function of Loqs2, we investigated the effect of the loss-of-function mutants on the development of the mosquitoes. We were able to demonstrate a significant difference in the fertility of the mosquitoes lacking Loqs2, in which we observed 20% less progeny than in the wild-type, despite no significant difference in the number of eggs laid per female. This seems to indicate that Loqs2 is actively involved in the development of eggs, as already suggested by high levels of *loqs2* mRNA detected in the early embryos, indicating a possible maternal deposition (Akbari *et al.*, 2013; Estevez-Castro *et al.*, 2021). This importance of Loqs2 in the oogenesis reminds that of another dsRBP, Staufen, which has been shown to be is essential to the determination of the egg polarity, through the transport of specific mRNAs from one pole of the egg to the other (Johnston *et al.*, 1991; Tian *et al.*, 2004). Although we do not know at the moment the mechanisms by which Loqs2 is involved in the fertility, it is still clear that this protein plays an important role in this process. Also, it has been shown that viral infections had a toll on different aspects of the life of mosquitoes, from their overall survival, to their fecundity (Styer *et al.*, 2007; Kramer and Ciota, 2015). It is possible that Loqs2 functions in a way that allows the mosquitoes to counterbalance this cost of the infection.

Following the development of these mosquitoes into adulthood, we were also able to demonstrate a slight reduction in the survival of the mosquitoes lacking Loqs2, hinting towards a possible involvement of this protein at later developmental stages. In this case most death events occurred either at the transition between the larval and the pupal stage, or at the transition to adults. These results remind the observations made in mosquitoes overexpressing *loqs2* constitutively, in which development was arrested at the just prior to pupation (Estevez-Castro *et al.*, 2021). In this case, it was shown that this interruption of the development was likely due to a metabolic imbalance, although the actual role of Loqs2 in this process was undetermined. Further investigations on the possibility that the loss of Loqs2 triggered the same type of response as its overexpression in the mosquito would be required. This phenotype was however not expected, considering that Loqs2 is only expressed at very low levels during development, and that its expression does not seem to be affected during the different phases (Harker *et al.*, 2013; Estevez-Castro *et al.*, 2021).

We were able to demonstrate that the natural role of Loqs2 seemed to be rather oriented towards development rather than the antiviral response, which was also

corroborated by our analyses of the interactome of the protein in the ovaries. We did not identify any known antiviral protein among the potential partners of Loqs2 in this tissue, except for Loqs, although it was not as highly enriched as in our analysis in the midgut. Our analyses also showed that approximately a third of the candidates identified were involved in splicing, a few of them being among the most significant and most enriched interactants. Some proteins involved in this biological process were also identified in our analyses of Loqs2 interactome in the midgut. Interestingly, it has been shown that some proteins with antiviral functions are involved in the splicing, such as Adar and mle, which were identified as partners of Loqs2 in its interactome in the ovaries and in the midgut, respectively (Cugusi *et al.*, 2015; Deng *et al.*, 2020). Also, in *D. melanogaste*r, Dcr-2 has been shown to interact with spliceosomal proteins, such as Syp, yps and nonA, whose orthologues were detected in our interactome of Loqs2 in the midgut (Rousseau, 2022). It would be interesting to further characterize the interactions of Loqs2 with this pathway, and investigate any potential effect it could have on its antiviral functions. Towards this aim, the transcriptome of our Loqs2 loss-of-function mutants could be studied, to identify any defects in splicing.

Loqs2 seems to interact with a large variety of proteins, and in particular, it seems that some of these potential partners mainly localize in different cellular compartments, with partners of the RNAi pathway localizing mainly in the cytoplasm, while spliceosomal proteins are rather nuclear. We were able to investigate the cellular localization of Loqs2 *in vivo*, in the ovaries, using specific antibodies. These first results suggest that Loqs2 localizes mainly in the cytoplasm, mostly in the nuclear periphery. Although this experiment was preliminary and did not allow us to have a great resolution on the exact subcellular localization, the absence of detection in the nucleus was clear, raising the question of how Loqs2 could be interacting with nuclear proteins. In *Ae. aegypti* Aag2 cells, on the contrary, it was shown that when overexpressed, Loqs2 was rather nuclear (Estevez-Castro *et al.*, 2021). Further studying the sequence of Loqs2, bioinformatic tools allowed us to identify predicted nuclear localization signals, suggesting that Loqs2 indeed has the ability to enter the nucleus. The difference observed in the localization of Loqs2 between those two conditions, *in vivo* and *in cellulo*, could be explained, in part, by the difference of expression levels, considering that the protein was overexpressed in cells, while it was expressed at physiological levels in the ovaries. Another hypothesis would be that Loqs2 can indeed enter the nucleus, under specific conditions, as it is observed for the PIWI proteins. These proteins are found in the cytoplasm until a piRNA gets loaded into them, at which point they are transported to the nucleus, where they can carry their function (Ramos-Nino *et al.*, 2022). It is possible that Loqs2 functions in a similar manner, and needs to be activated in some way, so that it can be transported in the nucleus, and that the conditions were not met for this to happen in our *in vivo* experiment. It would be interesting to further characterize this ability of Loqs2 to potentially localize to different cellular compartments, and identifying its RNA targets could also help understand the mechanisms by which the protein could be activated.

To further specify the role of Loqs2 in the biology of the mosquitoes, we also investigated the effect of the loss of Loqs2 on the transcriptome in ovaries. Our analyses revealed that the absence of Loqs2 seemed to have a very mild effect on gene expression,

with just above a dozen genes being significantly up- or down-regulated, when compared to the transcriptome of the WT line. The same was observed when comparing the transcriptome of Loqs2 KO ovaries to that of the Het mosquitoes, with the expression of only a small subset of genes being disrupted. This would thus indicate that a single copy of Loqs2 could be enough to carry most of Loqs2 functions in the ovaries. Overall, few pathways seem to have been affected by the loss of Loqs2, however, among the ones that were affected, several were also shown to be disrupted by the constitutive overexpression of Loqs2 in larvae, such as the fatty acid degradation pathway, that is down-regulated in both conditions (Estevez-Castro *et al.*, 2021).

Among the genes that have been affected by the loss of Loqs2 in ovaries, comparing to both WT and Heterozygous, we observed numerous uncharacterized genes, most of which seemed to have nucleic acid-binding activity, and to localize in the nucleus, further corroborating the hypothesis that Loqs2 could travel between cellular compartments. Among the characterized gene, whose expression was up-regulated in our KO mutants, we identified several gene encoding spliceosomal RNAs, coinciding with our findings that Loqs2 possibly interacts with proteins involved in splicing in ovaries. Also, we observed the down-regulation of multiple histone genes in the mutants lacking Loqs2. These results imply a potential role for Loqs2 in the regulation of the expression of these genes, although our analyses of the interactome of the protein in ovaries did not indicate any significant enrichment of these proteins. Interestingly, histone gene expression, in particular that histone H4, has been suggested to be possibly regulated by the piRNA pathway, with histone-derived piRNAs, harboring a ping-pong signature, having been detected in *Ae. aegypti* Aag2 cells (Girardi *et al.*, 2017). Considering the results previously obtained on the cellular localization of Loqs2, it is possible that its effect on the expression of histone genes is based on a similar mechanism, by which Loqs2 would bind a small RNA and be translocated to the nucleus to regulate the expression of those genes. Further studies on the effects of Loqs2 on small RNAs abundance are required, that would likely shed light on the involvement of Loqs2 in these mechanisms.

Furthermore, histone proteins (H4 and H2B) have been identified among the semen proteins that are transferred in *Ae. aegypti* females during mating (Sirot *et al.*, 2011). At this point, it would be interesting to investigate the protein interactome of Loqs2 in testes to decipher its interaction with this type of proteins. This knowledge could further shed light on the mechanisms by which Loqs2 affects the fertility of mosquitoes, which could also be imputable to an effect of the protein on male reproductive abilities. Histones are also widely expressed during oogenesis, with mRNA levels accumulating rapidly near the end of the process, largely contributing to the pool of maternally deposited mRNA, that are then essential for the early stages of embryogenesis (Anderson, 1980; Ruddell and Jacobs-Lorena, 1985). The effect of Loqs2, increasing the expression of histone genes, in this context of reproduction, could provide a first insight on the advantages that this protein provides to the mosquitoes, possibly giving a first explanation as to why it became fixated in the genome despite its effect on survival.

In summary, we devised a new approach for the generation of loss-of-function mutants in *Ae. aegypti*, that will add to the already available tools for the genetic study of this mosquito. Using this method, we were able to generate loss-of-function mutants of Loqs2, which we used to demonstrate that Loqs2 seems to have a cost for the organism of the mosquitoes, reducing its fitness and resistance to at least one type of stress. We also showed that Loqs2 substantially affected fertility in mosquitoes, although it still remains to be determined by which mechanisms it occurs. Finally, our results allowed us to expend the horizons of Loqs2 functions, by suggesting an important role in the regulation of gene expression, thus giving new perspectives for the further characterization of this protein.

Conclusion

The main focus of this thesis was to delve deeper into the newly discovered *Aedes*-specific dsRBPs, Loqs2. The objective was to study this protein and gain a comprehensive understanding of its function, and to characterize its role in the mosquito biology. By conducting further investigations into Loqs2, we aimed to shed light on its significance, with the ultimate goal of contributing to the development of anti-viral strategies.

Through phylogenetic analyses, we were able to determine that the *loqs2* gene originated approximately 67 MYA, likely in the last common ancestor of the *Stegomyia* subgenus. We also demonstrated that the gene originated from two independent duplication events of the first domain of *loqs*, a key co-factor of the RNAi pathway known for its involvement in antiviral immunity. Our work further demonstrated that *loqs2* is still evolving rapidly, alongside multiple genes of the RNAi pathway, suggesting a need for rapid adaptation to external threats, such as viral infections, corroborating again the initial hypothesis that Loqs2 is involved in the antiviral defence in mosquitoes.

Although we were unable for the time being to establish a direct connection between Loqs2 and the RNAi pathway through our experiments, we did find further evidence suggesting that it can play a role in the mosquito immune response, under specific conditions. Specifically, we discovered that when ectopically expressed in the midgut, where it has been shown to have antiviral properties, Loqs2 interacts with numerous antiviral protein partners, including other mosquito-specific proteins that still remain to be characterized. However, we showed that this antiviral function does not seem to extend to the tissues where Loqs2 is naturally expressed. Our preliminary results rather hint towards the possibility of an involvement of Loqs2 in the maintenance of germline integrity, through the control of vertical transmission of these viruses.

One important aspect of this work was also the development of a new tool for the genetic modification of mosquitoes. We developed a new approach for the generation of loss-of-function mutants using the CRISPR-Cas9 method, based on the generation of transgenic mosquitoes simultaneously expressing both the Cas9 proteins and the sgRNAs targeting the gene of interest. This method allows for a great efficiency, while requiring less resources and materials, quickening the process of selecting mutants, and can now be added to the continuously expanding list of tools available to study *Ae. aegypti* mosquitoes, complementing other already described CRISPR-Cas9 methods. This approach allowed us to generate a Loqs2 loss-of-function mutant that was substantial for the further characterization of the protein.

Our work also allowed us to start uncovering the potential roles of Loqs2 outside of its antiviral function in the midgut. We were able to demonstrate a substantial effect of this protein in the development of mosquitoes, and in particular, that it played a key role in the fertility. Interestingly, we also showed that this protein had detrimental effects on the mosquitoes, negatively affecting their survival and resistance to at least one type of stress, thus highlighting the multifaceted roles of this protein in the biology of these mosquitoes. Additionally, we discovered that Loqs2 is likely involved in a range of other biological processes, as its interactome in ovaries is very diverse, detecting proteins with various, seemingly unrelated, functions. Finally, analyses of the ovarian

transcriptome of our Loqs2 loss-of-function mutants revealed a very mild effect of the absence of this protein on gene expression, but demonstrated the up-regulation of spliceosomal RNAs, and the down-regulation of histone genes, implying a possible function in the transcriptional or post-transcriptional gene regulation.

In conclusion, this thesis provides valuable insights into the characterization of the *Aedes*-specific protein Loqs2, and its origin, evolutionary history and potential functions in mosquitoes. Overall, this research highlights the potential of Loqs2 as a candidate for dual targeting of mosquito population reduction and reduction of virus transmission. Our work allowed us to start elucidating some of the characteristics of Loqs2, while raising numerous new exciting questions, and further studies are now necessary to investigate the precise mechanisms by which this protein functions, and to fully understand its potential and limitations in the continuous struggle against arboviruses.

Materials and methods

I. Molecular and Cellular Biology

1. gDNA extraction

gDNA was extracted from whole mosquitoes or single mosquito legs using the Phire Tissue Direct PCR Master Mix kit (Thermo Fisher) following manufacturer's instructions, diluting the final product 1:5 for further applications.

Alternatively, gDNA was extracted by adding 0.2M NaOH on the sample, followed by a 10 min incubation at 75ºC. The reaction was then stopped by diluting the sample 1:10 in 0.04M Tris-HCl [pH 7.5].

2. PCR amplification

For applications where high-fidelity was necessary (clonings and dsRNA preparation), the PCR amplifications were performed using the Phusion High-Fidelity DNA polymerase (Thermo Scientific) according to manufacturer's instructions. Correct amplification was then verified by gel electrophoresis, using 1% agarose gels in 1X TAE. Purification of the PCR fragments was then performed differently, depending on the experiment, described in the corresponding section.

For other applications, the PCR amplifications were performed using the DreamTaq™ Green PCR Master Mix (2X; Thermo Scientific), following manufacturer's recommendations. PCR products were then verified by gel electrophoresis, using 2% agarose gels in 1X TAE.

Primers used in this study and their applications are listed on **Table 1**.

3. Cloning for transgenesis

Assembly of plasmids for transgenesis was performed in multiple steps.

Firstly, inserts of interest were cloned into the SmaI or EcoRV site of a pBluescriptSK- (Stratagene) in which the BsaI site had been mutated. A restriction-ligation reaction was performed by mixing the plasmid with the PCR-amplified fragments as well as the restriction enzyme Anza™ 22 SmaI (Invitrogen) or Anza™ Eco32I (Invitrogen) and T4 DNA ligase (Thermo Scientific). Ligation mix was prepared in Anza™ Digestion buffer, supplemented with 1 mM ATP (Thermo Scientific), and incubated at RT for at least 1h. Following inactivation of the enzymes, empty vectors were cleaved by additional SmaI/EcoRV for 30 min.

GoldenGate cloning (Engler and Marillonnet, 2013) was used for the assembly of the final transgenesis plasmid. Destination vector and all plasmids prepared previously were mixed in a concentration of 40 fmol each with Anza™ 36 Eco31I (Invitrogen) and T4 DNA ligase (Thermo Scientific). As previously, the Anza™ Digestion buffer in which the reaction was assembled was supplemented with 1 mM ATP (Thermo Scientific).

Restriction-ligation was achieved by running 3 to 5 cycles of 10 min 37 ºC incubations followed by 20 ºC incubations. Reaction mix was then incubated at 20 ºC for 50 min, followed by enzymatic inactivation at 80 ºC.

Table 1. List of primers used in this study and their applications

Application	Target	Sense	Sequence
Genotyping	Loqs2 KO region	Forward	CCTCGTCCAGTTCCAACGAA
	Loqs2 KO region	Reverse	ACACTCTATAGCGAAACGTC
dsRNA preparation	Loqs Strand 1	Forward	taatacgactcactatagggagaGGCAAATCGAAAAAGGAGGC
	Loqs Strand 1	Reverse	GGATGATCTGGTTCGGTTCC
	Loqs Strand 2	Forward	GGCAAATCGAAAAAGGAGGC
	Loqs Strand 2	Reverse	taatacgactcactatagggagaGGATGATCTGGTTCGGTTCC
	Loqs2 Strand 1	Forward	taatacgactcactatagggagaAGGCCCTAATTGACAAGATG
	Loqs2 Strand 1	Reverse	ACCTGGGTATGTTGATTACG
	Loqs2 Strand 2	Forward	AGGCCCTAATTGACAAGATG
	Loqs2 Strand 2	Reverse	taatacgactcactatagggagaACCTGGGTATGTTGATTACG
	GFP Strand 1	Forward	taatacgactcactatagggagaCCTGAAGTTCATCTGCACCA
	GFP Strand 1	Reverse	GTTGTGGCGGATCTTGAAGT
	GFP Strand 2	Forward	CCTGAAGTTCATCTGCACCA
	GFP Strand 2	Reverse	taatacgactcactatagggagaGTTGTGGCGGATCTTGAAGT
	mle Strand 1	Forward	taatacgactcactatagggagaTTACATCAATTCCGGACAGG
	mle Strand 1	Reverse	CGTGGTATCGATAGTAGCTG
	mle Strand 2	Forward	TTACATCAATTCCGGACAGG
	mle Strand 2	Reverse	taatacgactcactatagggagaCGTGGTATCGATAGTAGCTG
	Dip1 Strand 1	Forward	taatacgactcactatagggagaGGCTTCCTATGCTCTGTACA
	Dip1 Strand 1	Reverse	TTTAGTTGTGGGATCGGATGA
	Dip1 Strand 2	Forward	GGCTTCCTATGCTCTGTACA
	Dip1 Strand 2	Reverse	taatacgactcactatagggagaTTTAGTTGTGGGATCGGATGA
	aBravo Strand 1	Forward	taatacgactcactatagggagaCTATCCATTGGGCTATGTTACTTTG
	aBravo Strand 1	Reverse	CTGCTCTTCAGTTTCGTAGAAGATT
	aBravo Strand 2	Forward	CTATCCATTGGGCTATGTTACTTTG
	aBravo Strand 2	Reverse	taatacgactcactatagggagaCTGCTCTTCAGTTTCGTAGAAGATT
	Ago2 Strand 1	Forward	taatacgactcactatagggagaCAGTTCAAGCAGACGAACCA
	Ago2 Strand 1	Reverse	TGATGTAGACGCGTCCTCTG
	Ago2 Strand 2	Forward	CAGTTCAAGCAGACGAACCA
	Ago2 Strand 2	Reverse	taatacgactcactatagggagaTGATGTAGACGCGTCCTCTG
	AAEL022113 Strand 1	Forward	taatacgactcactatagggagaGCCAAGATCATGTTCACCAC
	AAEL022113 Strand 1	Reverse	GCTAACGTTTTGGGCAATCT
	AAEL022113 Strand 2	Forward	GCCAAGATCATGTTCACCAC
	AAEL022113 Strand 2	Reverse	taatacgactcactatagggagaGCTAACGTTTTGGGCAATCT
GoldenGate Assembly	Ligated product GoldenGate	Forward	GCATCAAAATATCTATATTGGATCTTCTTCAACACCTCTCCATG
	Ligated product GoldenGate	Reverse	CATACATTATACGAAGTTATACCGACATTGATGAGTTTGGACAA
	Linker GGATC GoldenGate	Forward	GGATGGCGCGTCGCAAGAGCACCCTCACGG
	Linker GGATC GoldenGate	Reverse	AACTCCGTGAGGGTGCTCTTGCGACGCGCC
Intermediate cloning pKSB-	Loqs2 gRNA 1	Forward	GTTGTACGATACCCGGTAGCTGA
	Loqs2 gRNA 1	Reverse	AAACTCAGCTACCGGGTATCGTA
	Loqs2 gRNA 2	Forward	AATGCGGAAGCAAATCGTATTG
	Loqs2 gRNA 2	Reverse	AAACCAATACGATTTGCTTCCG
	Loqs2 gRNA 3	Forward	GATGACTCAACAGCTCGTACAGA
	Loqs2 gRNA 3	Reverse	AAACTCTGTACGAGCTGTTGAGT

PCR was then performed to amplify part of the ligated product, followed by a digestion of the original plasmid with Anza™ 10 DpnI (Invitrogen). Final vector, already containing the sequence for Cas9 expression, was digested with BamHI (Thermo Scientific) and purified from an agarose gel using the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL). Both linear DNA, vector and PCR amplified product, were ligated using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs), according to manufacturer's recommendations.

The ligation reaction was then mixed with competent DH5α bacteria, in a ratio of 1:10, and after resuspension, placed on ice for 3 min. The bacteria were then spread on solid LB medium supplemented with ampicillin, and incubated at 37ºC over-night.

Several colonies were then selected and grown in liquid LB medium supplemented with ampicillin at 37 ºC over-night. On the next day, plasmids were extracted using the NucleoSpin® Plasmid Easy Pure kit (MACHEREY-NAGEL), following manufacturer's instructions.

All plasmids were then sequenced by Eurofins genomics, using appropriate primers either provided by their facilities or designed by us.

Plasmids and constructs used in this study and their applications are listed on **Table 2**.

Origin of U6 promoters and gRNA sequences are listed in **Table 3**.

Table 2. Plasmids and constructs used in this study and their applications

Plasmid	Application	Origin
pKSB- U6::Loqs2 gRNA1	Intermediate cloning of U6::Loqs2 gRNA1	This work
pKSB- U6::Loqs2 gRNA2	Intermediate cloning of U6::Loqs2 gRNA2	This work
pKSB- U6::Loqs2 gRNA3	Intermediate cloning of U6::Loqs2 gRNA3	This work
pKSB- Pub	Intermediate cloning of Pub	Eric Marois
pKSB- nlsYFP-sv40	Intermediate cloning of nlsYFP	Eric Marois
pENTR piggyBac attP loxP-[Exu::3xFLAG-Cas9]-loxP	piggyBac construct designed for the expression of the spCas9 protein under the control of Exuperentia promoter (expression in the germ-line).	Eric Marois
pENTR [Pub::pBtransposase]	Helper plasmid for piggyBac transposition expressing a codon-optimized transposase with NLS under the control of the PolyUbiquitin promoter	Eric Marois
pENTR piggyBac attP loxP-[Exu::3xFLAG-Cas9; U6::Loqs2 gRNA1; U6::Loqs2 gRNA2; U6::Loqs2 gRNA3; Pub::nlsYFP]-loxP	piggyBac construct designed for the expression of the spCas9 protein under the control of Exuperentia promoter (expression in the germ-line), combined with the expression of 3 gRNAs targeting the Loqs2 gene, driven by 3 different *Ae. Aegypti* promoters. nlsYFP is expressed under the conrtol of PolyUbiquitin promoter (PUb) to allow for selection of the transgenic mosquitoes.	This work

Table 3. Origin of U6 promoters and gRNA sequences.

U6 promoter origin	gRNA sequence +*PAM (5′ - 3′)*
Chr3. U6 AAEL017763	GACTCAACAGCTCGTACAGA *AGG*
Chr.3 U6 AAEL017905	CGCGGAAGCAAATCGTATTG *CGG*
Chr.1 U6 AAEL017702	GTACGATACCCGGTAGCTGA *AGG*

4. Cloning for dsRNA preparation

All dsRNA sequences amplified by PCR were cloned into pJET plasmids using the CloneJET PCR Cloning Kit (Thermo Scientific) according to manufacturer's instructions.

Plasmids were then amplified, purified and sequenced as described previously.

5. dsRNA preparation

All dsRNAs were produced to target the genes of interest and silence their expression in mosquitoes. Both strands of the dsRNA were synthesized independently, and then annealed together, to ensure an equimolar ratio of each.

For each target, the sequence of interest was amplified by PCR, as described previously. The DNA was then precipitated by adding ammonium acetate to a final concentration of 2.5 M to the PCR product, followed by 1 volume of isopropanol. After a 2h incubation at -20ºC, the sample was centrifuged at 14,000 x*g* for 30 min, at 4 ºC, and washed once with 70% EtOH. The pellet was then air-dried for 5-10 min, before being resuspended in RNase-free water. Concentration was measured using the NanoDrop OneC instrument (Thermo Scientific), before proceeding to the next step. Each PCR amplified fragment was then cloned into a pJET plasmid, as described previously and sequenced.

In vitro transcription (IVT) was then performed using the MEGAScript™ T7 Transcription kit (Invitrogen), following manufacturer's instructions.

ssRNAs were then purified by adding ammonium acetate to a final concentration of 2.5 M, followed by 1 volume of acid Phenol:Chloroform:Isoamyl alcohol (125:24:1). The samples were then mixed thoroughly and centrifuged at 16,000 x*g* for 15 min, at 4 ºC. The aqueous phase was transferred into a new tube, and 1 volume of isopropanol was added before incubating at -20 ºC for 2 h. After precipitation, the samples were again centrifuged at 14,000 x*g* for 30 min, at 40 ºC, and washed once with 70 % EtOH. The pellet was air-dried for 5 to 10 min, and resuspended in RNase-free water.

Purified ssRNAs were then mixed in equimolar ratios, to reach a final concentration of 7.2µg/µL, in 1X annealing buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA [pH 8]). The samples were then incubated at 95 ºC for 5 min and then left to cool down at room temperature (RT) for 1 h. dsRNAs were then stored at -80 ºC until further use.

6. RNA extraction

RNA extractions were performed on either whole mosquitoes, or various dissected mosquito tissues, that were flash-freezed on dry-ice and stored at -80 ºC.

Samples were grinded in 200 µL of TRIzol® Reagent (Ambion) using a Precellys® Evolution homogenizer (Bertin technologies), for 3x 20 s cycles at 6,800 rpm, with 30 s pause between each cycle. After the addition of 40 µL chloroform, the samples were incubated at RT for 10 min, and then centrifuged at 15,000 x*g*, for 15 min at 4 ºC. The aqueous phase was transferred into a fresh tube, and 1 volume of isopropanol was added to it, as well as 20 µg of glycogen. The samples were then incubated at -20 ºC for 2 h, before a new centrifugation step in the same conditions as earlier. The samples were then washed with ice-cold 70 % EtOH, and centrifuged at 15,000 x*g* for 5 min, at 4 ºC. The pellets were then air-dried for 5 to 10 min, and resuspended in RNase-free water.

Alternatively, for large experiments, the extraction was automatized using the KingFisher™ Apex instrument (Thermo Fisher). In this case, RNA was extracted using the Mag-Bind® Total RNA 96 kit (Omega Bio-Tek), following manufacturer's instructions, reducing the volumes by half.

7. Reverse transcription

Reverse transcription was performed using the RevertAid H Minus Reverse Transcription kit (Thermo Scientific), following manufacturer's instructions, using half of the recommended volume of enzyme. Samples were diluted 1:10 for further applications.

8. qPCR amplification

Quantitative PCRs (qPCRs) were performed on complementary DNA (cDNA) using the PowerSYBR® Green PCR Master Mix (Applied biosystems), following manufacturer's recommendations, reducing the volumes by half. Two negative controls (no template and no reverse transcriptase) were included for each experiment, as well as 3 standard dilutions.

qPCRs were performed either on the QuantStudio 5 (Thermo Fisher) or on the 7500 Fast Real Time PCR system (Thermo Fisher).

Primers for qPCR used in this study and their applications are listed on **Table 4**.

9. Protein extraction

Protein extractions were performed by grinding flash-freezed samples in a Precellys® Evolution homogenizer (Bertin technologies) containing dry-ice, for 3 x 20 s cycles at 6,800 rpm, with 30 s pause between each cycle. This program was repeated a second time, before adding 200 µL to 1 mL of Lysis Buffer (1X NP40, 50 mM Tris-HCl [pH

7.8], 50 mM NaCl, 2 mM $MgCl_2$, 1X cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack (Roche)). After addition of the lysis buffer, the program was repeated twice.

Table 4. List of qPCR primers used in this study

Target	Sense	Sequence
Loqs2 3' UTR	Forward	ACGTGAGTTGACCAATGCCA
Loqs2 3' UTR	Reverse	CGAACAACCGCAAGCTAACC
Loqs2 KO region	Forward	TCGCCTCCTTCTTCGATTGG
Loqs2 KO region	Reverse	TACGATTTGCTTCCGCGTGA
RpL32	Forward	AGCCGCGTGTTGTACTCTG
RpL32	Reverse	ACTTCTTCGTCCGCTTCTTG
SINV	Forward	CGTTCAGCTGCAAGACCA
SINV	Reverse	TACCGGCCGTGGCTAGTAT
ZIKV	Forward	CAGACTGCGACAGTTCGAG
ZIKV	Reverse	CGCGTTTTAGCATATTGACAATC
HTV	Forward	TAGGTTACGGGCCAGATGGT
HTV	Reverse	ATCCTCATAGGCGTCGGTGT
PCLV	Forward	AGACAAGAAGAGTTGAGTGTGCT
PCLV	Reverse	AGCAACGTAATTAGTCCCTCCA
mle	Forward	AATAGTTTCCGCGCGTTTGC
mle	Reverse	TCAAGAAGCCTCCGGTGAAA
Ago2	Forward	AGATTGACAAGCAGAAAATCCAC
Ago2	Reverse	CATTTGGACGCATCAGCA
Loqs	Forward	GAGCTGACGGGTACGAACAA
Loqs	Reverse	TCCCTCGACCTGGATCAAGT
Dip1	Forward	CCCTGCGAGATCTGGTCATC
Dip1	Reverse	GAGCCGTTGTTGCCATTGTT
r2d2	Forward	ATTGCTCTGGACGAAACGCT
r2d2	Reverse	CCAGATCGCTGTTGTCTCGT
GFP	Forward	CGACCACTACCAGCAGAACA
GFP	Reverse	TCTCGTTGGGGTCTTTGC
Nop56	Forward	AAAGCGCAGGCCATTATCGA
Nop56	Reverse	GCAGGTACTCAGCTAGCTGG
aBravo	Forward	GTGTATTGAACGGCATCGCC
aBravo	Reverse	ATTGGGAACGAAGCCTCCAG
AAEL022113	Forward	CCGAAAACCAGTCCTGTCGA
AAEL022113	Reverse	CACAGTGTGCGTTTCGGTTT

Samples were then clarified by centrifugation at 12,000 x*g* for 20 min, at 4 ºC, and the supernatant were transferred into a fresh tube for further applications. Protein concentration was measured by Bradford assay, using the Quick Start Bradford 1X Dye Reagent (BioRad).

Alternatively, proteins were extracted from whole mosquitoes or tissues by directly adding 1X Leammli Buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercapto-ethanol, 0.01% bromophenol blue) and grinding the tissues by hand with a pestle.

10. Western Blot

Protein samples were prepared by adding 1X Leammli Buffer to the desired amount of protein extracted previously, and boiled at 95ºC for 5 min.

Samples were then loaded onto a Mini-PROTEAN® TGX Stain-Free™ Precast Gel (BioRad), with a 4-15% polyacrylamide gradient. Unused wells were loaded with 1X Leammli Buffer, and the PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) was used to visualize the sizes later in the process. Gel electrophoresis was performed in 1X Tris-Glycine SDS, for 45 to 60 min at 180 V. Proteins were then transferred from the gel onto a 0.2 µm nitrocellulose membrane using the Transblot® Turbo™ transfer system (BioRad), for 7 min, at 25 V, 2.5 A.

Proper transfer was verified through the staining of the membrane for 5 min in Ponceau S solution (Sigma), followed by repeated washes with deionized water. After a picture was taken, the membranes were washed in PBS-T (PBS + 0.1% Tween 20) for 5 min to remove all traces of Ponceau.

Membranes were incubated in 3.5 % blocking buffer (3.5% BSA in PBS-T) for 30 min, and then incubated with the primary antibodies, diluted in 1.5% blocking buffer, overnight, at 4 ºC, shaking. Membranes were then washed twice in PBS-T for 15 min, before adding the secondary antibodies, diluted in 1.5 % blocking buffer, for 1 h 30, at RT, shaking. Membranes were finally washed again in PBS-T three times, for 10 min.

Proteins were revealed using the Clarity™ Western ECL Substrate (BioRad), following manufacturer's instructions, and incubated for 5 min in the mix before pictures were taken on the ChemiDoc™ Imaging System (BioRad).

Antibodies in this study and their dilutions are listed on **Table 5**.

11. Virus propagation

ZIKV viral isolates (strain PE243/2015) were propagated in C6/36 *Ae. albopictus* cells, in L-15 Leibovitz medium (Gibco) supplemented with 5 % FBS, streptomycin, gentamycin and penicillin. Cells were seeded to 70 % confluency, and infected at a MOI of 0.01. Supernatant was collected after 9 days at 28 ºC, and cells were lysed by 3 cycles of freezing and thawing, in order to release viral particles. Lysed cells were then mixed

with the supernatant, and clarified by centrifugation. Virus stocks were then stored at -80 ºC until further use.

Table 5. List of antibodies used in this study and corresponding dilutions.

Antibody	Origin	Reference	WB	IP	IFA
anti-Flag	Mouse	Abcam (ab1162)	1:2,000	-	-
anti-tubuline	Mouse	Abcam (ab7291)	1:2,000	-	-
anti-Loqs2	Guinea-pig	Produced by Proteogenix	1:2,000	1:200	1:50
anti-FHV	Guinea-pig	Produced by Proteogenix	-	1:200	-
anti-rabbit-HRP	Horse	Amersham (NA934)	1:10,000	-	-
anti-mouse-HRP	Sheep	Amersham (NA931)	1:10,000	-	-
anti-guinea-pig-HRP	Rabbit	Sigma (A5545)	1:10,000	-	-
anti-guinea pig-568	Goat	Invitrogen (A-11075)	-	-	1:500

SINV viral isolates (strain AR339) were generously provided by Sebastien Pfeffer. Briefly, they were produced by performing an IVT using the mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen), following a XhoI (Thermo Scientific) linearization of the pTR339-GFP/2A plasmid (Thomas *et al.*, 2003). RNA was then transfected into BHK21 cells using Lipofectamin™ 3000 reagent (Invitrogen), and supernatant was collected after 48 h. After a centrifugation step, virus was titrated in Vero cells by plaque assay. BHK21 cells were then infected again, at a MOI of 0.01, and collected again at 48 h to be centrifuged and titrated in Vero cells by plaque assay. Stocks were then stored at -80 ºC until further use.

HTV and PCLV were prepared from extracts of naturally infected *Ae. aegypti* mosquitoes, since it was not possible to produce these viruses in cell culture. Stocks were produced from pools of 15 females, grinded using pestles in 1.2 mL of L-15 Leibovitz medium (Gibco) supplemented with 10 % FBS. Samples were clarified by centrifugation at 3,000 x*g* for 15 min at 4 ºC, and supernatant were collected to be filtered through a 0.22 µm filter. Virus stocks were then stored at -80 ºC until further use.

II. Mosquitoes

1. Rearing

Mosquitoes were reared in an insectarium, in climatic chambers with a humidity rate of 60-80 %, a temperature of 26-28 ºC and a 14 h day/10 h night cycle. Larvae were grown in deionized water for seven to nine days, and fed daily with grinded Goldfish flakes (Tetra). As soon as pupation started, pupae were collected daily and placed in cages until emergence of the adults. Mosquitoes were then offered 10 % sucrose solution, renewed weekly, until their death.

For experiments (no infection) and maintenance of the lines, mosquitoes were offered bloodmeals on anesthetized CD1 mice. Mice received an intraperitoneal injection of 8.5 mg/kg of xylazine (Rompun) and 42.5 mg/kg of a mic of tiletamine and zolazepam

(w/w 1:1, Zoletil) diluted in saline solution (85 µL of the mix injected per 10g of mouse). They were then offered to the mosquitoes for 5 to 15 min, until they were gorged. After 72 h, females were allowed to lay eggs on a humidified filter paper, and again three days after this, the eggs were retrieved and either dried to be stored for later use, or directly hatched in deionized water by placing them under a vacuum bell, at -70 hPa for 30 min, twice.

All experiments using mice were performed at the Institut de Biologie Moléculaire et Cellulaire (Strasbourg, France), using facilities and protocols adhering to national regulation of laboratory animal welfare in France. Facility and protocols have received certification from the regional veterinary services (authorization № I67-4822) and from the national ethics committee board in animal experimentation (authorization APAFIS-20562-2019050313288887 v3).

2. Aedes aegypti lines

The wild-type Bangkok strain, isolated in Thailand in 2015, was used for most experiments, and transgenic lines referred to in this work were generated in this wild-type background (**Table 6**).

Table 6. List of transgenic mosquitoes used in this study

Transgene	Background	Marker	Reference
CP::3xFLAG-Loqs2	Bangkok	Pub::mTurquoise	Olmo et al., 2018
Exu::3XFLAG-spCas9; U6::Loqs2 gRNA 1; U6::Loqs2 gRNA 2; U6::Loqs2 gRNA 3	Bangkok	Pub::nlsYFP	This work

3. Dissection of mosquito tissues

Different organs of the mosquitoes were dissected for the experiments, namely the intestines (midguts), the ovaries and the testis. When mentioned, “carcasses” refers to the body of the mosquito without ovaries and midgut.

For small scale experiments, mosquitoes were collected 3 to 5 days after emerging, and were anesthetized on ice, and taken one by one to dissect the tissues in 1X PBS drops under an Olympus SZ61 stereomicroscope (Olympus). Once extracted from the body, the organs were directly place on a tube on dry ice to flash-freeze the sample. Depending on the experiment, tissues were either placed in tubes individually, or several were gathered in a same tube, when large amounts of material were necessary. For large scale experiments, dissections were performed as described previously, with the difference that mosquitoes were first frozen and preserved at -80 ºC before dissection.

For mass spectrometry analyses, female mosquitoes were collected 3 to 5 days after emerging and offered a bloodmeal. After 24 h, mosquitoes were sorted and the ones that were engorged were further processed. Dissection was performed as described

above, with the difference that the midguts were opened with SuperFine Vannas Scissors (0.015 mm x 0.015 mm tip; World Precision Instruments) and the blood was removed as much as possible (faint traces of blood were sometimes left in the samples) in 1X PBS. Further processing of the samples was done as described above.

4. dsRNA injections

All dsRNA injections were performed on female mosquitoes, aged 3 to 5 days.

Mosquitoes were anesthetized on ice, and kept there for the duration of the injections. Micro-injections were performed with NanoJect-III micro-injector (Drummond Scientific), using glass capillaries. Capillaries were filled with the dsRNA prepared as described previously, and mosquitoes were injected below the wing, in the mesopleuron with 500 ng dsRNA final, in a volume of 69 nL. Additional mosquitoes were injected with the same volume of 1X annealing buffer as injection controls.

Mosquitoes were allowed to recover for 2 days in regular rearing conditions before being offered a bloodmeal, and were finally collected or dissected at different time-points, with the earlier one being 4 days post-bloodmeal. For the whole duration of the experiments, mosquitoes were offered 10 % sucrose solution, until collection.

5. Micro-injections of viruses

Experiments necessitating the use of human pathogens were performed at the Institut de Biologie Moléculaire et Cellulaire (Strasbourg, France), in the Bio-safety Level 3 facility.

Viruses were diluted to the appropriate titer in L-15 Leibovitz medium (Gibco) and micro-injected in female mosquitoes with the NanoJect-III micro-injector (Drummond Scientific), to inject 1 pfu (SINV) or 10 pfu (ZIKV) per mosquito, under the same conditions as the dsRNA injections, with the exception of the volume injected, which was elevated to 100 nL per mosquito.

Mosquitoes were then placed into glove boxes reproducing the rearing conditions in terms of day/light cycle, temperature and humidity rate, and offered 10 % sucrose solution until collection, at different times after injections

Dissections were performed on these mosquitoes as described previously, and the leg of each dissected mosquito was retrieved for genotyping.

6. Infectious bloodmeal

To study the natural route of infections, experiments were also conducted by offering infectious bloodmeals to the mosquitoes.

To that effect, viruses (titer 2 x 10^7 pfu/mL) were diluted 1:1 (v/v) with sheep blood (Thermo Fisher) or human blood provided by the Etablissement Français du sang (EFS;

Convention ALC/PIL/DIR/AJR/FO/606 version 4), and placed at 37 ºC for 5 to 10 min. The infectious blood was then loaded into feeding units covered with pork intestine membranes, and pre-heated at 37 ºC on a HemoTek™ PS6 (Hemotek). Mosquitoes were then offered the infectious bloodmeal for 40 min, and finally anesthetized on a cold block to collect the ones that ingested the blood.

Mosquitoes were then placed into glove boxes reproducing the rearing conditions in terms of day/light cycle, temperature and humidity rate, and offered 10 % sucrose solution until collection, at different times after the bloodmeal.

Dissections were performed on these mosquitoes as described previously, and the leg of each dissected mosquito was retrieved for genotyping.

III. Transgenesis

1. Micro-injection in eggs

Transgenic mosquito lines were generated by micro-injecting the construct of interest in embryos from the Bangkok backgrounds.

Prior to injections, a mix was prepared, containing a 100 ng/µL of a home-designed transposase helper-plasmid and 400 ng/µL of the construct of interest, in 0.5X PBS.

Female mosquitoes were allowed to lay eggs, 3 days after a bloodmeal, which were collected 30 to 45 min after being deposited on the filter paper, just as they started to melanize. Eggs were then aligned in parallel against a nitrocellulose membrane, that was kept wet in deionized water. When the eggs turned dark grey, they were placed under a Nikon Eclipse TE2000-S inverted microscope and injected into the posterior pole with the injection mix prepared previously, using a Femtojet injector (Eppendorf) and a TransferMan NK2 micromanipulator (Eppendorf). Micro-injected eggs were then kept moist for 3 days before being hatched under vacuum, to proceed with the selection of the newly generated mosquito line.

2. Selection of transgenic mosquitoes

Following micro-injections, surviving larvae showing transient expression of the fluorescent marker were isolated and back-crossed into the Bangkok background. In the next generation, the integration of the plasmid was determined by the constitutive expression of the fluorescence marker, and mosquitoes were selected as neonate larvae, using the COPAS™ Select (Union Biometrica). Transgenic females were then individually crossed to wild-type males to start isofamilies.

In the case of the Loqs2 KO line, the transgene was removed from the population by selecting non-fluorescent larvae with the method described previously, and mosquitoes were genotyped regularly for experiments in each generation.

Lines were kept as heterozygous, to allow or the use of the non-transgenic siblings as controls in experiments, except for the Loqs2 KO line, which was also separated as individual lines for experiments.

IV. Phenotypic characterization of the Loqs2 KO line

1. Isolation of the mosquitoes

To prepare mosquitoes for crosses and experiments, males and females were separated at the pupal stage, by observing the difference in size. After emergence, the virgin mosquitoes were isolated in 24-well plates and the hindleg was retrieved for genotyping.

Genotyping of the mosquitoes was performed by PCR, as described previously, and mosquitoes of each genotype were then crossed or dissected accordingly.

To study the fertility and fecundity of the mosquitoes from the Loqs2 KO line, females were allowed to mate with the males for a few days and were isolated in 24-well plates containing a small filter paper to lay eggs (Ioshino *et al.*, 2018). Eggs were them hatched as usual under vacuum.

2. Tracking of the development

For each female that laid eggs, the number of eggs was counted, as well as the number of neonate larvae, to calculate the hatching rate.

Larvae were then reared in the same volume of deionized water and offered the same amount of food once a day, until all pupae were collected and placed in cages.

Every day after the hatching, the number of progenies at each developmental stage was counted, until they all reached adulthood, usually after 12 days maximum. At this stage, the mosquitoes were anesthetized on ice, and after counting the ratios of male and females in each progeny, a leg was retrieved from all mosquitoes from the KO group to use for genotyping by PCR, as described previously.

3. Starvation assay

As soon as adult mosquitoes emerged, they were separated by genotype and gender in three to five groups of 10 individuals. This virgin mosquitoes were then offered only water for the whole duration of the experiment, through a soaked cotton pad, that was humidified daily to keep them from dehydration. Control mosquitoes were offered 10 % sucrose for the duration of the experiment.

Dead mosquitoes were counted and removed daily in each group until all mosquitoes were dead, except for the control mosquitoes, for which no death was observed.

4. Feeding behaviour assay

Female mosquitoes were separated by genotype in three groups of 15 individuals. Each group of females was allowed to mate with 5 males for 3 days before being offered a bloodmeal. Mosquitoes were offered 10% sucrose on soaked cotton pads in the meantime, which was removed 5 h prior to the bloodmeal.

Female mosquitoes were then offered a bloodmeal, on 5 months-old anesthetized CD1 mice, as described previously. Each mouse was used to feed two groups, one from each genotype, for 7 min 30 s each.

Mosquitoes were then anesthetized on ice and the gorged mosquitoes were counted in each group.

5. Virus vertical transmission assay

Vertical transmission assay was performed by injecting female mosquitoes from each genotype with insect-specific viruses, HTV and PCLV, in the same conditions as described previously.

Females were offered a bloodmeal 7 days after injection, and after 3 more days, were isolated into 24-well plates to lay eggs individually, as described previously. After another 3 days, eggs from each individual female were hatched and the progeny was reared until pupal stage in regular conditions.

Pupae from each progeny were then collected in two groups of 5, when possible, and RNA was extracted as described previously, and qPCR was performed on each group of pupae to assess for the presence of both viruses.

V. Immunostaining

1. Preparation of the samples

Immunostaining was performed on ovaries, using ovaries from the Loqs2 KO females as a negative control.

Ovaries were dissected cautiously in the same conditions as described previously, with the difference that 4X PFA was used instead of 1X PBS.

Samples were fixed for 20 min in 4X PFA, and washed three times in 1X PBS, for 5 min, before being incubated for 15 min in PTX 1 % BSA (1X PBS, 0.1 % Triton-X100, 1 % BSA) to be permeabilized. Samples were then incubated with primary antibodies diluted

in PTX 1 % BSA overnight, at 4 ºC, shaking. After three more washes with PTX 1 % BSA, of 10 min each, samples were incubated for 2 h with secondary antibodies, diluted in PTX 1 % BSA, at RT, shaking. Samples were then washed again, three times for 10 min each with PTX 1 % BSA, and incubated for 15 min with Phalloïdin-TRITC diluted 1: 500 in PTX 1 % BSA, at RT, shaking. Finally, samples were washed one last time in PTX 1 % BSA, for 5 min.

Samples were then mounted onto a microscope slide, by retrieving the ovaries one by one and placing then in Vectashield with DAPI H-1200 (Vector Laboratories), diluted 1:4 in Vectashield H-1000 (Vector Laboratories). Cover slips were glued on each slide of the samples, using transparent nail polish, to avoid crushing the samples, by elevating the last cover slip, placed over it. After 1 h, the samples were sealed using transparent nail polish, and placed in a dark box at 4 ºC until further use.

2. Microscopy

Images were acquired using a spinning disk confocal microscope (Zeiss Axio Observer Z1 Yokogawa spinning disk system) with a ×40/1.4 Plan-Apochromat objective.

VI. Proteomics

1. Co-immunoprecipitation

Protein extracts were prepared as described previously, in large amounts to reach at least 500 µg of protein per replicate, with a final concentration between 1 and 3 µg/µL.

Co-immunoprecipitations were performed, following manufacturer's instruction, using the µMACS™ DYDDDDK Isolation kit (Miltenyi Biotec) for 3XFLAG-tagged proteins and the µMACS™ Protein A MicroBeads (Miltenyi Biotec) for untagged proteins, with the exception that an air blower bulb was used to collect all the remaining buffer and beads from the column. In the case of untagged protein, the incubation of the protein extracts with the magnetic beads was done for 16 h at 4 ºC, on a rotating wheel.

After elution, samples were placed at 95 ºC for 5 min at 1,000 rpm, for a last denaturation step, and centrifuged at 12,000 x*g* for 3 min.

Before being used for mass spectrometry analyses, 10 % of the final elution volume was kept aside for Western blotting.

2. Mass spectrometry analysis

Mass Spectrometry analyses were performed at the Proteomics platform in the Institut de Biologie Moléculaire et Cellulaire (Strasbourg, France).

Following co-immunoprecipitation, proteins were precipitated with 0.1M ammonium acetate in 100% methanol, and then resuspended in 50 mM ammonium

bicarbonate. After a reduction-alkylation step (5 mM dithiothreitol, 10 mM iodoacetamide), proteins were digested overnight with sequencing-grade porcine trypsin (Promega) diluted 1:25. The resulting peptides were dried and resuspended in 25 µL of water, containing 0.1% formic acid (solvent A). The peptide mixtures were then analyzed by nanoLC-MS/MS on a TripleTOF 5600 mass spectrometer (Sciex) coupled to nanoLC-ultra-2D-plus liquid chromatography (Eksigent) operating in positive mode with a nanoelectrospray source. A ChIP C-18 pre-column (300 µm ID x 5 mm ChromXP; Eksigent) was loaded with 5 µL of each sample, at 2µL/min in solvent A. Desalting and concentration of the samples were performed for 10 min, before the pre-column was switched online with the analytical ChIP C18 analytical column (75 µm ID x 15 cm ChromXP; Eksigent), equilibrated in solvent A: solvent B (95:5). Solvent B is composed of formic acid: acetonitrile (0.1:100). Peptides were then eluted using a 5%-40% gradient of solvent B for 120 min at a flow rate of 300 nL/min.

The TripleTOF 5600 was operated in data-dependant acquisition mode with Analyst software (v1.6, AB Sciex). Survey MS scans were acquired for 250 ms in the 150-1250 m/z range. If they exceeded the 150 counts per second intensity threshold, up to 20 of the most intense multiply charged ions (2+ to 5+) were selected for CID fragmentation. Ions were fragmented using a rolling collision energy script within a 60 ms accumulation time and an exclusion time of 15 s. This method, with a constant cycle time of 1.5 s, was set in high sensitivity mode.

Mass spectrometry data were then processed as described in the appropriate bioinformatics section.

VII. Transcriptomics

1. Libraries preparations for long RNA sequencing

Ovaries from Loqs2 KO mosquitoes and wild-type mosquitoes were dissected in groups of 10 (three replicates), and total RNA was extracted using TRIzol® Reagent (Ambion), as described previously.

rRNA were depleted from the samples using the riboPOOL™ kit (siTOOLs Biotech), following manufacturer's instruction. Depletion of rRNA was then assessed on the Agilent 2100 Bioanalyzer System (Agilent Technologies), using an RNA 6000 Nano kit (Agilent Technologies). The depletion was confirmed by the loss of 18S/28S rRNA peaks in all samples.

Long RNA libraries were then prepared from these rRNA depleted samples, using the NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina (New England Biolabs), with NEBNext Multiplex Oligos for Illumina (New England Biolabs), according to manufacturer's instructions.

Quality of the libraries was then assessed on the Agilent 2100 Bioanalyzer System (Agilent Technologies), using the DNA 1000 kit (Agilent Technologies), which also

allowed for quantification of each library. Indexed libraries were then diluted and pooled in similar concentrations, and sent to the sequencing platform.

2. Illumina sequencing

Paired-end Illumina high-throughput long RNA sequencing of the libraries was conducted at the GenomEast platform at the Institut de Génétique, Biologie Moléculaire et Cellulaire (Illkirch-Graffenstadten, France).

VIII. Evolution of Loqs2

1. Retrieval of the sequences from databases

Public high-throughput RNA-seq and DNA-Seq libraries from various mosquitoes were obtained from the NCBI/SRA database. The libraries were mapped to the *Ae. aegypti* and *Ae. albopictus* reference genomes (Vectorbase release 52) (Amos *et al.*, 2022) using STAR v2.7.9a (Dobin *et al.*, 2013), with a maximum alignment mismatch percentage allowed of 50%, to overcome the divergence between the RNA-seq data and the reference genomes. To avoid false positives, mapping coverage comprising regions of *loqs* and *loqs2* were manually inspected using IGV v2.8.3 (Robinson *et al.*, 2011).

2. Transcriptomic analyses of tissue-specific libraries

Quantification of the expression profiles of *loqs*, *loqs2* and *r2d2* among different tissues from *Ae. aegypti*, *Ae. albopictus* and *An. gambiae* mosquitoes necessitated the use of public RNA-seq libraries, obtained at NCBI/SRA as describe above. Reads were mapped to the decoyed reference transcriptome of each species (Vectorbase release 52) using Salmon v.1.5.2 (Patro *et al.*, 2017). Quasi-mapping quantifications were imported into R v4.1.0, and data normalization was performed using the packages EdgeR v3.34.0 and TMM (Robinson and Oshlack, 2010; Robinson *et al.*, 2010).

3. Phylogenetic analyses

Phylogenetic analyses were performed on a set of mosquitoes and flies, to infer the evolutionary history of *loqs2* at three different evolutionary ranges: *(i)* the dsRBP orthologs (InterPRO accession IPR014720) (Blum *et al.*, 2021), *(ii)* the close related evolutionary history of *loqs2*, including *loqs* and *r2d2* and, *(iii)* the *loqs2*, *loqs* and *r2d2* single domain evolutionary history. Input sequences were aligned using either PRANK+F (Löytynoja and Goldman, 2009), for strategies *(i)* and *(iii)*, or MAFFT-E-INS v7 (Katoh and Standley, 2013) for strategy *(ii)*, and the substitution model and phylogenetic relationships were inferred using IQ-tree2 (Minh *et al.*, 2020).

The maximum-likelihood consensus tree was generated with 1000 ultra-fast bootstraps iterations. Trees were rooted at the midpoint, using Figtree v1.4.4 for visualization purposes. For the dsRBP evolutionary range, amino acid sequences of the dsRBPs (InterPRO accession IPR014720), including Dicer-2, were retrieved using

VectorBase (release 52). The WAG amino acid exchange matrix under FreeRate heterogeneity model with 5 categories (WAG+R5) was determined to be the best fit model based on the Bayesian information criterion (BIC). Coding sequences from the orthologs belonging to the same set of species of strategy *(i)* were used for the *loqs2* closely related tree.

The KOSI07 empirical codon model with discrete Gamma model with 4 rate categories (KOSI07 + G4) was determined to be the best fit model base on the BIC. Coding sequences of each domain from the *loqs2*, *loqs* and *r2d2* belonging to *Ae. aegypti* and *Ae. albopictus* were used for the single domain tree. The KOSI07 empirical codon model with amino-acid frequencies given by the protein matrix (KOSI07 +FU) was determined to be the best fit model based on the BIC.

4. Alignment and comparisons of dsRBDs

Homology models of the dsRBDs of *Ae. aegypti* and *Ae. albopictus* Loqs and Loqs2 were built using a template-based method. Structure of the *D. melanogaster* Loqs-PD dsRBD1 (PDB id: 5NPG) was used as the structural template to model the dsRBD1 of Loqs and the dsRBDs of Loqs2. The *D. melanogaster* Loqs-PD dsRBD2 structure (PDB id: 5NPA) was used as a template for Loqs dsRBD2 models (Tants *et al.*, 2017). Models were made with MODELLER v9.24 (Webb and Sali, 2016) using the automodel class. For each of them, 100 models were produced, and the one with lowest MODELLER DOPE score was selected. Ramachandran plots were built with PROCHECK (Laskowski *et al.*, 1993) for each selected model, to assess their quality. At least 88% of the residues of all selected models were located in the most favored regions of the plot, and no more than 1 residue was located in a disallowed region. Models were visualized using pymol v2.4.0.

For structural comparisons, the modeled *Ae. aegypti* and *Ae. albopictus* Loqs2 and Loqs dsRBDs were compared using the DALI server (Holm, 2020). Amino acid sequences of *Ae. aegypti* and *Ae. albopictus* Loqs and Loqs2 dsRBDs and Staufen C were aligned pairwise with BLASTp under the BLOSUM62 (Altschul *et al.*, 1990) substitution matrix.

IX. Bioinformatics

1. Online ressources

For all experiments that required the sequences of the genes of interest, such as clonings, designing of primers or gRNA, the fasta sequences were retrieved using Vectorbase release 52, from the *Ae. aegypti* Liverpool_AGWG reference strain (AaegL5.0 assembly).

Primers for cloning, qPCR and production of dsRNA were designed using the online tool Primer3Plus. Settings were changed depending on the application of the primers, namely the length of the expected product, the length of the primer as well as the T_m. In the case of the production of dsRNA, the expected product was then blasted

against the genome of *Ae. aegypti* on Vectorbase release 52, to verify that the potential siRNAs generated would not have off-targets.

gRNAs were designed using an online tool called CRISPOR, by providing a genomic sequence of the gene of interest and selecting the PAM adapted to the Cas9 protein used. Predicted guide sequences were then provided by the software, and each of them was verified individually for number off-targets with increasing mismatches, as well as the location of the off-targets, until 3 gRNAs were selected. These gRNAs were then ordered as primers and annealed to be cloned as described previously.

2. Proteomics analyses

Mass spectrometry data were searched against the UniProtKB database (*Ae. aegypti*, taxon 7159, release 2018_03, 24927 sequences) with a decoy strategy. Human keratins and porcin trypsin fasta sequences were added to the *Ae. aedypti* sequences as well as their corresponding decoy entries (Database toolbox from MSDA, http://msda.unistra.fr/). Database search was performed using the Mascot algorithm (version 2.5, Matrix Science) with a decoy strategy. Further post-processing was performed after importing the .dat Mascot files into Proline v1.4 package (http://proline.profiproteomics.fr/).

Proteins were validated on Mascot pretty rank equal to 1, 1 % FDR on both peptide spectrum matches (PSM) and protein sets (based on score). Raw Spectral Count values were then imported into R v3.2.5, to be normalized, and analyzed using the R package IPinquiry4 v0.4.6. Variability between the replicates was visualized on a multidimensional scaling plot, generated with the cmdscale() function. Spectral count values were then submitted to a negative-binomial test using an edgeR GLM regression. Results, generated by the msms.edgeR() function, were exported into a table using the xlsx library. For each identified protein, an adjusted P-value corrected by Benjamini-Hochberg was calculated, as well as a protein FC.

3. Transcriptomics analyses

Sequences reads with an average quality score of above phred 25 had adaptors removed using Trimmomatic v0.39 and were mapped to the decoyed transcriptome of *Ae. aegypti* (Vectorbase release 52) using Salmon v1.5.2 (Bolger *et al.*, 2014; Patro *et al.*, 2017). Quasi-mapping quantifications were imported into R v4.1.0 (R Core Team 2021) and data normalization was performed using the packages EdgeR v3.28.1 and TMM (Robinson and Oshlack, 2010; Robinson *et al.*, 2010). Principal component analysis (PCA) comparing all libraries was performed using the R package mixOmics v6.24.0, using the pca() function (Rohart *et al.*, 2017).

Differential gene expression was analyzed for both comparisons (KO against WT and KO against Het) with the ExacTest using the R package EdgeR v3.28.1 (Robinson *et al.*, 2010), and represented using the R package EnhancedVolcano v1.4.0. For each differentially expressed gene, a Z-score was calculated and data was represented, clustered by genes and libraries, using the R package ComplexHeatmap v2.16.0 (Gu *et*

al., 2016; Gu, 2022). Ranked lists of gene expression for the comparison KO against WT was used as input for gene set enrichment analyses (GSEA) (Subramanian *et al.*, 2005), using the R package fgsea v1.12.0 (Korotkevich *et al.*, 2016) and in-house developed gene-sets comprising gene ontology annotation (GO), KEGG pathways, and genes of interest. Sets with adjusted FDR *q*-value < 0.05 were considered in our analysis and redundancy was removed if overlapping genes within given gene-sets were higher than 90 %.

4. Image processing

Agarose gel pictures were taken using the GelDoc™ EZ Imager (BioRad) and western blot membrane pictures were taken using the ChemiDoc™ Imaging sytem (BioRad). Images were saved in .scn format and further processed using ImageLab v6.0.0 (BioRad).

Fluorescence microscopy images were processed using ImageJ2 v1.3.0/1.53q.

5. Statistical analyses

Statistical analyses of the proteomic and transcriptomic data, as well as the data on the evolution of *loqs2*, were performed as described in the corresponding sections.

Statistical analyses for other experiments were performed on GraphPad Prism v9.0.0, on each dataset to be subsequently plotted. Before each analysis, normality tests were run to assess the gaussian distribution of the samples, and according to the results, either parametric or non-parametric tests were further performed. In the case of prevalence analyses, a two-tailed Fischer exact test was performed to compare datasets two by two. For other analyses requiring comparisons of samples two by two, two-tailed Mann-Whitney tests were performed. Survival experiments were displayed as Kaplan-Meier curves, and statistical significance was determined using a Gehan-Breslow-Wilcoxon test.

Statistical tests performed for each experiment are indicated and explained on each figure in this manuscript.

6. Graphical representations

Data were imported into R v4.1.0 to generate plots and graphical representations of the results, using the R package ggplot2 v3.3.3. In the case of the mass spectrometry and transcriptomics results, the R package EnhancedVolcano v1.4.0 was used to represent the data.

Plots of the qPCR data and data from the phenotypic characterization of the Loqs2 KO line were generated on GraphPad Prism v9.0.0, where statistical analyses were performed.

Figures were formatted using Affinity Designer v1.10.6.

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Résumé en français

Introduction

Il est souvent fait référence aux moustiques comme les animaux les plus meurtriers, un titre acquis il y a plus de 300 ans, lorsqu'il a pour la première fois été suspecté que les moustiques ont la capacité à transmettre des pathogènes responsables de maladies chez les humains (Klaasen *et al.*, 2011). De nos jours, les moustiques sont présents dans tous les environnements de par le monde, et il est possible d'en retrouver, comme la majorité des insectes, dans les régions tropicales et subtropicales, cependant, de nombreuses espèces se sont également établies dans des régions tempérées. A l'exception de l'Antarctique et de quelques îles, les moustiques sont actifs dans tous les pays du globe, et il est estimé qu'il existe plus de 4 000 espèces de moustiques, dont seulement 3 500 ont été identifiées (Rueda, 2008).

La particularité de la majorité des espèces de moustiques est la nécessité pour les femelles de prendre un repas sanguin afin de débuter leur cycle gonotrophique et donner naissance à leur progéniture. Les sources de ces repas sanguins sont très variables selon les espèces de moustiques, et certaines en particulier ont développé un régime presque exclusivement anthropophile, comme c'est le cas d'espèces telles que *Anopheles gambiae*, *Aedes aegypti* et *Aedes albopictus* (Rueda, 2008 ; Stone et Gross, 2018 ; Egid *et al.*, 2022). Ce régime préférentiel vient ainsi avec des risques de transmission de maladies, et ces risques sont associés à plus de 200 espèces de moustiques (Reidenbach *et al.*, 2009).

Ces maladies transmises par les moustiques représentent plus de 17 % des maladies infectieuses de par le monde, d'après l'Organisation Mondiale de la Santé (OMS), et résultent en plus de 700 000 fatalités chaque année, dont plus de la moitié est liée à la transmission d'un parasite par les moustiques du genre *Anopheles*, causant le paludisme (Talapko *et al.*, 2019).

Lorsque l'origine de ces maladies infectieuses est virale, on parle alors d'arbovirus (*arthropod-borne viruses*), et parmi ceux-ci on retrouve le virus de la Dengue (DENV), dont on estime qu'il cause plus de 96 millions de cas symptomatiques par an, en plus de 294 millions d'infections asymptomatiques. Les moustiques responsables de la transmission de ce virus, *Ae. albopictus* et *Ae. aegypti*, étendent continuellement leur territoires, colonisant de nombreux environnements naïfs, et il est estimé qu'à ce jour, près de 3,9 milliards de personnes sont à risque d'infection par ce virus, ce qui représente environ la moitié de la population mondiale (Bhatt *et al.*, 2013 ; Messina *et al.*, 2019). En plus de la Dengue, d'autres virus constituent un sujet important de santé publique, tels que les virus de la fièvre jaune, Zika (ZIKV) ou encore Chikungunya (CHIKV).

Ces maladies représentent environ 20 % de la charge mondiale de morbidité, principalement en raison des conséquences à long-terme de ces infections (Carvalho et Long, 2021). En effet, ces infections peuvent résulter en des handicaps importants, affectant sévèrement la qualité de vie des patients, avec des pathologies telles que la microcéphalie chez les nouveau-nés dont les mères ont été infectées par ZIKV, ou une arthrite chronique chez les patients infectés par CHIKV (Carvalho et Long, 2021).

Les principaux vecteurs des virus mentionnés précédemment sont deux moustiques du genre *Aedes*, *Ae. albopictus* and *Ae. aegypti*. Les impressionnantes capacités vectorielles de ces deux espèces sont responsables de la propagation des virus susmentionnés de par le monde, et peuvent être attribuées à plusieurs facteurs.

En particulier, ces moustiques possèdent un génome exceptionnellement large, 2,5 à 5 fois plus grand que les autres espèces de moustiques majeures, bien que le nombre de gènes qu'il contient reste similaire (Nene *et al.*, 2007 ; Matthews *et al.*, 2018). En réalité, près de la moitié du génome d'*Ae. aegypti* et constitué d'éléments transposables (*transposable elements*, TE), une proportion largement supérieure à celle des autres moustiques, et proche de celle observée chez l'humain (Nene *et al.*, 2007 ; Arensburger *et al.*, 2010 ; Ayarpadikannan et Kim, 2014 ; Matthews *et al.*, 2018). La présence d'un si grand nombre de TE a nécessairement participé à l'évolution de cet organisme, en modulant ses capacités d'adaptation aux différents environnements auxquels il a dû faire face. Les extraordinaires capacités d'adaptation des moustiques *Aedes* sont également des facteurs de sa capacité vectorielle remarquable, en ce qu'ils ont pu au cours de ces dernières années, coloniser de nombreux nouveaux environnements, en termes climatiques, et également en termes d'urbanisation, se rapprochant toujours plus de leurs populations cibles (Gubler, 2011 ; Kraemer *et al.*, 2019). Ces moustiques ont ainsi théoriquement développé une capacité à transmettre plus de 22 arbovirus, bien qu'une partie ne soit pas observée dans la nature, mais uniquement en laboratoire, en raison des hôtes de ces virus, qui ne sont pas des cibles des moustiques pour leurs repas sanguins (Lwande *et al.*, 2020).

Les interactions entre ces moustiques et les virus ont évolué au fil du temps, et leur évolution a été modulée en particulier par les réponses immunitaires du vecteur, qui pousse à de nouvelles adaptations de part et d'autre de ces interactions.

Chez les moustiques, de même que chez les autres insectes, de nombreuses voies immunitaires participent à la réponse antivirale, y compris celles impliquées dans la réponse antibactérienne et antifongique, mais une voie en particulier est considérée comme la réponse antivirale par excellence. Il s'agit de la voie des petits ARN interférents (*small-interfering RNAs*, siRNAs) qui permet la reconnaissance par une protéine appelée Dicer-2 (Dcr-2) et son co-facteur Loquacious (Loqs), d'un ARN double-brin (ARNdb), qui sera ensuite clivé en duplexes siRNAs de 21 nucléotides. Ces siRNAs, avec l'aide d'un second co-facteur principal, r2d2, sont par la suite chargés dans un complexe protéique dont le principal effecteur est la protéine Ago2. La reconnaissance de la séquence complémentaire à celle du siRNA permettra l'hybridation de ce dernier avec la séquence cible, qui sera finalement clivée par Ago2, puis dégradée (Olson et Blair, 2015). Une autre voie appelée la voie des *PIWI-interacting RNAs* (piRNAs), qui est habituellement associée au maintien de l'intégrité des cellules germinales, au travers du contrôle de l'expression des TE, a également été associée à la réponse antivirale contre plusieurs arbovirus chez le moustique (Wu *et al.*, 2010 ; Morazzani *et al.*, 2012 ; Vodovar *et al.*, 2012 ; Schnettler *et al.*, 2013 ; Varjak *et al.*, 2017).

Des études réalisées par notre équipe ont cependant démontré que la réponse antivirale induite par la voie des siRNAs n'est pas efficace pour contenir l'infection virale

dans les intestins des moustiques *Ae. aegypti*, tandis qu'elle est en mesure de jouer son rôle habituel lorsque la source d'ARNdb n'est pas d'origine virale (Olmo *et al.*, 2018). Des analyses du génome de ces moustiques nous a permis d'identifier une nouvelle protéine de liaison à l'ARNdb (*dsRNA-binding protein*, dsRBP), dont l'absence dans l'intestin pose la question de sa possible implication dans la discrimination des ARNdb d'origine virale dans ce tissu (Olmo *et al.*, 2018). L'étude de cette nouvelle protéine, renommée Loqs2, en raison de sa similarité avec Loqs et R2d2, a révélé ses propriétés antivirales. En effet, nos recherches ont permis de démontrer que l'expression ectopique de cette protéine dans les intestins du moustique permet le contrôle de la réplication virale de DENV et ZIKV, et perturbe ainsi la dissémination des particules virales dans le reste du corps du moustique (Olmo *et al.*, 2018). L'étude plus poussée de cette protéine *in cellulo* a mis en avant son interaction avec deux protéines de la voie des siRNA, Loqs et R2d2, ainsi que sa possible interaction avec plus de 70 autres protéines (Olmo *et al.*, 2018). Bien que n'étant pas suffisant pour prouver l'implication de Loqs2 dans la voie des siRNA, ces résultats ont permis de montrer une interaction complexe entre cette protéine et les principaux co-facteurs de cette voie immunitaire, et de démontrer l'importance des dsRBP dans la réponse antivirale, encourageant à continuer l'étude et la caractérisation de cette protéine.

Il est estimé qu'environ une douzaine de dsRBP sont encodée par le génome des moustiques *Aedes*, dont près de la moitié sont des composants des différentes voies de l'ARN interférence (ARNi). Ces protéines sont caractérisées par la présence d'un domaine consensus de liaison à l'ARNdb (*dsRNA-binding domain*, dsRBD), découvert il y a plus de 30 ans (Johsnton *et al.*, 1991).

Ces dsRBP présentent différents rôles dans différents processus biologiques chez les moustiques, montrant une grande versatilité de ce type de protéines. En plus d'être des effecteurs importants ou co-facteurs de voies antivirales, ces protéines peuvent également participer à la régulation de l'expression des gènes, comme c'est le cas de plusieurs dsRBP impliquées dans la voie des micro-ARNs (Olson et Blair, 2015). D'autres dsRBP, telles que l'Adenosine deaminase acting on RNA (ADAR), sont responsables de l'édition de certains ARNdb, participant ainsi à l'altération de la structure des ARNdb et de l'épissage, en plus d'affecter le développement, notamment au niveau neurologique (Deng *et al.*, 2020). Il a également été démontré que d'autres protéines contenant ces dsRBDs sont impliquées dans le développement et la régulation de l'expression des gènes, comme c'est le cas de Disconnected-interacted protein 1 (Dip1) et maleless (mle)(Kuroda *et al.*, 1991 ; DeSousa *et al.*, 2003). Enfin, plusieurs dsRBPs semblent également être impliquées dans d'autres processus biologiques primordiaux, tels que l'ovogénèse. Une des premières dsRBP identifiées chez la drosophile en particulier, Staufen, est impliquée dans le processus d'établissement de la polarité des embryons, au travers du transport d'ARN messagers spécifiques du pôle antérieur au pôle postérieur de l'ovocyte (Johnston *et al.*, 1991). D'autres protéines jouent également un rôle important dans les processus reproductifs, comme Blanks, une dsRBP impliquée dans la maturation du sperme chez la drosophile, ou encore la protéine Belle, impliquée dans fertilité des femelles et la spermatogénèse chez la drosophile (Johnstone *et al.*, 2005 ; Gerbasi *et al.*, 2011 ; Sanders et Smith, 2011 ; Poynter *et al.*, 2019). De façon intéressante, plusieurs protéines de la voie des siRNA, telles que Loqs et Ago2, semblent également

affecter la reproduction, leur absence menant à des défauts du maintien des cellules germinales ou possédant un possible rôle spécifique aux testicules chez quelques espèces de drosophiles (Fösterman *et al.*, 2005 ; Lewis *et al.*, 2016).

Considérant les rôles variés et l'importance des fonctions ces dsRBPs dans tous les organismes, une caractérisation approfondie de Loqs2, cette nouvelle dsRBP identifiée par notre équipe, ainsi que de ses fonctions permettrait probablement une avancée considérable dans la compréhension des mécanismes régissant les interactions virus-vecteurs.

L'objectif de ces travaux de thèse est donc de caractériser de manière exhaustive la protéine Loqs2, en se concentrant sur trois aspects clés qui nous aideront à mieux comprendre son rôle dans la biologie des moustiques. Tout d'abord, nous nous sommes penchés sur son origine et son évolution, afin de mieux comprendre son développement jusqu'à aujourd'hui. Deuxièmement, nous avons exploré les propriétés antivirales de Loqs2 dans l'intestin, un aspect important compte-tenu de son potentiel en tant que cible pour le contrôle des arbovirus. Enfin, nous avons étudié sa fonction naturelle, qui pourrait fournir des informations précieuses sur ses rôles potentiels dans la biologie des moustiques, au-delà de sa fonction antivirale.

Discussions et conclusions

1. Origine et évolution de loqs2

La découverte de *loqs2*, une nouvelle dsRBP spécifique aux moustiques *Aedes*, paralogue de *loqs*, qui n'est pas exprimée dans le tissu où le traitement des ARNdb d'origine virale est compromis, a constitué une première étape dans la mise en lumière du processus de discrimination de l'origine des ARNdb (Olmo *et al.*, 2018).

L'étude des processus ayant permis l'apparition et la stabilisation de *loqs2* dans le génome des moustiques *Aedes*, nous a permis de dater son origine autour de 67 millions d'années coïncidant avec la ramification du sous-genre *Stegomyia* (Estevez-Castro *et al.*, 2021). L'absence de séquences génomiques accessibles au public ou de qualité pour de nombreux autres moustiques de ce genre nous a empêchés de préciser davantage l'origine de *loqs2*.

Nos analyses ont également permis de démontrer que *loqs2* provenait de deux duplications indépendantes d'un domaine de *loqs*, et non d'une duplication complète du gène. Ce type d'évènement est considéré comme relativement rare, car les dsRBP sont généralement le produit de duplication de gènes entiers, suivis d'une spéciation du nouveau gène ainsi créé (Tian et Matthews, 2003 ; Dias *et al.*, 2017). De plus, tous les dsRBD n'ont pas la même fonction, et sont subdivisés en deux groupes, avec les dsRBDs de type A, capables de se lier l'ARNdb avec une grande affinité, et les dsRBDs de type B, présentant une affinité réduite pour l'ARNdb, mais étant préférentiellement impliqués dans des interactions protéine-protéine (Krovat et Jantsch, 1996 ; Hitti *et al.*, 2004 ; Chang et Ramos, 2005).

De nombreuses dsRBPs contiennent plusieurs de ces domaines, et il a été démontré que les protéines contenant plusieurs dsRBD de type A, peuvent se lier plus efficacement à l'ARNdb (Krovat et Jantsch, 1996). Il est intéressant de noter que dans le cas de *loqs*, le premier domaine est de type A, tandis que les deux autres domaines sont associés à la liaison aux protéines (Miyoshi *et al.*, 2010). Considérant le fait que les deux domaines de *loqs2* dérivent du premier domaine de *loqs*, cela suggère que la protéine pourrait présenter une très forte affinité avec l'ARNdb, et que sa fonction pourrait reposer sur la force de sa liaison à ce type d'acides nucléiques, plutôt que sur sa capacité à interagir avec d'autres protéines. Cette différence dans la constitution du gène, ainsi que le peu de conservation observé entre *loqs2* et *loqs* en dehors de ces domaines suggèrent que la fonction de Loqs2 a évolué pour diverger de celle de son prédécesseur.

Nous avons par la suite réalisé des analyses complémentaires, qui ont appuyé cette hypothèse d'une diversification de la fonction de Loqs2. Nous avons pu montrer que ce gène évoluait sous une pression de sélection positive et relâchée, qui permet généralement l'accumulation libre de mutations, qui se fixent dans la population lorsqu'elles sont avantageuses (Lynch et Conery, 2000 ; Prince et Pickett, 2002). Dans ce cas, le gène dupliqué acquiert en règle générale une nouvelle fonction, qui peut compléter celle du gène d'origine ou être entièrement différente. Nous avons également pu démontrer que *loqs2* évolue encore rapidement sur une échelle plus récente, suggérant ainsi que le gène est toujours en train de se diversifier activement, pour soit continuer à affiner sa fonction, ou bien car il doit être en mesure de s'adapter rapidement à des pressions externes. Chez la drosophile, plusieurs composants de la voie de l'ARNi, à savoir *dcr-2*, *ago2* et *r2d2*, figurent parmi les 3 % de gènes qui évoluent le plus rapidement, similairement à ce que nous avons observé pour *loqs2* (Obbard *et al.*, 2006). Bien qu'il reste possible que l'évolution rapide de *loqs2* soit également motivée par cette course à l'armement avec les virus, comme c'est probablement le cas des autres gènes de l'ARNi mentionnés précédemment, il est également possible qu'elle résulte de son implication potentielle dans d'autres processus biologiques importants.

2. Propriétés antivirales de Loqs2

Nos recherches ont permis de démontrer des propriétés antivirales de Loqs2, dans certaines conditions particulières. En effet la réduction de l'expression de ce gène suivie d'une infection par DENV conduit à une augmentation de la charge virale dans la carcasse, et les moustiques exprimant Loqs2 de manière ectopique dans l'intestin présentent une diminution de la charge virale dans ce tissu, ainsi qu'une dissémination moindre que les moustiques de référence (Olmo *et al.*, 2018). Ces expériences ont été à la base de notre hypothèse selon laquelle Loqs2 est une protéine antivirale, et nous avons cherché à caractériser davantage cette protéine, et à comprendre les mécanismes par lesquels elle peut être impliquée dans ce processus.

Une première étude de la fonction de Loqs2, a été réalisée qui a permis l'identification de 79 potentiels interactants de cette protéine, dans des cellules Aag2 surexprimant Loqs2, parmi lesquels les partenaires les plus significatifs étaient les deux principaux cofacteurs de la voie des siRNA, Loqs et R2d2 (Olmo *et al.*, 2018). Ces résultats ont à nouveau corroboré nos hypothèses selon lesquelles Loqs2 était fortement lié à la

défense antivirale, et en particulier à la voie de l'ARNi. Afin de préciser ces résultats, nous avons répété ces expériences *in vivo*, utilisant des moustiques surexprimant Loqs2 dans les intestins, ces mêmes moustiques transgéniques chez lesquels nous avions initialement identifié les propriétés antivirales de Loqs2. De nouveau, Loqs est ressorti comme un des principaux interactants parmi les 15 candidats identifiés. Nous avons choisi d'étudier en particulier 6 de ces candidats pour commencer, ayant tous des propriétés antivirales démontrées ou supposées : deux protéines de la voie de l'ARNi, Loqs et Dcr-2, deux dsRBP ayant des orthologues chez la drosophile, AAEL004859 (orthologue de mle) et AAEL012964 (orthologue de Dip1), et enfin deux protéines inconnues, spécifiques des moustiques Aedes, ou Culicidae, AAEL004699 (aBravo) et AAEL022113, respectivement. Ce dernier candidat sélectionné, AAEL022113, n'a pas encore été décrit dans la littérature, mais ses caractéristiques particulières, telles que la présence de motifs de liaison à l'ARN, et son profil d'expression spécifique dans les tissus de moustiques rappellent ceux observés pour Loqs2, et font de cette protéine un candidat intéressant à caractériser également.

L'étude en particulier de l'orthologue de maleless (mle) et de AAEL022113, deux protéines dont l'implication dans la réponse antivirale n'avait pas encore été démontrée expérimentalement, nous a permis de confirmer les propriétés antivirales de mle chez *Ae. aegypti*, et son implication dans le contrôle de la réplication virale dans l'intestin, cependant son rôle dans le contrôle de l'infection systémique reste à déterminer. Nous avons également caractérisé un premier rôle pour la protéine AAEL022113, précédemment inconnue, en démontrant sa capacité à réguler la réplication virale dans l'intestin des moustiques *Ae. aegypti*.

Le but de ces travaux était également de déterminer si cette fonction antivirale que nous avons observée s'étendait aux tissus où Loqs2 est naturellement exprimé. Pour ce faire, nous avons généré des moustiques dans lesquels l'expression du gène Loqs2 est inactivée. Grâce à ce nouvel outil, nous avons pu étudier l'implication de Loqs2 dans la réponse antivirale dans d'autres tissus. Nous avons montré que chez les moustiques infectés par ZIKV lors d'un repas sanguin, suivant la voie naturelle d'infection, Loqs2 ne semble pas jouer un rôle dans la défense antivirale, dans aucun des tissus testés. Nos résultats indiquent également que même dans le cas d'une infection systémique directe, suite à l'injection des virus dans le thorax des moustiques, Loqs2 ne contrôle la réplication virale ou la dissémination dans d'autres tissus.

Étant donné que c'est dans les ovaires que Loqs2 présente la plus forte expression, nous avons également étudié la possibilité que cette protéine soit impliquée dans le contrôle de la transmission verticale des virus. Il a été démontré que les moustiques *Ae. aegypti* sont capables de transmettre des arbovirus, tels que le DENV et le ZIKV, à leur progéniture (Sánchez-Vargas *et al.*, 2018 ; Lai *et al.*, 2020 ; Manuel *et al.*, 2020). Bien que très variables, dépendant surtout des procédures expérimentales utilisées, ces taux étaient systématiquement assez faibles. Nous avons choisi de travailler avec deux virus spécifiques aux insectes, HTV et PCLV, pour tester cette hypothèse. Ces virus sont présents dans la nature, infectant la plupart des populations d'*Ae. aegypti*, et sont transmis verticalement (Olmo *et al.*, 2023). Nous avons pu montrer que, bien que n'affectant pas la charge virale globale dans la descendance, l'absence de Loqs2 semblait

affecter la prévalence de l'infection. Pour les deux virus, nous avons observé deux fois plus de progénitures porteuses des virus dans la lignée n'exprimant plus Loqs2, par rapport à la lignée de type sauvage. Nous supposons que les tests statistiques effectués n'ont pas considéré cette différence comme étant significative en raison du faible nombre de moustiques testés, et ces expériences devront être répétées afin de confirmer nos observations.

3. Fonction naturelle de Loqs2

Comme nous avons pu le démontrer, la fonction antivirale observée pour Loqs2 lorsqu'elle est exprimée de manière ectopique dans l'intestin moyen ne semble pas s'étendre aux tissus où Loqs2 est naturellement exprimée. Compte tenu de la variété habituelle des fonctions observées pour les dsRBPs, un autre objectif de notre travail était d'étudier et de caractériser d'autres rôles possibles pour cette protéine, dans le contexte de son expression naturelle.

Nous avons observé que les niveaux d'expression de *loqs2* varient considérablement entre tous les tissus, avec l'expression la plus élevée dans les ovaires et le corps gras, suivis de près par les embryons précoces, tandis que les niveaux d'expression diminuent de manière significative de la fin de l'embryogénèse à l'âge adulte (Akbari *et al.*, 2013 ; Matthews *et al.*, 2016 ; Estevez-Castro *et al.*, 2021). De manière intéressante, il a également été observé que la surexpression de *loqs2* sous le contrôle d'un promoteur ubiquitaire chez les moustiques conduisait à un arrêt du développement au stade larvaire, entravant la nymphose (Estevez-Castro *et al.*, 2021). Les analyses du transcriptome de ces larves ont révélé un déséquilibre du métabolisme cependant, aucun changement majeur dans l'abondance des petits ARN dérivés de la voie de l'ARNi n'a été observé (Estevez-Castro *et al.*, 2021). Ces résultats ne permettent pas de définir un rôle clair pour Loqs2 dans cet arrêt du développement, et l'étude de l'abondance des petits ARN (miARN, siARN et piARN) n'a pas mis en évidence de changements significatifs, suggérant ainsi que cette fonction ne serait pas dépendante des voies de l'ARNi (Estevez-Castro *et al.*, 2021).

Pour mieux caractériser cette fonction, nous avons développé une nouvelle méthode pour inactiver des gènes d'intérêt chez les moustiques, à ajouter au répertoire des outils disponibles pour l'étude des moustiques *Aedes*. Deux méthodes, utilisant la technique CRISPR-Cas9, ont été précédemment décrites dans la littérature, reposant soit sur l'injection d'ARN guides dirigés vers le gène cible en plus d'une protéine Cas9 purifiée, soit sur des moustiques transgéniques exprimant déjà la protéine Cas9 dans la lignée germinale (Kistler *et al.*, 2015 ; Li *et al.*, 2017). Nous avons cherché à développer une nouvelle approche pour la génération de ces moustiques dits « *knock-out* » (KO) pour nos gènes d'intérêt, toujours basée sur la méthode CRISPR-Cas9, qui contournerait les limitations liées aux quantités de matériel injecté, et le risque de leur dégradation avant qu'ils n'atteignent leur cible. Nous avons conçu des constructions exprimant la protéine Cas9 sous le contrôle du promoteur germinal *Exuperentia*, ainsi que 3 ARNs guides sous le contrôle de différents promoteurs U6, et un marqueur de fluorescence et les avons insérés dans le génome de nos moustiques par transgénèse. Ce marqueur de fluorescence nous a permis d'effectuer une première sélection dans la génération G0, en

sélectionnant seulement les larves présentant une expression transitoire, et de les croiser entre-elles. Dans la génération suivante, nous avons pu utiliser la fluorescence pour sélectionner les moustiques exprimant la construction et les génotyper pour trouver des mutants. Ces deux cycles de sélection avant l'étape de génotypage nous ont permis de réduire considérablement le nombre de moustiques à génotyper avant de trouver un moustique chez lequel le gène serait inactivé sur les deux allèles. D'autres croisements nous ont permis de débarrasser les moustiques de la construction, et d'obtenir une lignée dans laquelle le gène d'intérêt est inactivé. Nous avons constaté que cette méthode nécessitait moins de ressources et de temps que celles que nous avions expérimentées précédemment, et nous avons pu utiliser cette approche pour générer une lignée dans laquelle loqs2 est inactivé, à laquelle il sera fait référence sous le nom de Losqs2 KO.

Ce nouvel outil que nous avons généré a été essentiel pour la caractérisation de la fonction de Loqs2. Tout d'abord, nous avons pu démontrer que le KO était transmis aux descendants suivant une héritabilité mendélienne chez les femelles, avec environ 20 % de moustiques présentant le génotype sauvage (WT), 50 % d'hétérozygotes et 30 % de KO. Chez les mâles, en revanche, les taux d'héritabilité étaient radicalement différents, et nous avons été surpris de n'y retrouver que 2 % de KO. Une hypothèse possible pour expliquer ce biais lié au sexe serait la localisation génomique de loqs2. Ce gène est en effet localisé sur le premier chromosome d'*Ae. aegypti*, à proximité immédiate du locus déterminant le sexe m/M, une région de 63 Mbp contenant de multiples gènes spécifiques au sexe, tels que *myo-sex* et *nix*, et connue pour présenter une recombinaison quasi nulle (Fontaine *et al.*, 2017). Afin de préserver cette région, il est probable que les taux de recombinaison des gènes situés à proximité soient également perturbés, par rapport au reste du génome. Le fait que notre lignée ait été générée par un premier croisement de femelles KO avec des mâles WT est susceptible d'expliquer la diminution du nombre de mâles porteurs des mutations dans ce contexte. Cependant, il est intéressant de noter que dans la génération suivante, le pourcentage de mâles KO dans la population a été multiplié par 4.

Nous avons également pu montrer une augmentation de la survie des moustiques dépourvus de Loqs2, par rapport aux moustiques de type sauvage, ce qui suggère que la présence de Loqs2 pourrait avoir un effet préjudiciable sur la survie des moustiques. Il est important de noter que lors de nos premiers essais, en raison du nombre extrêmement faible de mâles homozygotes KO, nous n'avons pas été en mesure de générer une lignée homozygote KO. Compte tenu du biais sexuel que nous avions observé, nous avons décidé de générer notre lignée KO en croisant des femelles KO homozygotes avec des mâles hétérozygotes, de manière à conserver les femelles KO homozygotes au fil des générations et à espérer augmenter la proportion de mâles KO dans la population au fil du temps. Pour l'expérience susmentionnée, la survie des moustiques a été évaluée dans son ensemble, sans séparation des mâles et des femelles. Nous ne savons donc pas si cette amélioration de la survie des moustiques est imputable à un sexe spécifique et à son génotype correspondant, mais nous soupçonnons que cette survie est globalement une conséquence de l'absence de Loqs2. Il serait nécessaire de générer une lignée dans laquelle les mâles et les femelles sont homozygotes KO, afin de confirmer ces résultats.

Il est intéressant de noter que nous avons également pu démontrer une meilleure résistance au stress chez les moustiques dépourvus de Loqs2. Pour cette expérience en particulier, nous avons séparé les moustiques par génotype et par sexe, et nous avons surveillé individuellement leur survie en l'absence de nourriture. Nous avons constaté une différence importante entre la survie des mâles KO et celle des autres groupes, ces moustiques montrant une résistance accrue à ce stress particulier, bien qu'aucun moustique n'ait survécu plus de 10 jours dans ces conditions. Ces résultats suggèrent à nouveau que la présence de Loqs2 affecte négativement la survie des moustiques, ce qui soulève la question de savoir comment ce gène a pu être sélectionné et fixé dans la population en premier lieu. Il serait intéressant d'évaluer la résistance de ces moustiques à d'autres types de stress, tels que la sécheresse, les changements de température, ainsi que leur résistance à différents types d'insecticides. Il est possible que, bien que Loqs2 semble réduire leur capacité à résister au stress auquel nous les avons soumis, il en aille autrement dans le cas d'un autre type de stress.

Pour mieux caractériser la fonction de Loqs2, nous avons étudié l'effet des mutants de perte de fonction sur le développement des moustiques. Nous avons pu montrer une différence significative en termes de fertilité de ces moustiques dépourvus de Loqs2, chez lesquels nous avons observé une diminution de 20 % de leur progéniture, en comparaison avec les moustiques de type sauvage, bien qu'il n'y ait pas de différence significative dans le nombre d'œufs pondus par femelle. Cela semble indiquer que Loqs2 est activement impliqué dans le développement des œufs, comme le suggéraient déjà les niveaux élevés d'ARNm de Loqs2 détectés dans les embryons précoces, indiquant un possible dépôt maternel (Akbari *et al.*, 2013 ; Estevez-Castro *et al.*, 2021). Cette importance de Loqs2 dans l'ovogenèse rappelle celle d'une autre dsRBP, Staufen, dont il a été démontré qu'elle est essentielle à la détermination de la polarité de l'œuf, à travers le transport d'ARNm spécifiques d'un pôle de l'œuf à l'autre (Johnston *et al.*, 1991 ; Tian *et al.*, 2004). Bien que nous ne connaissions pas encore les mécanismes par lesquels Loqs2 est impliquée dans la fertilité, il est clair que cette protéine joue un rôle important dans ce processus. Il a également été démontré que les infections virales avaient un impact sur différents aspects de la vie des moustiques, de leur survie globale à leur fécondité (Styer *et al.*, 2007 ; Kramer et Ciota, 2015). Il est possible que Loqs2 fonctionne d'une manière qui permette aux moustiques de contrebalancer ce coût de l'infection.

En suivant le développement de ces moustiques jusqu'à l'âge adulte, nous avons également pu mettre en évidence une légère réduction de la survie des moustiques dépourvus de Loqs2, ce qui laisse supposer une implication possible de cette protéine à des stades de développement plus tardifs. Il est intéressant de noter que dans ce cas, la plupart des décès ont eu lieu soit à la transition entre le stade larvaire et le stade nymphal, soit à la transition vers l'âge adulte. Ces résultats rappellent les observations faites chez les moustiques surexprimant loqs2 de manière constitutive, chez lesquels le développement a été arrêté juste avant la nymphose (Estevez-Castro *et al.*, 2021). Dans ce cas, il a été démontré que cette interruption du développement était probablement due à un déséquilibre métabolique, bien que le rôle réel de Loqs2 dans ce processus n'ait pas été déterminé. Il serait intéressant d'étudier la possibilité que la perte de Loqs2 déclenche le même type de réponse que sa surexpression chez le moustique. Ce phénotype est cependant surprenant, étant donné que Loqs2 n'est exprimé qu'à de très

faibles niveaux au cours du développement, et que son expression ne semble pas être affectée au cours des différentes phases (Harker *et al.*, 2013 ; Estevez-Castro *et al.*, 2021).

Nous avons pu démontrer que le rôle naturel de Loqs2 semblait plutôt orienté vers le développement que vers la réponse antivirale, ce qui a également été corroboré par nos analyses de l'interactome de cette protéine dans les ovaires. Nous n'avons identifié aucune protéine antivirale connue parmi les partenaires potentiels de Loqs2 dans ce tissu, à l'exception de Loqs, bien qu'elle ne soit pas aussi fortement enrichie que dans notre analyse des partenaires de Loqs2 dans l'intestin. Nos analyses ont également montré qu'environ un tiers des candidats identifiés étaient impliqués dans l'épissage, quelques-uns d'entre eux étant parmi les interactants les plus significatifs et les plus enrichis. Certaines protéines impliquées dans ce processus biologique ont également été identifiées dans nos analyses de l'interactome de Loqs2 dans l'intestin. De manière intéressante, il a été montré que certaines protéines ayant des fonctions antivirales sont impliquées dans l'épissage, comme Adar et mle, qui ont été identifiées comme partenaires de Loqs2 dans son interactome dans les ovaires et dans l'intestin, respectivement (Cugusi *et al.*, 2015 ; Deng *et al.*, 2020). Par ailleurs, il a été montré que Dcr-2 interagit avec des protéines impliquées dans l'épissage, telles que Syp, yps et nonA, dont nous avons détecté les orthologues dans l'interactome de Loqs2 dans l'intestin moyen (Rousseau, 2022). Il serait intéressant de caractériser davantage les interactions de Loqs2 avec cette voie, et d'étudier tout effet potentiel qu'elle pourrait avoir sur ses fonctions antivirales. Dans ce but, le transcriptome de nos mutants de perte de fonction de Loqs2 pourrait être étudié, afin d'identifier d'éventuels défauts d'épissage.

Il est intéressant de constater la variété des partenaires avec lesquels Loqs2 semble interagir, et en particulier, il semble que certaines de ces protéines se localisent principalement dans différents compartiments cellulaires, les partenaires de la voie de l'ARNi se localisant principalement dans le cytoplasme, tandis que les protéines liées à l'épissage sont plutôt nucléaires. Nous avons pu étudier la localisation cellulaire de Loqs2 in vivo, dans les ovaires, en utilisant des anticorps spécifiques à cette protéine. Ces premiers résultats suggèrent que Loqs2 se localise principalement dans le cytoplasme, particulièrement à la périphérie du noyau. Bien que cette expérience soit préliminaire et ne permette pas une grande résolution concernant la localisation subcellulaire exacte, l'absence de détection dans le noyau était claire, soulevant la question de savoir comment Loqs2 pourrait interagir avec les protéines nucléaires. Il est intéressant de noter qu'il a été précédemment démontré que Loqs2 était préférentiellement nucléaire lorsque la protéine était surexprimée dans les cellules Aag2 (Estevez-Castro *et al.*, 2021). En étudiant plus en détail la séquence de Loqs2, les outils bio-informatiques nous ont permis d'identifier des potentiels signaux de localisation nucléaire, suggérant que Loqs2 a effectivement la capacité d'entrer dans le noyau. La différence observée concernant la localisation de Loqs2 entre ces deux conditions, *in vivo* et *in cellulo*, pourrait être expliquée, en partie, par la différence des niveaux d'expression, étant donné que la protéine a été surexprimée dans les cellules, tandis qu'elle a été exprimée à des niveaux physiologiques dans les ovaires. Une autre explication probable est que Loqs2 peut effectivement entrer dans le noyau, mais uniquement dans des conditions spécifiques, comme c'est le cas pour les protéines PIWI. Ces protéines se trouvent dans le cytoplasme jusqu'à ce qu'un piRNA y soit chargé, après quoi elles sont

transportées vers le noyau, où elles peuvent fonctionner (Ramos-Nino *et al.*, 2022). Il est possible que Loqs2 fonctionne de manière similaire et que la protéine nécessite d'être activée d'une manière ou d'une autre pour pouvoir être transportée dans le noyau, et que les conditions n'aient pas été réunies pour que cela se produise dans notre expérience in vivo. Il serait intéressant de caractériser davantage cette capacité de Loqs2 à se localiser potentiellement dans différents compartiments cellulaires, et l'identification de ses cibles ARN pourrait également aider à comprendre les mécanismes par lesquels la protéine pourrait être activée.

Pour mieux préciser le rôle de Loqs2 dans la biologie des moustiques, nous avons également étudié l'effet de la perte de Loqs2 sur le transcriptome des ovaires. Nos analyses ont révélé que l'absence de Loqs2 semblait avoir un effet très faible sur l'expression des gènes, avec un peu plus d'une douzaine de gènes significativement régulés à la hausse ou à la baisse, par rapport au transcriptome de la lignée WT. La même chose a été observée en comparant le transcriptome des ovaires Loqs2 KO à celui des moustiques Het, l'expression d'un petit sous-ensemble de gènes seulement étant perturbée. Cela indiquerait donc qu'une seule copie de Loqs2 pourrait suffire à assurer la plupart des fonctions de Loqs2 dans les ovaires. Dans l'ensemble, peu de voies semblent avoir été affectées par la perte de Loqs2, cependant, parmi celles qui ont été affectées, plusieurs se sont également révélées perturbées par la surexpression constitutive de Loqs2 dans les larves, comme la voie de dégradation des acides gras, qui est régulée négativement dans les deux conditions (Estevez-Castro *et al.*, 2021).

Parmi les gènes dont l'expression a été affectée par la perte de Loqs2 dans les ovaires, en comparant les gènes WT et hétérozygotes, nous avons observé de nombreux gènes non caractérisés, dont la plupart semblent avoir une activité de liaison aux acides nucléiques et se localiser dans le noyau, ce qui corrobore davantage l'hypothèse selon laquelle Loqs2 pourrait voyager entre les compartiments cellulaires. Il est intéressant de noter que, parmi les gènes caractérisés dont l'expression était régulée à la hausse dans nos mutants KO, nous avons identifié plusieurs gènes codant pour des ARN liés à l'épissage, ce qui coïncide avec nos conclusions selon lesquelles Loqs2 interagit probablement avec des protéines impliquées dans l'épissage dans les ovaires. Nous avons également observé une régulation négative de l'expression de plusieurs gènes d'histones chez les mutants dépourvus de Loqs2. Ces résultats impliquent un rôle potentiel de Loqs2 dans la régulation de l'expression de ces gènes, bien que nos analyses de l'interactome de Loqs2 dans les ovaires n'aient pas indiqué d'enrichissement significatif de ces protéines. Il est intéressant de noter que l'expression des gènes d'histones, en particulier de l'histone H4, a été suggérée comme pouvant être régulée par la voie des piRNA, des piRNA dérivés des histones, portant une signature ping-pong, ayant été détectés dans les cellules Aag2 d'*Ae. aegypti* (Girardi *et al.*, 2017). Compte tenu des résultats précédemment obtenus sur la localisation cellulaire de Loqs2, il est possible que son effet sur l'expression des gènes d'histones repose sur un mécanisme similaire, par lequel Loqs2 se lierait à un petit ARN et serait par la suite transporté vers le noyau pour réguler l'expression de ces gènes. D'autres études sur les effets de Loqs2 sur l'abondance des petits ARN sont nécessaires, ce qui permettrait de mettre en lumière l'implication de Loqs2 dans ces mécanismes.

Par ailleurs, des protéines histones (H4 et H2B) ont été identifiées parmi les protéines du sperme qui sont transférées chez les femelles *Ae. aegypti* lors de l'accouplement (Sirot *et al.*, 2011). À ce stade, il serait intéressant d'étudier l'interactome protéique de Loqs2 dans les testicules afin de déchiffrer son interaction avec ce type de protéines. Ces connaissances pourraient permettre de mieux comprendre les mécanismes par lesquels Loqs2 affecte la fertilité des moustiques, ce qui pourrait également être imputable à un effet de la protéine sur les capacités reproductives des mâles. Les histones sont également largement exprimées au cours de l'ovogenèse, les niveaux d'ARNm s'accumulant rapidement vers la fin du processus, contribuant largement au pool d'ARNm déposés par la mère, qui sont alors essentiels pour les premiers stades de l'embryogenèse (Anderson, 1980 ; Ruddell et Jacobs-Lorena, 1985). L'effet de Loqs2, augmentant l'expression des gènes d'histones, dans ce contexte de reproduction, pourrait donner un premier aperçu des avantages considérables que cette protéine a procurés aux moustiques, expliquant pourquoi elle s'est fixée dans le génome malgré son coût en termes de survie.

En conclusion, cette thèse a fourni des informations précieuses sur la caractérisation de la protéine Loqs2 spécifique aux moustiques *Aedes*, ainsi que sur son origine, son histoire évolutive et ses fonctions potentielles chez les moustiques. Dans l'ensemble, cette recherche met en évidence le potentiel de Loqs2 en tant que candidat pour le double ciblage de la réduction de la population de moustiques et de la réduction de la transmission du virus. Notre travail nous a permis de commencer à élucider certaines des caractéristiques de Loqs2, tout en soulevant de nombreuses nouvelles questions passionnantes, et d'autres études sont maintenant nécessaires pour étudier les mécanismes précis par lesquels cette protéine fonctionne, et pour comprendre pleinement son potentiel et ses limites dans la lutte continue contre les arbovirus.

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ORIGIN AND FUNCTION OF LOQS2, A MOSQUITO-SPECIFIC DOUBLE-STRANDED RNA-BINDING PROTEIN

RESUME

L'objectif de ce projet de thèse était de caractériser un gène récemment identifié, Loqs2, encodant une protéine de liaison à l'ARN double brin spécifique des moustiques Aedes, dont l'implication dans le contrôle de la réplication du virus de la dengue a été démontrée, lors de son expression ectopique dans l'intestin.

Dans ces travaux, nous avons étudié l'histoire évolutive de loqs2, dont l'apparition remonte à 67 millions d'années, suite à une duplication d'un domaine de son paralogue, loqs. Nous avons exploré les fonctions biologiques de Loqs2 et montré l'importance de son rôle dans la survie et la reproduction, ainsi qu'un potentiel effet sur la régulation de l'expression des gènes. Nos résultats suggèrent également que l'activité antivirale de Loqs2 est restreinte aux intestins.

Dans l'ensemble, nos travaux ont permis de mettre en lumière la complexité des multiples rôles de Loqs2, et a ouvert la voie pour de futures études visant à caractériser ses fonctions naturelles.

Mots-clés : Loqs2 - dsRBP - Antiviral - RNAi - Ovaires - Fertilité

SUMMARY

The aim of this thesis project was to decipher the interactions between arboviruses and their vectors (Ae. aegypti here), by focusing on the characterization of a recently identified protein, Loqs2. This Aedes-specific dsRBP, has been shown to be involved in the control of DENV replication when ectopically expressed in the midgut.

In this work, we studied the origin and the evolutional history of loqs2, that originated 67 MYA, from a domain duplication of its paralog loqs. We also further investigated the antiviral activity in this protein, that seems to be restricted to the midgut. Finally, we explored the other biological mechanisms that Loqs2 could be involved in and showed an important role in fitness and reproduction, and a potential effect on the regulation of gene expression.

Overall our work uncovered multiple layers of complexity on the roles of this protein, and led the way for further studies to characterize the mechanisms by which it functions.

Keywords: Loqs2 - dsRBP - Antiviral - RNAi - Ovaries - Fertility