

Nucleoporin 358: a novel factor in *Drosophila melanogaster* antiviral immunity

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“A espantosa realidade das coisas
É a minha descoberta de todos os dias.”

(The amazing reality of things
Is my everyday discovery.)

Alberto Caeiro

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The discovery process is innate to those who dare to be curious, to question themselves, and to let the answers shape their growth. Shall never this path be explored by a sole soul, as each new encounter unveils a new amazing reality of things.

No discovery made during this past semester would have ever been possible if the Saleh Lab had not accepted me as one of its members. To every single one of them, I could not be more grateful for their genuine kindness and support throughout my internship. To them I owe the discovery of a new way of making science, of a new research field of my interest, and, most importantly, of new friendships. I would like to specifically address to Professor Carla Saleh and thank her for all the inspiring advice she gave me and for helping me discover that there are no limits to what one can do in research other than those created by oneself. I shall never forget that “Anticipation is the key for success in academia”. I also want to thank Sabrina for teaching me everything I learned during this semester, for always being patient and interested in clarifying my curiosities, and for motivating me when science seemed to fail. To everyone in the Saleh Lab, I wish the best the future can offer them.

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Indubitavelmente, apenas se passaram cinco anos de descobertas de espantosas realidades.

ABSTRACT

Drosophila melanogaster is a model organism to study insect antiviral immunity, enabling to predict diverse virus-insect interactions, including those between arthropod-borne viruses (arboviruses) and mosquitoes. The small-interfering RNA (siRNA) pathway is the main antiviral response in *Drosophila* and is dependent on the recognition of virus-derived double-stranded RNA (dsRNA) structures to exert its function. dsRNA is also necessary to attain systemic antiviral immunity in the fruit fly, emphasising its role as a hallmark of viral infection. Unpublished data from the Saleh Lab identified several *Drosophila* proteins that bound to dsRNA, therefore with the potential to participate in the response to viral infection. Among them, Nucleoporin 358 (Nup358), a component of the nuclear pore complex (NPC) was identified. Here, an antiviral effect of Nup358 is described for the first time in *Drosophila* S2 cells infected with Sindbis virus (SINV) and *Drosophila* C virus (DCV). Indeed, following the knock down of Nup358 mRNA in cell culture, increased levels of SINV and DCV replication were observed, confirming a role of this protein in the antiviral response in *Drosophila*. Resorting to two lines of Nup358-silenced mutant flies, the antiviral effect of Nup358 *in vivo* was also tested by infecting the fly lines with SINV and DCV. These results were not conclusive, mainly due to technical limitations and lack of time to perform all the required experiments. Collectively, my data identify a new factor, Nup358, that is required to control virus infection in *Drosophila* cells.

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ABBREVIATIONS

Abbreviation	Explanation
Ago2	Argonaute-2 (<i>Drosophila melanogaster</i>)
AL	Annulate lammellae
AMP	Antimicrobial peptide
ANE	Acute necrotising encephalopathy
CrPV	Cricket paralysis virus
DAV	<i>Drosophila</i> A virus
DCV	<i>Drosophila</i> C virus
dsAgo2	dsRNA targeting Ago2 mRNA
dsControl	dsRNA targeting GL3 mRNA
dsDNA	double-stranded DNA
dsNup358	dsRNA targeting Nup358 mRNA
dsRBD	dsRNA binding domain
dsRBP	dsRNA binding protein
dsRNA	double-stranded RNA
DXV	<i>Drosophila</i> X virus
ER	Endoplasmic reticulum
FG	Phe-Gly repeats
FHV	Flock House virus
GFP	Green fluorescent protein
GL3	<i>Photinus pyralis</i> luciferase protein
HIV-1	Human immunodeficiency virus type 1
HsAgo	<i>Homo sapiens</i> proteins of the Argonaute family
HsAgo2	<i>Homo sapiens</i> Argonaute-2 protein
HsNup358	<i>Homo sapiens</i> Nup358
IR	Internal repeat
JAK-STAT	Janus kinase – signal transducer and activator of transcription

Loqs-PB/PD	Loquacious isoform B/D
miRNA	microRNA
mRNP	messenger ribonucleoprotein
NE	Nuclear envelope
NPC	Nuclear pore complex
NTR	Nuclear transport receptor
Nup	Nucleoporin
Nup358/RanBP2	Nucleoporin 358 (<i>Drosophila melanogaster</i>)
PAMP	Pathogen-associated molecular pattern
PAZ	Piwi/Argonaute/Zwille domain
P body	Processing body
PFU	Plaque forming unit
piRNA	Piwi-interacting RNA
PRR	Pattern recognition receptor
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase III	Type III ribonuclease
SG	Stress granule
shNup358	short hairpin targeting Nup358 mRNA
SINV	Sindbis virus
SINV-RLuc	SINV expressing <i>Renilla</i> luciferase
SINV-WT	SINV wild type
siRNA	small interfering RNA
TCID₅₀	Median tissue culture infectious dose
UAS	Upstream activation sequence

CHAPTER I: INTRODUCTION

Drosophila melanogaster as a model for insect viral infection

Drosophila melanogaster, commonly known as the fruit fly, has been the model insect of choice since its use in genetic research carried on by Thomas Morgan at the beginning of the 20th century. Fruit flies are easy and inexpensive to culture and have short life cycles – around 10 days from the embryo to the adult stage – which allow a fast scale-up (Fig. I). Also, their simple and non-redundant genome enabled the creation of a well-established genetic toolbox (Hales et al., 2015). These characteristics have rendered *D. melanogaster* extremely useful to address fundamental questions in cell and molecular biology, developmental biology, neurobiology, and immunity over the past 100 years.

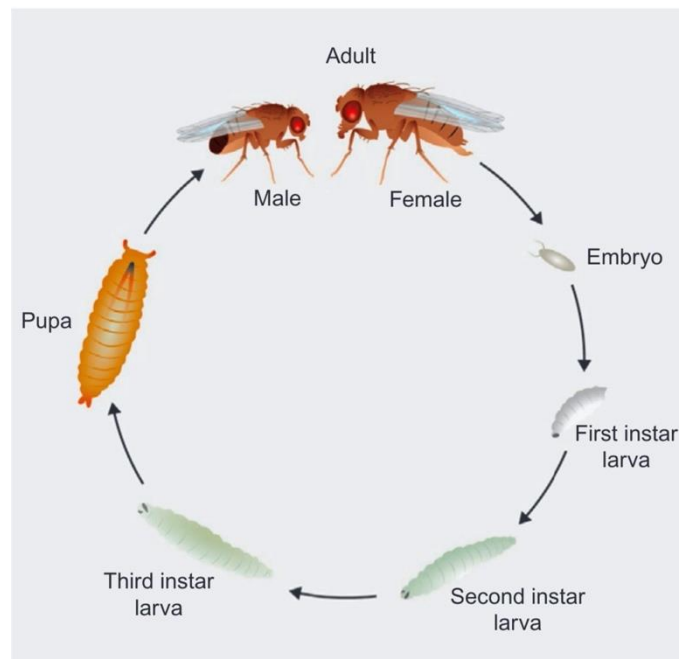


Figure I | *Drosophila melanogaster* life cycle. *Drosophila* is a holometabolous organism with complete metamorphosis. The life cycle includes an embryo, different larval stages (instar), pupa, and finally emergence as an adult. Illustration from Mondotte & Saleh, 2018.

Insects lack adaptive immunity, contrarily to vertebrates, and they are completely dependent on their innate immune responses to be protected against pathogens. The basis of such responses generally, but not exclusively, involves host pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPs) and trigger both humoral and cellular defence mechanisms (Buchon et al., 2014). *Drosophila melanogaster* has been crucial to describe these

immune pathways, which are highly conserved among insects. This is particularly relevant to predict and establish how the immune system of mosquitoes works, and further understand the interactions between the mosquito host and the mosquito-borne human-infecting pathogens. Mosquitoes act as vectors of pathogens that cause severe diseases to humans, but not to the vector itself. These pathogens can be bacteria, parasites, or viruses known as arthropod-borne viruses (arboviruses) such as Zika, Dengue and Chikungunya viruses. The genome sequencing of different mosquito species (Holt et al., 2002; Nene et al., 2007) allowed to identify genes that are orthologous to antiviral immunity-related genes in *D. melanogaster*, further enabling the description of the immune response against viruses in mosquitoes (Waterhouse et al., 2007). The ability to trigger a persistent infection of an arbovirus in *D. melanogaster* cells (Mudiganti et al., 2006) was revolutionary and led to widespread use of this model in insect-arbovirus research (Chotkowski et al., 2008; Mukherjee & Hanley, 2010).

Drosophila melanogaster's antiviral immune response

The main antiviral immune response in insects is the RNA interference (RNAi) mechanism, responsible for silencing nucleotide structures via the production of small non-coding RNAs. So far, three main pathways have been described based on the origin, length and function of the small RNAs (Fig. II). The micro-RNA (miRNA) pathway regulates gene expression at the post-translational level and mainly participates in developmental and homeostatic processes (Carrington & Ambros, 2003). The Piwi-interacting RNA (piRNA) pathway is crucial for protecting the germline against nuclear transposable elements (Aravin et al., 2001). Lastly, the small interfering RNA (siRNA) pathway is the one specifically involved in broad antiviral defence (Hammond et al., 2000; Kingsolver et al., 2013; Parrish et al., 2000). These pathways crosstalk and some miRNAs have been reported to exert a role against viruses in *Drosophila* (Monsanto-Hearne et al., 2017), while piRNAs were shown to be antiviral in mosquitoes (Suzuki et al., 2020).

The siRNA mechanism is initiated in the cytoplasm when the RNase III endonuclease Dicer-2 binds to long double-strand RNA (dsRNA) molecules (Bernstein et al., 2001) from either exogenous or endogenous sources. Exogenous dsRNAs are mainly of viral origin. The types of genomes of many viruses – dsRNA, single-stranded RNA or DNA – lead to the production of intermediate dsRNA forms during replication. This allows Dicer-2 to recognise these viral dsRNA and cleave them into smaller RNA duplexes of approximately 21 nucleotides (nt) with 2-nt overhangs at the 3'-end of both strands – the siRNA (Elbashir, Lendeckel, et al., 2001; Elbashir, Martinez, et al., 2001). Dicer-2 and its cofactor, R2D2 (Q. Liu et al., 2003), then load the viral siRNA onto an Argonaute-2(Ago2)/pre-RNA-induced silencing

complex (RISC), which unwinds the siRNA duplexes into two individual strands. One of them – passenger strand – is removed while the other – guide strand – remains bound to the catalytic core of RISC – the Ago2/holo-RISC (Pham et al., 2004). Via sequence complementarity, the guide strand binds to its viral RNA targets, and Ago2 cleaves them leading to their degradation (Hammond et al., 2001). The sequence-specificity of the siRNA response renders it an effective broad antiviral response against all types of viruses.

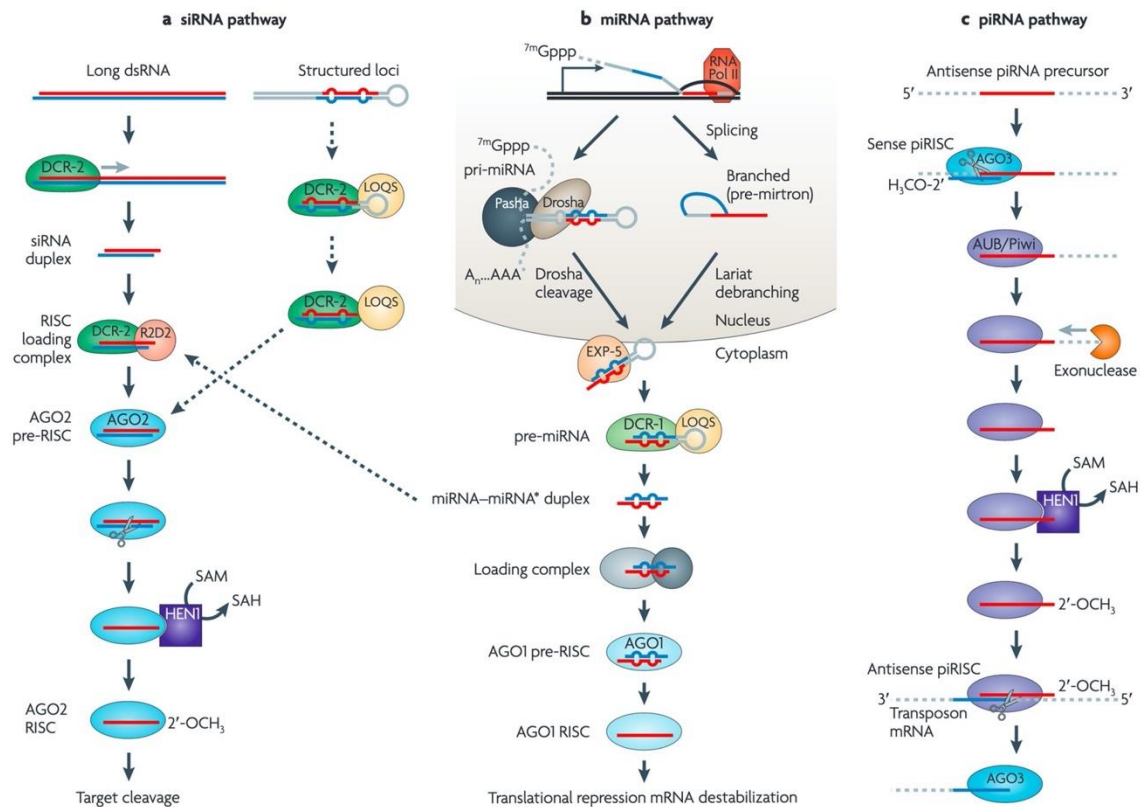


Figure 11 | RNA interference (RNAi) pathways in *Drosophila*. Small-interfering RNA (siRNA) (a), microRNA (miRNA) (b) and Piwi-interacting RNA (piRNA) (c) pathways are the three small RNA silencing pathways. The siRNA pathway (a) is the main mediator of the antiviral response, being activated upon recognition of long dsRNA from viral origin. Illustration from Ghildiyal & Zamore, 2009.

The siRNA pathway constitutes the main antiviral mechanism in *Drosophila*. Indeed, mutant flies for the genes coding for Dicer-2 (Galiana-Arnoux et al., 2006), R2D2 (Wang et al., 2006) or Ago2 (van Rij et al., 2006) are more susceptible to infection with different RNA viruses and die faster than their wild type counterparts. This premise is further confirmed by suppressors of RNAi in the virus genomes, allowing the virus to bypass or inhibit the siRNA pathway at different levels (Chao et al., 2005; van Mierlo et al., 2012; van Rij et al., 2006).

Exogenous dsRNAs of non-viral origin (i.e., cytosol-delivered dsRNA) are also processed by the siRNA machinery, with Dicer-2 also relying on another cofactor – Loquacious (Loqs)-PD – to produce siRNA duplexes before associating with R2D2

(Marques et al., 2010). Experimental introduction of dsRNA in *Drosophila* cells (e.g., by transfection *in vitro*) is particularly convenient to silence endogenous mRNA and study the effects in diverse cellular mechanisms. In fact, the same post-transcriptional gene silencing strategy is used by host cells to target protein-coding genes and transposable elements (Czech et al., 2008). Endogenous siRNA synthesis is also Dicer-2/Loqs-PD-dependent and these duplexes are originated from structured genomic loci or convergent transcription units in the fly genome.

Some immune signalling pathways have also been proposed to be antiviral against certain *Drosophila*-specific viruses and arboviruses (Fig. III). The Toll and Imd pathways are mostly responsible for detecting and mounting an immune response against bacteria and fungi that greatly relies on the production of antimicrobial peptides (AMPs). However, mutant flies for genes encoding components of the Toll pathway exhibited an increased susceptibility to *Drosophila* C virus (DCV) and other RNA viruses (Gil Ferreira et al., 2014; Zambon et al., 2005). By applying the same strategy to Imd components, this pathway was found to mediate an antiviral response in flies against the replication of Sindbis virus (SINV), an arbovirus (Avadhanula et al., 2009). Among others, the *Drosophila* JAK-STAT pathway (Dostert et al., 2005) and Toll-7-mediated autophagy (Nakamoto et al., 2012) are additional examples of alternative host cells mechanisms that only are antiviral against specific viruses, in contrast to RNAi.

One fascinating characteristic of the antiviral RNAi response, is its capacity to confer systemic immunity to infected flies, i.e. not only protecting the site of infection but also distal locations. This is attained by the systemic spread of the siRNA response (Saleh et al., 2009). It has been hypothesised that during spread of the RNAi response, viral dsRNAs from infected cells are released following cell lysis and uptake by non-infected cells in distant sites to confer protection. An alternative, and not mutually exclusive, mechanism has also been proposed: the uptake of viral dsRNA or viral particles by circulating immune cells in *Drosophila* allows them to convert viral RNA molecules into viral DNA, which is further used to synthesise *de novo* secondary siRNAs. Such siRNAs are finally secreted in exosome-like vesicles that fuse with the membranes of cells in distal tissues eliciting an antiviral response (Tassetto et al., 2017). These findings highlight the role of dsRNA as a hallmark of viral infection in *Drosophila*. Although it is known that dsRNA is internalised in the cells via the endocytic pathway (Saleh et al., 2006), the identity of the dsRNA cell surface receptor has remained elusive.

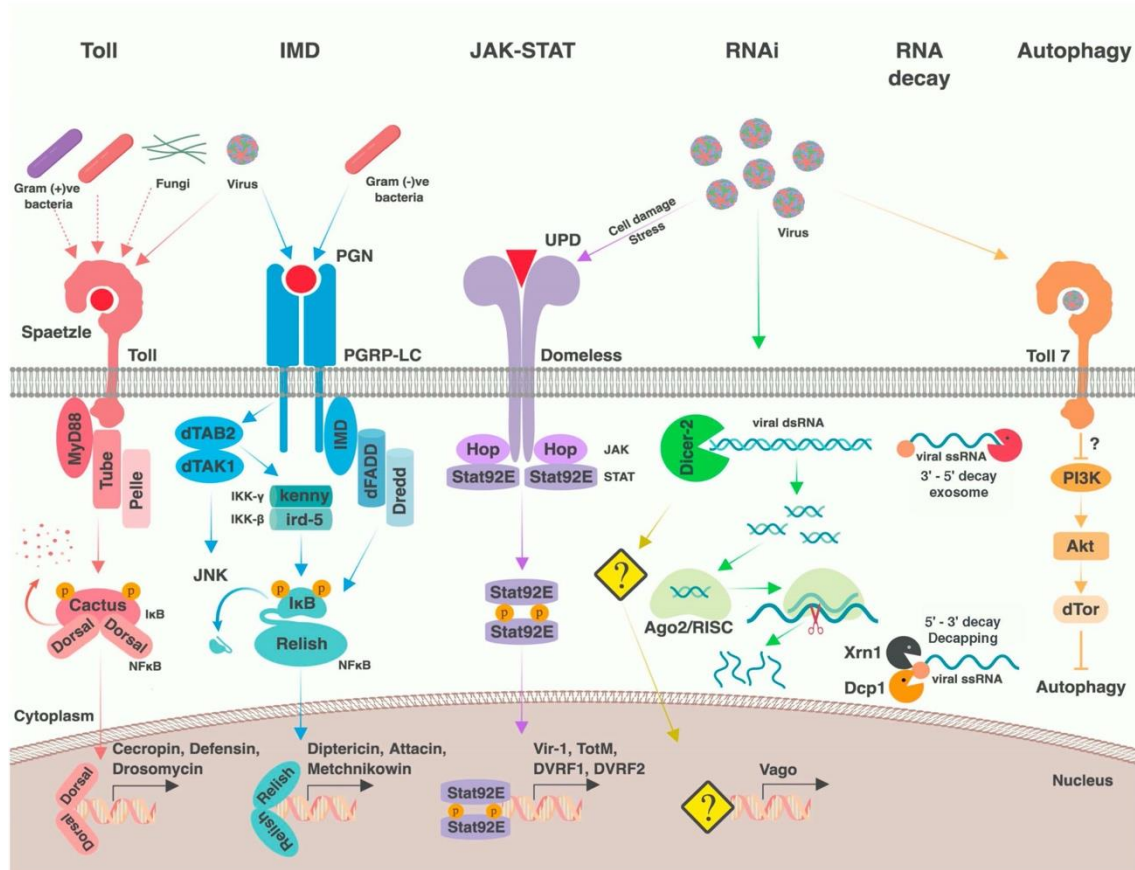


Figure III | Additional immune pathways in *Drosophila* with antiviral roles. The Toll, Imd and JAK-STAT pathways participate in the antiviral response. Viral infection can also be controlled by promoting the decay of viral single-stranded RNA forms or via Toll-7-mediated autophagy. However, the siRNA remains as the prime defence against viruses as it is a broad sequence-specific response against a wide range of viruses. Illustration from Palmer et al., 2018.

In order to identify this receptor, the Saleh Lab performed a co-immunoprecipitation designed to identify dsRNA-binding proteins in *Drosophila* cells. Subsequent mass-spectrometry analysis revealed the identity of these proteins (unpublished data), with many of them described for the first time to have the capacity to bind dsRNA. These results raised the following question: since dsRNA is a feature of viral infection, could at least one of these newly identified dsRNA-binding proteins have a role during viral infection?

dsRNA binding proteins in *Drosophila melanogaster*

Double-stranded RNA binding proteins (dsRBPs) are proteins that bind RNA duplex structures. Normally, such interactions are mediated by small domains that preferentially recognise dsRNA by their A-form RNA helix, even though sequence-specificity and other RNA features may also be determinant (Masliah et al., 2013). These domains are canonical dsRNA binding domains (dsRBDs), which comprise a sequence of approximately 65 to 70 amino acids displayed in an α - β - β - β - α fold, with the α -helixes packed against the anti-parallel β -sheets. In addition to binding dsRNA,

dsRBDs also mediate protein-protein interactions (Doyle & Jantsch, 2002). dsRBDs are believed to be conserved across species as they were first described upon alignment of protein sequences of known and putative dsRBPs belonging to different species (St Johnston et al., 1992). dsRBDs are present in proteins involved in many cellular activities including post-transcriptional gene silencing (RNAi), RNA trafficking, RNA editing, and the antiviral response (Saunders & Barber, 2003).

Up to 2006, fourteen dsRBPs had been identified in *Drosophila melanogaster*, their respective functions and expression patterns were extensively reviewed in (Gamberi et al., 2006). Among those dsRBPs, both Dicer proteins (Dicer-1 and Dicer-2), that play major roles in the RNAi mechanism, were present. Dicer proteins share two dsRBDs within their structure: an atypical central dsRBD (also known as DUF283) and a C-terminal dsRBD (Welker et al., 2011). Dicer-1 is another RNase III enzyme with endonuclease activity which participates in the miRNA pathway. It is responsible for cleaving nucleus-exported precursor miRNA (pre-miRNA) into miRNA-miRNA* duplexes that will further be processed into miRNA, which are responsible for silencing endogenous mRNA (Tsutsumi et al., 2011). The central dsRBD of Dicer-1 is essential, along with other domains, to process pre-miRNA (Ye et al., 2007). The C-terminal dsRBD of Dicer-2 is indispensable for efficient binding of long dsRNA to Dicer-2 and is responsible for the production of 21-nt siRNA with high fidelity (Kandasamy et al., 2017).

Both Dicer rely on cofactors to fully execute their functions: Dicer-2 requires either R2D2 or Loqs-PD while Dicer-1 depends on a longer isoform of the Loquacious protein – Loqs-PB (Saito et al., 2005). Such cofactors also belong to the dsRBPs family as dsRBD are present within their structure. Mutant R2D2 proteins for the two dsRBD at the N-terminus revealed that both domains are required for siRNA binding to Dicer-2/R2D2 and subsequent loading to RISC; however, only the first dsRBD can bind long dsRNA (Liu et al., 2006). Loqs-PB contains three dsRBDs, the first two of which interact with dsRNA throughout Dicer-1-mediated cleavage and RISC assembly (Förstemann et al., 2005). Its C-terminal dsRBD (third) allows Loqs-PB to heterodimerise with Dicer-1, impacting its activity, or to form homodimers in the absence of Dicer-1 (Jakob et al., 2016). Loqs-PD does not possess this C-terminal dsRBD.

It is important, however, to mention that dsRBDs are not the sole domains that bind dsRNA. Dicer-2 perfectly illustrates this premise as its Platform-PAZ (Piwi/Argonaute/Zwille) domains and its helicase domain specifically recognise and bind dsRNA termini (Sinha et al., 2018). The structures of other domains such as zinc fingers (Lu et al., 2003), RNA recognition motifs (Oubridge et al., 1994), the sterile alpha motif (Oberstrass et al., 2006), and the Z-DNA binding domain (Placido et al.,

2007), have also been determined to bind double-helix RNA in other species. This possibly expands the repertoire of putative dsRBPs in *Drosophila* since the absence of a canonical dsRBD within a protein's structure may not be indicative of a lack of interaction with RNA duplexes. Interestingly, among the proteins that were identified in the co-immunoprecipitation experiment mentioned in the previous chapter, a nucleoporin, specifically Nucleoporin 358, was found to presumably bind to dsRNA (data not shown). So far, no nucleoporin from any species has been reported to interact with dsRNA molecules.

Nuclear pore complex and Nucleoporin 358

The nuclear pore complex (NPC) is a multiprotein structure embedded in the nuclear envelope (NE) which acts as the main gate between the cytoplasm and the nucleus. Although the structure of the NPC has been resolved only for a few species, most of its architecture is thought to be conserved among eukaryotes (DeGrasse et al., 2009). The NPC is an assembly of diverse copies of approximately 30 different nucleoporins (Nups) displayed in three regions: an outer cytoplasmic region, a central core, and an inner nuclear portion (Fig. IV). The cytoplasmic ring and the nuclear ring along with the transmembrane Nups of the central core delineate the outer and inner borders of the NPC across the envelope bilayer. Nups rich in Phe-Gly (FG) motifs cover the NPC channel by binding to linker Nups that anchor them to the inner surface of the central core. The remaining Nups of the nuclear portion assemble in a basket-like shape while the Nups in the cytoplasmic side form filaments (Raices & D'Angelo, 2012).

Initially, the NPC was believed to serve only as a bilateral transport mechanism between the nucleus and the cytoplasm; however, in the recent years, strong evidence suggests additional roles linked to both cellular compartments. In fact, the Nups in each portion of the NPC execute distinct functions, which depend on their placement within the complex's structure. The Nups in the nuclear basket are mostly involved in nucleoplasm-related activities including epigenetic and post-transcriptional control of gene activity, repair and maintenance of chromatin, and RNA biogenesis. The FG Nups in the central core further extend to both sides of the NPC spanning their reach throughout the whole channel. Such feature renders these Nups the primary line of transport regulation across the NPC. The NPC allows the passive diffusion of small molecules, ions and metabolites (smaller than 40 kDa) but becomes strictly selective towards macromolecules. These are only granted active passage if bound to nuclear transport receptors (NTRs) that recognise localisation signals within the structure of the macromolecules, which identifies them as cargo. The exclusive freedom of movement of the NTRs along the NPC relies on their affinity for the FG repeats of the FG Nups. The cytoplasmic region of the NPC is mostly

associated with the control of protein translation and establishes physical links with components of the cytoskeleton (Beck & Hurt, 2017; Raices & D'Angelo, 2012; Strambio-De-Castilla et al., 2010).

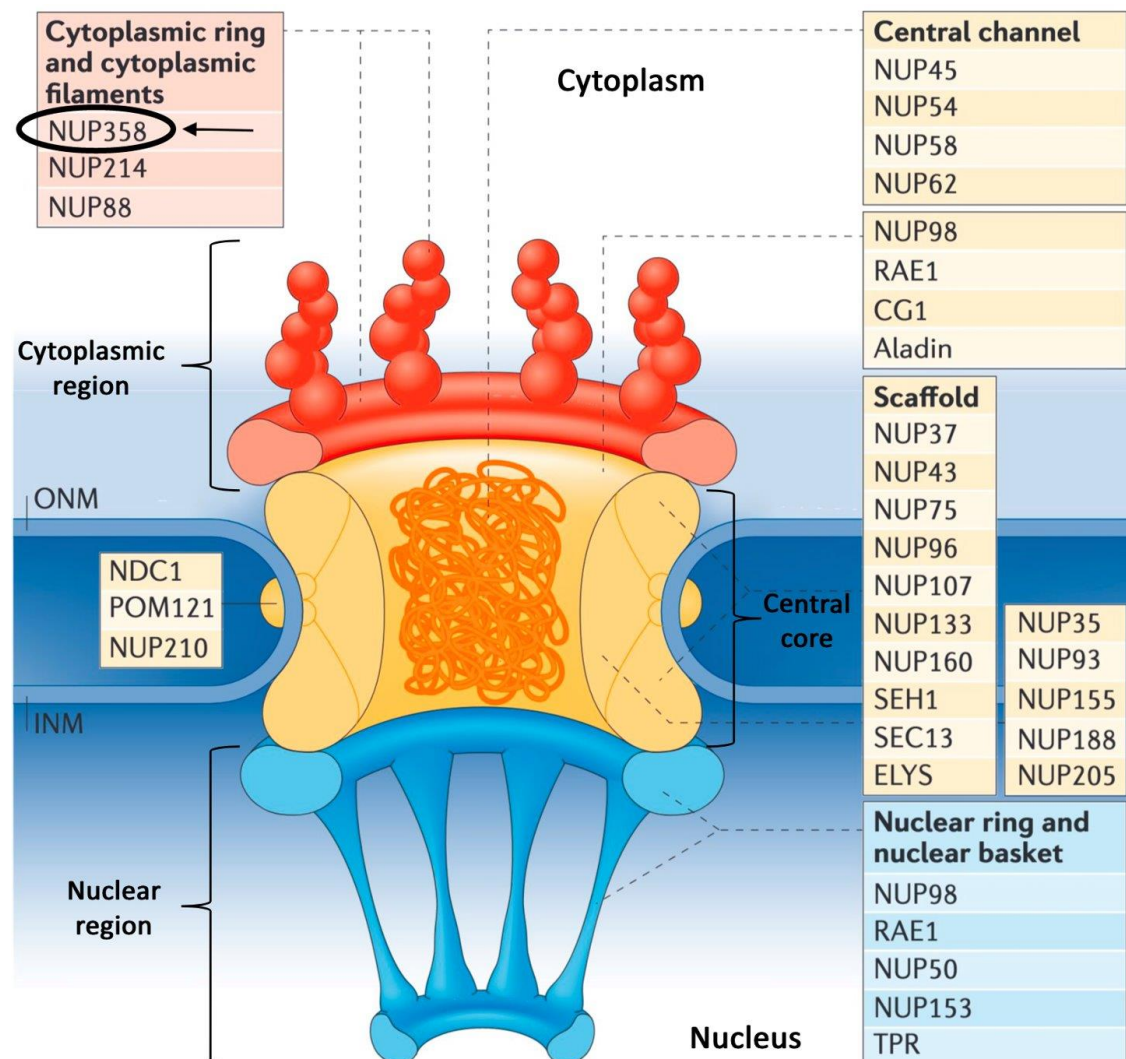


Figure IV | Structure and composition of the nuclear pore complex (NPC) in *Homo sapiens*. The NPC structure is conserved among eukaryotes and comprises three main regions: cytoplasmic, central core, and nuclear regions. *HsNup358*, the human Nucleoporin 358 (Nup358), is located on the distal portion of the cytoplasmic region (circle and arrow) and is a component of the cytoplasmic filaments. Likewise, *Drosophila* Nup358 is assumed to take the same position within the fruit fly NPC. Illustration modified from Raices & D'Angelo, 2012.

In the outermost region of the NPC, comprising one of the proteins that compose the cytoplasmic filaments, lies the Nucleoporin 358 (Nup358), also known as Ran-binding protein 2 (RanBP2) (Fig. IV). Over the last two decades, Nup358 was found to physically interact with several different proteins (Guruharsha et al., 2011; He et al., 2017; Parikh et al., 2018; Vinayagam et al., 2016), even though most of such interactions were predicted by co-affinity purification or co-immunoprecipitations coupled to mass spectrometry analysis and have not been studied with further detail. Some of these proteins are involved in countless signalling pathways with metabolic, immune, and tumour-suppressing activities, possess chromatin-

remodelling profiles, regulate the cell cycle, or mediate protein nuclear export/import, among other functions. The most remarkable interaction of Nup358 might be the one established with Piwi, a member of the Argonaute/Piwi family that participates in the piRNA pathway (Parikh et al., 2018). After being loaded with piRNA, Piwi localises to the nucleus to silence transposons, requiring Nup358 to cross the NE. Upon knockdown of Nup358 in germline cells, Piwi entry into the nucleus is impaired, as well as piRNA biogenesis and transposon silencing, denoting a direct role of Nup358 in the piRNA pathway, therefore, in the RNAi machinery.

Several reports have shown that Nup358 is also indispensable to recruit different NTRs and import them to the nucleus. These include importins – NTRs that recognise classical nuclear localisation signals in their cargo – like Imp7/8 (Sabri et al., 2007), and NTRs that translocate specific proteins, such as the receptor Met, which is involved in the *Drosophila* juvenile hormone signalling pathway and gene activation (He et al., 2017). Besides the nuclear import of NTR-bound proteins, Nup358 is indirectly responsible for the success of mRNA export to the cytoplasm. NXF1-p15 heterodimers assist the export of mRNA from the nucleus through the NPC, and stably bind to Nup358, which is hypothesised to subsequently recycle NXF1 to the nucleus (Forler et al., 2004). In the absence of Nup358, NXF1 freely diffuses to the cytoplasm leading to accumulation of mRNA in the nucleus due to a decrease in the availability of NXF1-p15. Additionally, Nup358 regulates NPC biogenesis during *Drosophila melanogaster* oogenesis in cytoplasmic organelles termed annulate lamellae (AL) (Hampoelz et al., 2019). The AL consist of stacked sheets of endoplasmic reticulum (ER)-like membranes in which NPCs are also embedded (besides the NE).

Curiously, the human Nup358 (*HsNup358*) was one of the few NPC elements that was found to not be shared with lower eukaryotes (DeGrasse et al., 2009). Nonetheless, it is accepted that Nup358 and *HsNup358* are homologous, which can be helpful to transpose knowledge and shed some light on undiscovered functions and properties of Nup358 in *Drosophila*. Remarkably, *HsNup358* supports the nuclear import of the human immunodeficiency virus type 1 (HIV-1), as *HsNup358*-depleted cells exhibited attenuated HIV-1 infectivity (Bichel et al., 2013; Meehan et al., 2014), which confers this protein a proviral role during HIV-1 infection. Concomitantly, the N-terminal region of *HsNup358* is required for the nuclear import of adenoviral (dsDNA) genomes (Carlón-Andres et al., 2020). Also, during Vaccinia virus infection, *HsNup358* localises to cytoplasmic viral replication sites and recruits other proteins to these structures, which become defective in number and size after *HsNup358* knockdown (Khuperkar et al., 2017). In contrast, a missense mutation in the *HsNup358* gene is the cause behind genetic susceptibility to acute necrotising encephalopathy (ANE), a rare disease that affects children after infection by

common viruses (Neilson et al., 2009). These contradictory data suggest that the role of *HsNup358* during viral infection is virus dependent.

HsNup358 interacts with the RNAi machinery through the miRNA pathway upon binding to proteins of the Argonaute family (*HsAgo*) via its internal repeats (IR) region (Sahoo et al., 2017). Abolishment of the motif within this region that promotes *HsNup358-HsAgo* interaction impaired the association of target mRNA with RISC. Also, *HsNup358* depletion disrupted the formation of processing bodies (P bodies). Along with stress granules (SGs), P bodies were shown to dynamically associate with *HsNup358*-positive structures (NE and AL) (Sahoo et al., 2017).

HsNup358 is also involved in centrosome regulation during mitosis (Splinter et al., 2010) and was proposed to bind to single-stranded RNA and DNA (Wu et al., 1995). Undoubtedly, knowledge gathered on *HsNup358* is vaster than that of its *Drosophila* homologous *Nup358*, and though some cellular mechanisms are preserved across species (cell cycle, nuclear transport), experimental validation in both organisms is still required.

CHAPTER II: OBJECTIVES

With the aim of finding the dsRNA receptor in insects, the Saleh laboratory has performed an immunoprecipitation and proteomics protocol that allowed to identify several dsRNA binding proteins (dsRBPs) in the insect model *Drosophila melanogaster* (unpublished data). Intriguingly, several of the proteins found were intracellular and most of them have not yet been described to have dsRNA affinity and are not known to have a role during viral infection. The hypothesis is that due to their affinity to dsRNA, a hallmark of viral infection, some of these proteins may play a role during viral infection as restriction factors – participating in the antiviral response – or could be recruited by the virus to facilitate its replication, having a proviral role.

Among the identified dsRBPs there was Nucleoporin 358 (Nup358), that is a component of the nuclear pore complex. As nucleoporins have never been reported to bind dsRNA structures, Nup358 was the ideal candidate to study.

The objectives of this project were:

- To test whether Nup358 has a role during viral infection *in vitro* in *D. melanogaster* cells.
- To test whether Nup358 has a role during viral infection *in vivo* in *D. melanogaster* lines lacking Nup358.

CHAPTER III: MATERIALS AND METHODS

Cells, viruses and plasmids

Drosophila Schneider 2 (S2) cells (Invitrogen, Carlsbad, CA) were cultured at 25°C in Schneider's *Drosophila* Medium (#21720-024, GIBCO) supplemented with 10% fetal bovine serum (FBS) (#10500-064, GIBCO), 2 mM L-glutamine (#25030149, GIBCO), 100 U.mL⁻¹ penicillin, and 100 mg.mL⁻¹ streptomycin (#15140122, GIBCO). Baby Hamster Kidney (BHK-21) cells (#CCL-10, American Type Culture Collection) and Vero-E6 cells (#CCL-81, American Type Culture Collection) were both grown at 37°C, 5% CO₂ in DMEM (1X) + GlutaMAX-I (#61965-026, GIBCO) supplemented with 10% FBS, 2 mM L-glutamine, 100 U.mL⁻¹ penicillin, and 100 mg.mL⁻¹ streptomycin.

Drosophila C virus (DCV) was obtained from Jean-Luc Imler (University of Strasbourg) (ENA: GCA_000850085). Viral titre was determined by end-point dilution assay (TCID₅₀/mL). Sindbis virus wild type (SINV-WT) (TR339) had been previously produced in the laboratory by in vitro transcription of the pTR339 construct followed by transfection (Klimstra et al., 1998; Mondotte et al., 2020). Titre was determined by plaque assay (PFU/mL).

Plasmid pTR₃₃₉-RL2A (Mondotte et al., 2020) was used to produce recombinant Sindbis virus expressing the *Renilla reniformis* luciferase (SINV-RLuc). Plasmid pMT-GL3 (Saleh et al., 2006) encoding the firefly (*Photinus pyralis*) luciferase (GL3) was used to produce dsRNA targeting GL3-encoding mRNA (dsControl).

Fly stocks and collection

Flies were reared on standard cornmeal medium (Bloomington recipe) at 25°C under a 12-hour photoperiod. Flies from the ***UAS-shNup358*** line (w¹¹¹⁸; P{GD7405}v38583, #38583, Vienna *Drosophila* Resource Center) contain a homozygous insertion in chromosome 2 encoding a short hairpin against the mRNA encoded by the *Nup358* gene (*shNup358*) regulated by the upstream activation sequence (UAS). This line was crossed with each of the following driver lines: (i) ***Act5C-GAL4***: y[1] w[*] ; P{w[+mC]=Act5C-GAL4}25FO1/CyO, y[+] (Bloomington *Drosophila* Stock Center), expressing GAL4 under an actin 5C promoter; (ii) ***tubP-GAL80^{ts}/GAL4***: ; P{w[+mC]=tubP-GAL80[ts]}10; P{w[+mC]=tubP-GAL4}LL7/TM6 Tb (gently provided by Professor François Schweisguth), that after several passages became homozygous for the expression of GAL4, and that expresses the temperature-sensitive GAL80 (GAL80^{ts}) which binds and inhibits GAL4 at temperatures below 29°C. Crosses of female virgins from the parental line *UAS-shNup358* and males from the driver line *Act5C-Gal4* were performed to generate offspring capable of expressing the *shNup358* through the GAL4/UAS system. Only flies from the F1 generation that did not have the balancer chromosome CyO (flies without the curly wings phenotype) were selected to be used since they had the complete GAL4/UAS system within their genome. Crosses of female virgins from the parental line *UAS-shNup358* and males from the *tubP-GAL80^{ts}/GAL4* driver line allowed the

generation of an offspring fully expressing the shNup358 through the GAL4/UAS system, when grown at 29°C.

SINV-RLuc production

SINV-RLuc was produced by transfecting BHK-21 cells with *in vitro* transcribed mRNA from pTR₃₃₉-RL2A. Briefly, the plasmid was previously linearised with the XhoI restriction enzyme (#10 899 194 001, Roche) and purified with Phenol:Chloroform:IAA pH 7.9 (#AM9730, Ambion). *In vitro* transcription was performed using the mMACHINE SP6 Transcription Kit (#AM1340, Life Technologies) following the kit's protocol. BHK-21 cells were transfected with SINV-RLuc mRNA using Lipofectamine 3000 Transfection Reagent (#L3000001, Invitrogen), and supernatant was collected after 24 hours of incubation at 37°C and centrifugation at 1000 x g for 10 minutes. Viral titre was determined by plaque assay (PFU/mL) on Vero-E6 cells. 700,000 Vero-E6 cells were seeded in a 6-well plate and incubated at 37°C for 24 hours. Serial 10-fold dilutions from 10⁻³ to 10⁻⁷ of SINV-RLuc in DMEM without FBS were prepared and added to the Vero-E6 cells. After a 1-hour incubation at 37°C, an overlay of DMEM 2%FBS/0.8% agarose solution was added to each well, and the plate was incubated at 37°C for 72 hours. Then, the Vero-E6 cells were fixed with 4% formaldehyde for 30 minutes. After fixation, the medium was discarded and the cells were stained with a 20% ethanol 0.23% crystal violet solution – prepared from a 2.3% crystal violet solution (#HT90132, Sigma-Aldrich) - for 10 minutes. The plate was washed twice with H₂O and left to dry. Plaques were counted and the viral titre was determined by quantifying Plaque Forming Units/mL (PFU/mL).

dsRNA production

RNA was extracted from S2 cells with TRIzol Reagent (#15596026, Life Technologies), following the manufacturer's instructions, and the concentration was determined with NanoDrop One (#ND-ONE-W, Thermo Scientific). RNA was treated with DNase RQ1 (#M6101, Promega), and reverse-transcribed into cDNA with SuperScript II Reverse Transcriptase (#18064022, Invitrogen). Amplification of the template cDNAs by PCR (DreamTaq Green DNA Polymerase; #EP0711, Thermo Scientific) was performed using primers containing the T7 RNA polymerase promoter at the 5'-end. *In vitro* transcription was performed with MEGAscript T7 Transcription Kit (#AM1334, Invitrogen), following the kit's protocol. RNA was purified with acid phenol:CHCl₃ pH: 4.5 (#AM9720, Life Technologies). Purified RNA was resuspended in 30 µL of H₂O. For the annealing of the two complementary strands, RNA was heated at 90°C for 15 minutes, and let cool down slowly until room temperature was attained. Synthesis was confirmed by running 3 µL of dsRNA with DNA Gel Loading Dye (6X) (#R0611, Thermo Scientific) in a 1% agarose gel. For dsRNA against the firefly luciferase (dsControl), T7_DNA was produced by using the pMT-GL3 plasmid as template. dsRNA concentrations were measured on a Qubit 3

Fluorometer (#15387293, Invitrogen) and dsRNA solutions were ultimately diluted to 50 ng/ μ L.

In vitro experiments control conditions

For all *in vitro* experiments, mock infected cells served as negative control for the infections; non-transfected and infected cells were used as control to normalise the remaining conditions (NT); cells transfected with dsRNA against the firefly luciferase's gene (dsControl) (not present in the *Drosophila melanogaster* genome or in the viruses used) were used as control of the effect of dsRNA per se; cells transfected with dsRNA targeting *Argonaute-2* (dsAgo2) were used as a positive control for mRNA silencing via the siRNA pathway.

RT-qPCR

To confirm silencing in S2 cells after dsRNA transfection, RNA was extracted from S2 cells 48 hours after transfection with corresponding dsRNA, using either Effectene Transfection Reagent (#301427, QIAGEN) or Lipofectamine 3000 (#L3000001, Invitrogen) as transfection agent. For flies, 3 pools of 3 female flies (*Act5C-GAL4* driver's crosses) or 3 individual male flies (*tubP-GAL80^{ts}/GAL4* driver's crosses) were collected from the parental lines and the F1 generation, and immediately frozen at -20°C for posterior analysis. For cells and flies, RNA extraction was performed with TRIzol, quantified with NanoDrop One. Equal amounts of RNA were used per experiment (500 ng for cells, 400 ng for *Act5C-GAL4* $\times$ *UAS-shNup358* crosses and 200 ng for *tubP-GAL80^{ts}/GAL4* $\times$ *UAS-shNup358* crosses). Prior to cDNA synthesis, DNase RQ1 treatment was performed. Reverse-transcription was done with either random hexamer primers or Oligo(dT)s primers using the Maxima H Minus First Strand cDNA Synthesis Kit (#K1682, Thermo Scientific). Quantitative PCR (qPCR) was performed using Luminaris Color HiGreen qPCR Master Mix kit (#K0374, Thermo Scientific), following the kit's protocol, and CT values were measured in a QuantStudio™ 7 Pro Real-Time PCR System (#A43165, Applied Biosystems). CT values were normalised to the housekeeping gene *Rp49*, allowing to obtain the Δ Cq value for each sample. $\Delta\Delta$ Cq value resulted from the difference between each sample's Δ Cq and the geometric mean of the Δ Cq of the NT (*in vitro*) or the *UAS-shNup358* parental line (*in vivo*). Fold change in the expression of the tested gene (relative to the control condition) for each sample corresponded to $2^{-\Delta\Delta Cq}$.

Cell viability assay

50,000 S2 cells/well were transfected with 10 ng of dsRNA using Effectene (#301427, QIAGEN) and incubated for 48 hours at 25°C. Cells were then transferred to a white 96-well plate, to which CellTiter-Glo Reagent – CellTiter-Glo Luminescent Cell Viability Assay (#G7571, Promega) – was added according to the manufacturer's recommendations. This kit measures the number of viable cells based on the quantitation of the ATP present. Experimental controls included wells without cells (luminescence negative

control), paraformaldehyde (PFA)-treated cells (non-viability control) and wells seeded with double the number of cells. Luminescence was measured immediately in a GloMax 96 Microplate Luminometer with an integration time of 3 seconds.

In vitro SINV-RLuc *Renilla* luciferase assay

In a 96-well plate, 50,000 S2 cells/well were transfected with 10 ng of dsRNA with Effectene (#301427, QIAGEN) following the manufacturers' recommendations, and incubated for 48 hours at 25°C. Before infection, the medium was changed for growth medium without FBS. Cells were infected with SINV-RLuc (MOI: 3 PFU/cell) or mock-infected (negative control) and incubated for 2 hours at 25°C. Then, the medium was replaced by normal growth medium with 10% FBS and cells were incubated for 24 hours. S2 cells were lysed with Passive Lysis Buffer (PLB) 1X. *Renilla* luciferase's activity was determined as an indirect indicator of viral replication using the *Renilla* Luciferase Assay System (#E2810, Promega), according to the manufacturer's instructions. Briefly, 20 µL of lysed sample were loaded into a white-walled 96-well plate, and 20 µL of *Renilla* Luciferase reagent were added. Luminescence was measured immediately in a GloMax 96 Microplate Luminometer (#E6521, Promega) with an integration time of 3 seconds.

SINV-WT plaque assay

In a 24-well plate, 100,000 S2 cells/well were transfected with 20 ng of dsRNA with Lipofectamine 3000 (#L3000001, Invitrogen) and incubated for 48 hours at 25°C. Before infection, the medium was changed for growth medium without FBS. Cells were infected with SINV-WT (MOI: 3 PFU/cell) or mock-infected and incubated for 2 hours at 25°C. Then, the medium was replaced by normal growth medium with 10% FBS and the cells were incubated for 48 hours. Supernatants (SN) were collected and diluted from 10⁻¹ to 10⁻⁶ in DMEM without FBS and added to 24-well plates with 170,000 Vero-E6 cells seeded the day before. After a 1-hour incubation at 37°C, an overlay of DMEM 2%FBS/0.8% carboxymethylcellulose (CMC) solution was added to each well, and the plate was incubated at 37°C for 72 hours. The crystal violet staining was performed as previously described for the titration of the SINV-RLuc stock produced, and SINV viral titres were quantified (PFU/mL).

DCV enzyme-linked immunosorbent assay (ELISA)

In a 96-well plate, 50,000 S2 cells/well were transfected with 10 ng of dsRNA with Effectene (#301427, QIAGEN), and incubated for 48 hours at 25°C. Before infection, the medium was changed for growth medium without FBS. Cells were infected with DCV (0.1 TCID₅₀/cell) or mock-infected and incubated for 2 hours at 25°C. Then, the medium was replaced for normal growth medium with 10% FBS and cells were incubated for 96 hours. Cells were lysed with 100 µL of 2X RIPA buffer/ 2X PIC (2% NP40 substrate, 1% Sodium Deoxycholate, 0.2% Sodium Dodecyl Sulfate, 0.3 M Sodium Chloride, 4 mM EDTA, 0.1 M Tris pH=8 – supplemented with 25X cOmplete Protease Inhibitor Cocktail

(PIC) (#11697498001, Roche)). 190 μ L of coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) and 10 μ L of protein extract from the cells lysate were added to a Nunc MaxiSorp flat-bottom plate (#439454), and incubated for 2 hours at room temperature (*rt*). Then, the wells were washed 3 times with 1X PBS-0.05% Tween (PBS-T), and blocked for 2 hours with blocking buffer (5% non-fat dry milk in PBS-T) at *rt*. Wells were washed again 3 times with PBS-T. Rabbit polyclonal primary antibody against DCV (previously produced for the laboratory by GroupBios, third immunisation) (1:5000 diluted in blocking buffer) was added and incubated at 4°C overnight (ON). Wells were washed 4 times with PBS-T and incubated with anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody (#NA934, GE Healthcare UK Limited) (1:5000 dilution in blocking buffer). After 4 washes with PBS-T, 100 μ L of 1-Step Turbo TMB-ELISA (#34022, Thermo Scientific) were added to the wells and the plate was incubated for 30 minutes at *rt*, protected from light. The reaction was stopped by adding the same volume of HCl 2N. Absorbance was measured at 450 nm in an Infinite M200 PRO microplate reader (TECAN), allowing to compare viral loads.

End-point dilution assay

In a 96-well plate, 50,000 S2 cells/well were transfected with 10 ng of dsRNA with the Lipofectamine 3000 (#L3000001, Invitrogen) and incubated for 48 hours at 25°C. Before infection, the medium was changed for growth medium without FBS. Cells were infected with DCV (0.1 TCID₅₀/cell) or mock-infected and incubated for 2 hours at 25°C. Then, the medium was replaced by normal growth medium with 10% FBS, and cells were incubated for 96 hours. After incubation, SN was collected and diluted from 10⁻¹ to 10⁻¹¹ in medium without FBS. 20 μ L of each SN dilution were added to 96-well plates pre-seeded with S2 cells prepared the day before, and were incubated at 25°C for 7 days, daily checking for cytopathic effect (CPE). When CPE was observed, 25 μ L of each well's content were transferred to a new 96-well plate with normal growth medium. These plates were incubated at 25°C for another 7 days, again checking daily for CPE. The median tissue culture infectious dose (TCID₅₀/mL) was estimated using the Spearman-Kärber method (Hamilton et al., 1977).

Wolbachia spp. detection *in vivo*

One pool of 3 female and 3 male flies from the lines *UAS-shNup358*, *Act5C-GAL4*, and F1 generation from the *tubP-GAL80^{ts}/GAL4* $\times$ *UAS-shNup358* crosses was collected, and DNA was extracted by squishing with pestle in squishing buffer (10 mM Tris-HCl pH=8.2; 25 mM NaCl; 1 mM EDTA; 200 μ g/mL Proteinase K (#EO0491, Thermo Scientific)). For the *tubP-GAL80^{ts}/GAL4* line, one pool of 3 females flies was used. After squishing, samples were incubated at 37°C for 30 min, followed by a 2 min incubation at 94°C. DNA samples were tested for the presence of *Wolbachia* spp. by touchdown PCR (DreamTaq Green DNA Polymerase; #EP0711, Thermo Scientific) using *Wolbachia*-specific primers (listed below) and adequate positive (12S rRNA) and negative controls (H₂O). PCR

products were run in a 1% agarose gel. Touchdown PCR programme: 95°C – 5 min ; 30 x [95°C – 30 s ; (65°C – 0.5°C/cycle) – 30 s ; 72°C – 45 s] ; 72°C – 5 min.

Drosophila viruses detection *in vivo*

One pool of 3 female and 3 male flies from the lines *UAS-shNup358*, *Act5C-GAL4*, and F1 generation from the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses was collected, squished with pestle in TRIzol Reagent (#15596026, Life Technologies), and RNA was extracted following the manufacturer's instructions. For the *tubP-GAL80^{ts}/GAL4* line, one pool of 3 females flies was used. Extracted RNA was DNase-treated (RQ1) and reverse-transcribed (SuperScript II) to cDNA. cDNA samples were tested for the presence of *Drosophila* viruses by PCR (DreamTaq Green DNA Polymerase; #EP0711, Thermo Scientific) using viruses-specific primers (listed below) and adequate positive (Rp49) and negative controls (RT-). PCR products were run in a 1% agarose gel. PCR programme: 95°C – 3 min ; 35 × (95°C – 30 s ; 58°C – 30 s ; 72°C – 1 min) ; 72°C – 10 min.

Flies injection

For the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses, male *tubP-GAL80^{ts}/GAL4* flies were crossed with virgin female *UAS-shNup358* flies. 9 days after the eggs hatched until the end of the experiments, flies of the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses were put at 29°C to induce shNup358 transcription in the F1 generation flies. For the *Act5C-GAL4* × *UAS-shNup358* crosses, male *Act5C-GAL4* flies were crossed with virgin female *UAS-shNup358* flies, and they were maintained at 25°C before and throughout the *in vivo* infections. 4-6-day old flies were CO₂-anesthetised and intrathoracically injected with 50.6 nL of solution using a nanoinjector Nanoject II (#3-000-205/206, Drummond Scientific). Female flies were used for the *Act5C-GAL4* × *UAS-shNup358* crosses. Male flies were used for the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses.

In vivo Renilla luciferase assay

10 flies were injected with a DMEM 5% FBS solution (mock) and 30 flies with SINV-RLuc (759 PFU/fly) per line. Individual flies were collected at 1 day post-infection (dpi), 2 dpi and 3 dpi, and mock-injected flies at 3 dpi. After being euthanised, the flies were lysed in PLB 1X using a Precellys Evolution homogeniser (#EQ02520-300-RD000.0, Bertin Technologies). *Renilla* luciferase's activity was measured exactly as described before for the *in vitro Renilla* luciferase assay.

DCV survival curve

30 flies were injected with a Tris 10 mM pH = 7.5 solution (mock) and other 30 with DCV (10 TCID₅₀/fly) per line. Flies were separated in groups of 10 flies per tube. Mortality due to injection was assessed on the following day and the surviving flies were

acknowledged as the initial population for the survival curve. Flies were flipped to new tubes every two days and their mortality rate was daily registered and plotted.

Statistical analysis

Differences between groups, both *in vitro* and *in vivo* (SINV-RLuc injection only), were assessed by ordinary one-way ANOVA followed by Dunnett's multiple comparison post-hoc test, unless otherwise stated. mRNA expression in S2 cells was compared by unpaired t test and in flies by ordinary one-way ANOVA. Comparison of the survival curves was performed using a log-rank (Mantel-Cox) test. Differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed in GraphPad Prism 9.2.0.

Oligonucleotide primers

T7-tailed primers (dsRNA production)

Nup358 Fw: 5'-TAATACGACTCACTATAGGGAGACCAACCTGTCGGCCAATAGC-3'

Nup358 Rv: 5'-TAATACGACTCACTATAGGGAGACAACCTTTACCCTCTGCGTCCAC-3'

GL3 Fw: 5'-TAATACGACTCACTATAGGGCGCCCTGGTTCCTGGAAC-3'

GL3 Rv: 5'-TAATACGACTCACTATAGGGAGAATCTCACGCAGGCAGTTC-3'

Ago2 Fw: 5'-TAATACGACTCACTATAGGGAGATACTATGGTGAAGAACGGGTCG-3'

Ago2 Rv: 5'-TAATACGACTCACTATAGGGAGAGAACATGTCCTCAATCTCCTCC-3'

qPCR primers

Ago2 Fw: 5'-GTGGTTTACACGCCTCCTCA-3'

Ago2 Rv: 5'-GGGTAGTTGCGACTGTGGAA-3'

Nup358 Fw: 5'-CTGGTGAAGGAGAACCAACGC-3'

Nup358 Rv: 5'-CGCAGGTTGAAGACTTGCTTG-3'

Rp49 Fw: 5'-CGGATCGATATGCTAAGCTGT-3'

Rp49 Rv: 5'-GCGCTTGTTGATCCGTA-3'

Wolbachia sp. (*Wsp*) and viruses detection

Wsp Fw: 5'-TGGTCCAATAAGTGATGAAGAAAC-3'

Wsp Rv: 5'-AAAAATTAAACGCTACTCCA-3'

WspB Fw: 5'-TTTGCAAGTGAAACAGAAGG-3'

WspB Rv: 5'-GCTTTGCTGGCAAAATGG-3'

Drosophila mitochondrial 12S rRNA Fw: 5'-AACTAGGATTAGATACCCTATTAT-3'

Drosophila mitochondrial 12S rRNA Rv: 5'-AAGAGCGACGGGCGATGTGT-3'

Nora virus Fw: 5'-ATGGCGCCAGTTAGTGCAGACCT-3'

Nora virus Rv: 5'-CCTGTTGTTCCAGTTGGGTTCTGA-3'

Sigma virus AP30 Fw: 5'-AAATCCGCGCCTACTGACCGA-3'

Sigma virus AP30 Rv: 5'-AGCGCAGCTTTCCATTCCAT-3'

Drosophila A Virus Fw: 5'-AGAGTGGCTGTGAGGCAGAT-3'

Drosophila A Virus Rv: 5'-GCCATCTGACAACAGCTTGA-3'

Drosophila C Virus Fw: 5'-GTTGCCTTATCTGCTCTG-3'

Drosophila C Virus Rv: 5'-CGCATAACCATGCTCTTCTG-3'
Drosophila X Virus segment B Fw: 5'-TCAAGGCATTCGATCCCTAC-3'
Drosophila X Virus segment B Rv: 5'-CCATACGCGTTGTGTATTTCG-3'
Drosophila X Virus segment A Fw: 5'-CGTCGAGTATTAGCGGCTTC-3'
Drosophila X Virus segment A Rv: 5'-GCCCTACGGAGTCCACATTA-3'
Flock House Virus RNA1 Fw: 5'-CCAGATCACCCGAACTGAAT-3'
Flock House Virus RNA1 Rv: 5'-CGACCGATGGAAACCAGCAGTTC-3'
Flock House Virus RNA2 Fw: 5'-CGTCACAACAACCCAAACAG-3'
Flock House Virus RNA2 Rv: 5'-CCACCGCTAGAACACCATCT-3'
Rp49 Fw: 5'-AAGGGTATCGACAACAGAGTGC-3'
Rp49 Rv: 5'-ACAAATGTGTATTCCGACCACG-3'

CHAPTER IV: RESULTS

dsRNA effectively silences Nup358 mRNA in *Drosophila* cells

In order to study the role of Nup358 during viral infection *in vitro*, the approach was to induce silencing of Nup358 mRNA via the siRNA pathway by transfecting cells with dsRNA targeting its mRNA (dsNup358), and then assess its effects in viral replication by quantification of viral titres. dsRNA against Ago2 (dsAgo2) was used as a control for the silencing of an antiviral protein.

To confirm that dsNup358 and dsAgo2 were effectively silencing Nup358 and Ago2 mRNA levels, *Drosophila* S2 cells were either non-transfected (NT) or transfected with dsNup358 or dsAgo2. Initially, the Effectene reagent was used for the transfections, but following an unexpected shortage of this kit in France due to the COVID-19 pandemic, Lipofectamine 3000 was used in some experiments. Therefore, *in vitro* silencing was checked using both transfection kits. RNA was extracted from cells 48 hours after transfection and fold change in the mRNA levels was assessed by RT-qPCR. It is worth mentioning that the qPCR primers bind to a region that is upstream of the dsRNA-targeted site. If one is to use random hexamer primers (Random primers) to generate the cDNA, the fragment upstream of the dsRNA target region will still be amplified by the PCR reaction even if the mRNA has been cleaved, generating a false positive detection. Considering this, two types of primers were separately used to generate the cDNA: Random primers or Oligo(dT)s. Theoretically, the Oligo(dT)s should only amplify the full-length mRNA or the 3'-end fragments of cleaved mRNA, which are not targeted by the qPCR primers.

For cells transfected with Effectene, there was a significant decrease of the Ago2 mRNA levels, either when Random primers or Oligo(dT)s were used, compared to non-transfected (NT) cells (Fig. 1a,b). Non-significant differences were observable for the dsNup358-transfected cells using the same transfection reagent (Fig. 1a,b). Even if there was no significant difference, there was a strong tendency to a decrease in the Nup358 mRNA levels when Oligo(dT)s were used ($P=0.06$; Fig. 1b). Probably, if this experiment was repeated, a significant decrease could be seen for this condition. In the cells transfected with Lipofectamine 3000, there were significant decreases of the levels of the targeted mRNA (both Ago2 and Nup358), using both RT primers sets (Fig. 1c,d). These results allowed to conclude that dsNup358 partially knocks down the Nup358 mRNA levels in cells transfected with Effectene, and that this decrease was significant in cells transfected with Lipofectamine 3000. Overall, there was a decrease of about 40% in the mRNA levels of Nup358.

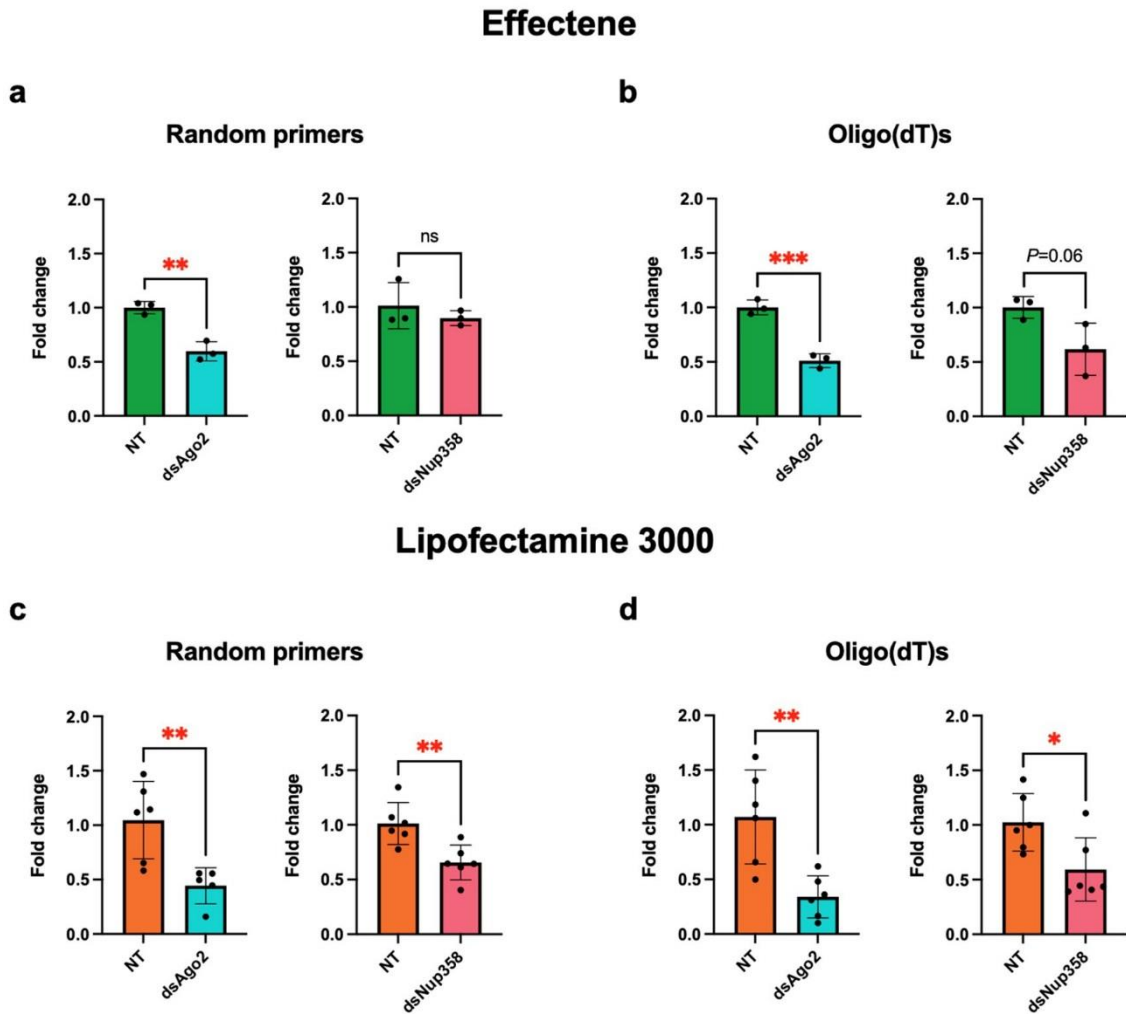


Figure 1 | dsRNA partially decreases the expression of its target mRNA *in vitro*. S2 cells were transfected for 48 hours with dsRNA targeting either Ago2 mRNA (dsAgo2) or Nup358 mRNA (dsNup358) using Effectene (**a,b**) or Lipofectamine 3000 (**c,d**) as transfection reagent. Extracted RNA was reverse-transcribed to cDNA using Random primers (**a,c**) or Oligo(dT)s (**b,d**) before qPCR. RT negative samples were used as control for DNA contamination. CT values were obtained and ΔCT were calculated by subtracting the CT values obtained for the housekeeping gene *Rp49*. For **a** and **b**, one experiment was performed with three biological replicates, and for **c** and **d**, two independent experiments were performed with three replicates each. Data are presented as mean $\pm$ standard deviation (s.d.) of $2^{-\Delta\Delta CT}$ normalised to non-transfected cells (NT). A *P* value <0.05 was considered to be significant. ns: non-significant, **P* <0.05 , ***P* <0.01 , ****P* <0.001 .

Nup358 silencing does not impair cell viability

We then explored whether knocking down Nup358 mRNA levels, and therefore decreasing the amount of available Nup358 protein, was affecting the viability of the S2 cells. To this end, S2 cells were transfected with dsRNA targeting different RNAs. Cells transfected with dsRNA against the firefly luciferase (dsControl) were used as a control for the effect of dsRNA per se. Cells transfected with dsAgo2 were the positive control for an antiviral protein, since Ago2 plays a major antiviral role in the siRNA pathway. Cell viability was assessed via a luminescence-based reaction, and luminescence measured

was proportional to the amount of ATP detected, which was used as an indicator of viable cells. For the experiment, the following control conditions with non-transfected cells were considered: 50,000 cells (to which the remaining conditions were compared); 100,000 cells (to confirm the correlation between the number of cells and the intensity of luminescence); 50,000 cells that were treated with paraformaldehyde, a fixing agent that kills cells, for 30 minutes prior measuring luminescence (to confirm that ATP was a reliable parameter to determine cell viability). The differences in arbitrary luminescence units (ALUs) between these conditions were significant (Fig. 2). When the number of transfected cells was duplicated, detected luminescence largely increased; also, for the paraformaldehyde-treated cells, a significant decrease was observed, compared to the 50,000 cells control. However, no significant differences in the luminescence levels were observed for any of the transfection conditions (dsControl, dsAgo2 and dsNup358) relative to the 50,000 cells control. These results indicate that none of the dsRNA used had an impact in S2 cells viability. Therefore, they can be further used to study the role of Nup358 on the antiviral response in S2 cells.

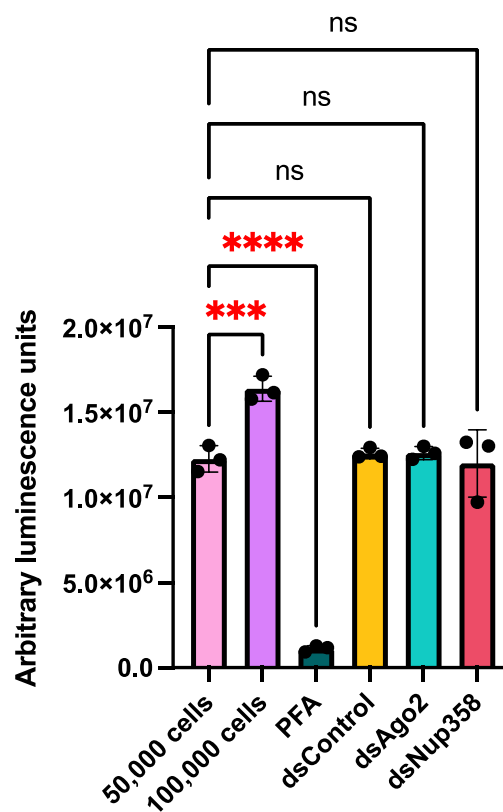


Figure 2 | Silencing of target transcripts does not affect the viability of transfected S2 cells. 50,000 S2 cells were transfected for 48 hours with dsRNA targeting either the firefly luciferase RNA (dsControl), dsAgo2 or dsNup358 using Effectene as transfection reagent. Cells were lysed and luminescence was measured. The intensity of luminescence is directly proportional to the amount of ATP in the samples, which roughly indicates the number of viable (metabolically active) cells. Experiment control conditions include non-transfected 50,000 cells, 100,000 cells or 50,000 cells that were treated with paraformaldehyde (PFA) for 30 minutes before measuring luminescence. One experiment with three replicates was performed. Data are presented as mean $\pm$ s.d. of measured luminescence values. A P value <0.05 was considered to be significant. ns: non-significant, *** $P<0.001$, **** $P<0.0001$.

Sindbis virus replication is increased in Nup358-silenced cells

The role of Nup358 during viral infection was studied *in vitro* in S2 cells challenged either with an arbovirus – Sindbis virus (SINV) – or a *Drosophila* natural virus – *Drosophila* C virus (DCV). A reporter SINV – SINV-RLuc – which possesses the gene encoding for the luciferase of *Renilla reniformis*, was produced to perform luminescence assays. In these viruses, production of luminescence is a surrogate of virus replication, allowing to compare viral replication in non-transfected (NT) cells to dsRNA-transfected cells (Fig. 3a). When cells were transfected with dsControl, no significant differences in luminescence fold change were detected comparatively to the NT cells. Cells knocked down for Ago2 (positive control condition) exhibited significantly increased luminescence values. Similarly, for dsNup358-transfected cells, the fold change in luminescence was significantly higher than the NT cells, suggesting an antiviral function for Nup358. To further confirm the obtained results, infective viral particles were titred, in PFU/mL, for each condition (Table S1). This was achieved by doing plaque assays (see Methods) (Fig. S1) using supernatants collected from cells infected with SINV wild type (SINV-WT). Surprisingly, a significant increase ($0.01<P<0.05$) in the SINV-WT titre of dsControl-transfected cells was registered (Fig. 3b). Nonetheless, the effects of silencing either Ago2 or Nup358 mRNA on viral titres were highly significant ($P<0.0001$) when compared to dsControl. These results suggest that Nup358 has an antiviral role upon SINV infection.

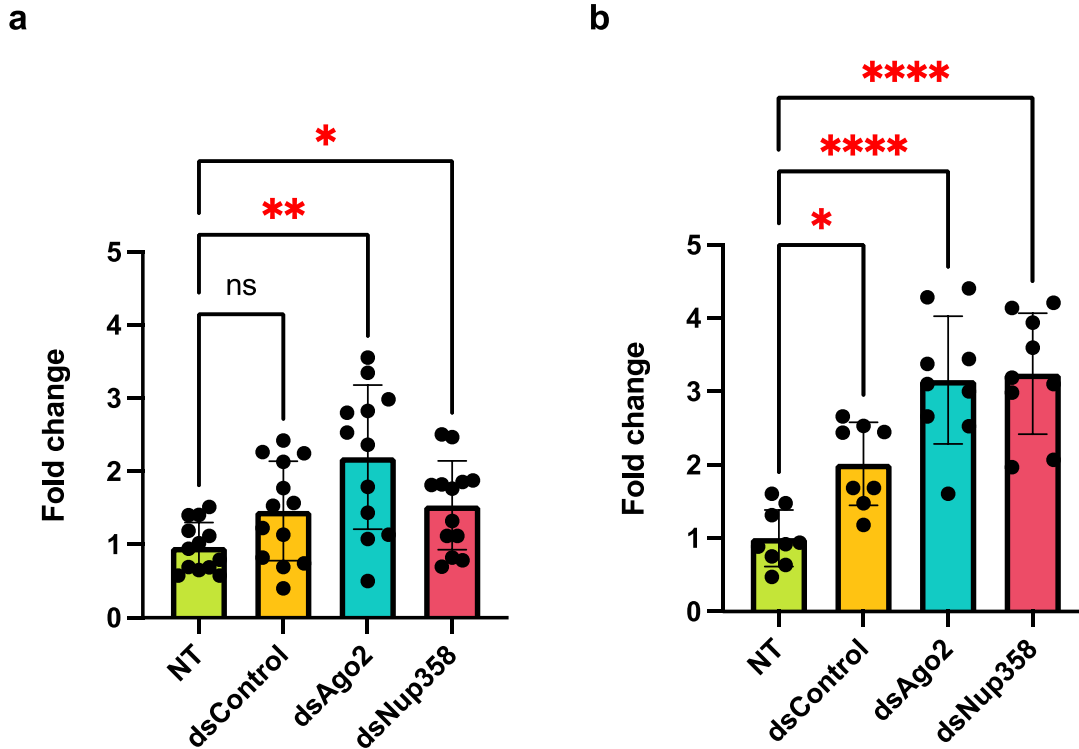


Figure 3 | Nup358-silenced S2 cells exhibit increased SIN V-RLuc and SIN V-WT replication. S2 cells were transfected for 48 hours with dsControl, dsAgo2 or dsNup358 using Effectene (a) or Lipofectamine 3000 (b), and infected with 3 PFU/mL of SIN V-RLuc for 24 hours (a) or SIN V-WT for 48 hours (b). Luminescence of SIN V-RLuc-infected cells was measured after lysis as a surrogate for viral replication (a). SIN V-WT viral titres in PFU/mL were determined by plaque assay using the supernatants of infected cells (b). For a, four experiments were performed, with three replicates each. For b, three independent experiments were performed, with three replicates each. For the SIN V-RLuc experiment, fold changes in luminescence were compared using Welch's ANOVA followed by Dunnett's T3 multiple comparison post-hoc test (a). Data are presented as mean $\pm$ s.d. of luminescence values (a) or PFU/mL (b) normalised to NT. A P value <0.05 was considered to be significant. ns: non-significant, * $P<0.05$, ** $P<0.01$, **** $P<0.0001$.

DCV replication is increased in Nup358-silenced cells

The aim of this experiment was to assess *in vitro* whether the silencing of Nup358 has an effect on a *Drosophila* natural pathogen, DCV, as observed with Sindbis. To assess DCV presence following infection, viral proteins were detected in cells lysates by ELISA and the absorbance values were normalised to those obtained for the NT condition (Fig. 4a). There were no significant differences in the absorbance fold change between the non-transfected cells and any of the dsRNA-transfected cells and the result of the positive control (dsAgo2-transfected cells) immediately invalidated the values obtained for the remaining conditions. Indeed, an increase in viral load should have been observed for this control due to the lack of the antiviral activity of Ago2 in the cells. We then decided to quantify the supernatants of infected S2 cells using end-point dilution assays, and the calculated TCID₅₀/mL values (Table S2) were relativised to the NT

condition (Fig. 4b). Significant increases of the DCV titres were only observed for supernatants collected from dsAgo2 and dsNup358-transfected cells, which suggests that Nup358, like Ago2, may have an antiviral effect against DCV.

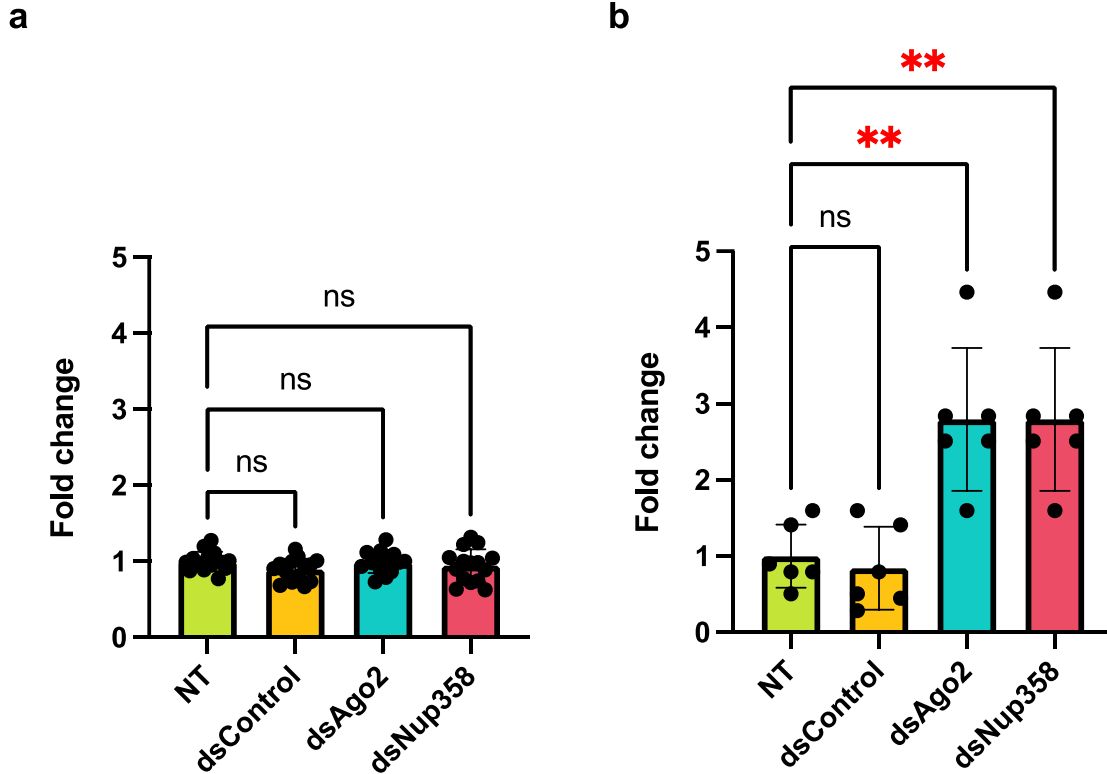


Figure 4 | Nup358-silenced S2 cells exhibit increased DCV titres upon infection. S2 cells were transfected for 48 hours with dsControl, dsAgo2 or dsNup358 using Effectene (a) or Lipofectamine 3000 (b), and infected with 0.1 TCID₅₀/mL of DCV for 4 days. After cell lysis with RIPA 2X, intracellular DCV loads were inferred by absorbance values determined by ELISA (a). The titres of infectious DCV particles present in the supernatants of infected cells, in TCID₅₀/mL, were calculated by end-point dilution assay (b). For a, five independent experiments were performed, with three replicates each. For b, two independent experiments were performed, with three replicates each. Data are presented as mean $\pm$ s.d. of luminescence values (a) or TCID₅₀/mL (b) normalised to NT. A *P* value <0.05 was considered to be significant. ns: non-significant, ***P*<0.01.

Detection of *Wolbachia* and common viruses in *Drosophila* stocks

Having shown that Nup358 play an antiviral role *in vitro* against SINV and DCV, the following step was to understand if this protein exhibited the same profile *in vivo*. To do so, three different fly lines were used: *Act5C-GAL4*, *tubP-GAL80^{ts}/GAL4* and *UAS-shNup358*. Before using any of these lines, they were tested for the presence of both *Wolbachia* spp. (Fig. 5a) and common *Drosophila* viruses (Fig. 5b). No *Wolbachia* or viruses were detected in the fly lines *Act5C-GAL4* and *UAS-shNup358*. However, flies

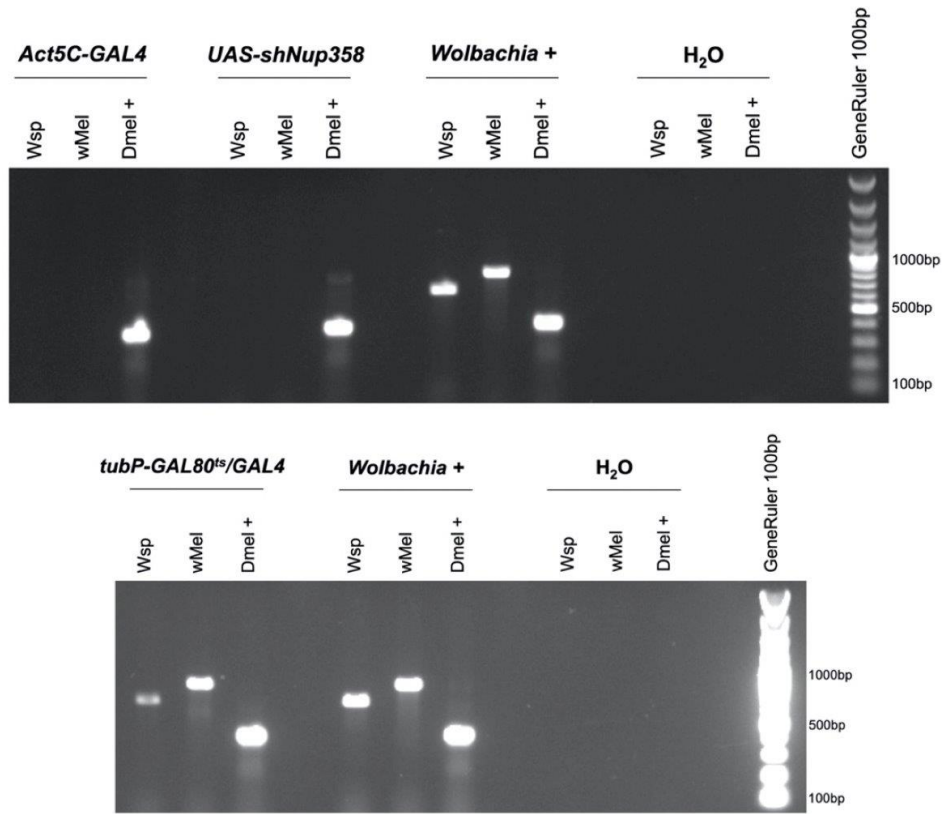
from the *tubP-GAL80^{ts}/GAL4* line were infected with *Wolbachia*, Nora virus and *Drosophila A virus* (DAV).

The purpose of using these lines was to produce a generation of flies that would be silenced for Nup358 mRNA. Crosses of *Act5C-GAL4* male flies with *UAS-shNup358* female virgin flies allowed to obtain a first (F1) generation of flies that expressed a short-hairpin against Nup358 mRNA (shNup358) via the GAL4/UAS system, regulated by the Actin 5C promoter (Fig S2). However, during the development of this project, other members of the laboratory using the *Act5C-GAL4* driver line realised that the driver was not working properly. The main problem resides on the fact that when the *Act5C-GAL4* line was crossed with a control line, it led to deadly phenotypes or, in other cases, phenotypes in which no silencing could be observed. To solve this, *tubP-GAL80^{ts}/GAL4* male flies were crossed with *UAS-shNup358* female virgin flies allowing to obtain an F1 generation of flies that expressed shNup358 via the GAL80^{ts}/GAL4/UAS system, regulated by the tubulin promoter, when grown at 29°C. GAL80^{ts} is an inhibitor of GAL4 but its activity can be repressed at temperatures from 29°C and above, enabling the binding of GAL4 to the UAS and the subsequent transcription of shNup358 (Fig. S2). Nonetheless, the *tubP-GAL80^{ts}/GAL4* driver line was infected with *Wolbachia* and two viruses, which affected the maintenance and normal response of this line during the experiments. As the time allowed for this project precluded me from obtaining clean flies, they were used even though they were infected. Interestingly, *Wolbachia*, Nora and DAV were not detected in the F1 generation (Fig. S3).

Nup358 silencing *in vivo*

Before using the parental and F1 generation lines to test the hypothesis of the antiviral role of Nup358 *in vivo*, the silencing of Nup358 mRNA was assessed in all the lines by RT-qPCR (Fig. 6). Once again, both primers sets – Random primers or Oligo(dT)s – were used to generate cDNA since shNup358 targets a region of the Nup358 mRNA that is downstream of the qPCR primers binding sites. For the *Act5C-GAL4* × *UAS-shNup358* crosses (Fig. 6a), a significant decrease of the Nup358 mRNA levels in the F1 generation was detected comparatively to the *UAS-shNup358* parental, only for Oligo(dT)s-reverse-transcribed cDNA. However, these results could not be confirmed and parallel independent experiments to silence Nup358 mRNA in the F1 generation did not show the same results (data not shown). No significant Nup358 mRNA silencing was observed for the F1 generation resultant from the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses when compared with each of the respective parental lines (Fig. 6b).

a



b

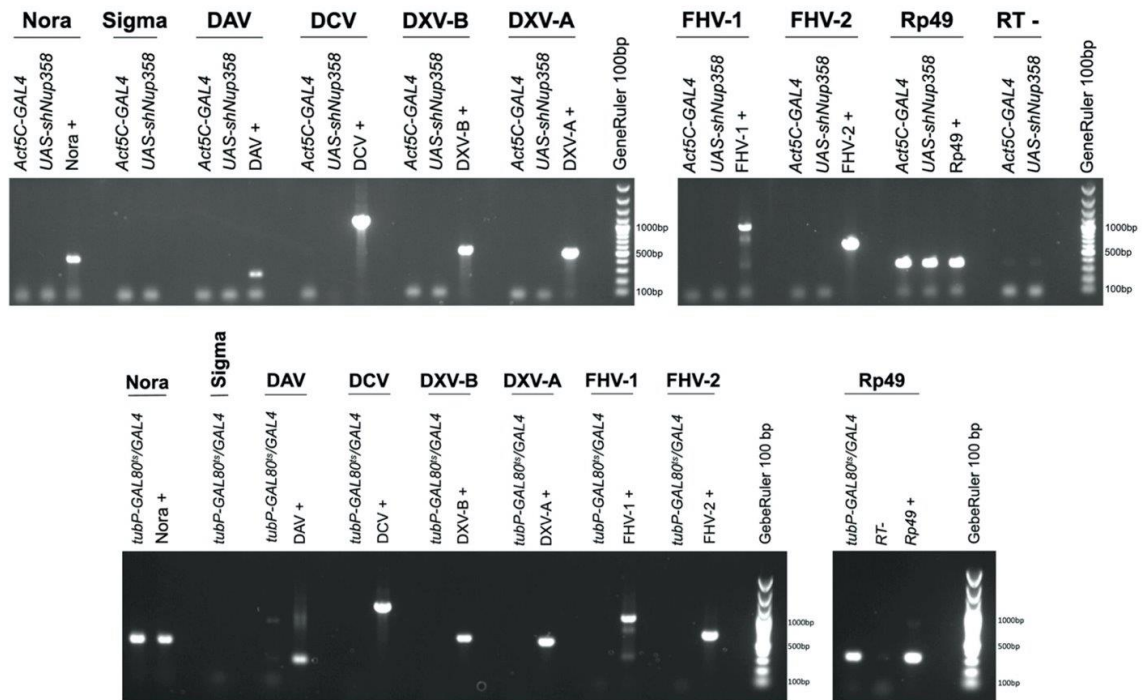


Figure 5 | *Wolbachia* spp. and viruses detection in *Drosophila* fly lines. One pool of three female and three male flies, each of *Act5C-GAL4* and *UAS-shNup358* lines, and one pool of three female flies of *tubP-GAL80^{ts}/GAL4* line were collected and assessed for the presence of both *Wolbachia* spp. (a) and *Drosophila*-specific viruses (b). Flies were lysed in Squishing Buffer supplemented with Proteinase K and the presence of *Wolbachia* DNA was assessed by PCR using pathogen-specific primers. DNA extracts from *Wolbachia*-infected flies were used as positive control and H₂O as negative control (a). Flies were lysed in TRIzol to extract RNA and reverse-transcribed to cDNA. Virus-derived cDNA was detected by PCR with viruses-specific primers. cDNA samples amplified with the housekeeping gene Rp49 primers were used as an endogenous positive control for the RT-PCR and the RT negative samples were used to assess DNA contamination (b). Wsp and wMel primers bind to genes encoding for *Wolbachia* surface proteins; Dmel+ is an endogenous positive control for PCR; Nora: Nora virus; Sigma: Sigma virus; DAV: *Drosophila* A virus; DCV: *Drosophila* C virus; DXV-B/A: *Drosophila* X virus Segment B/A; FHV-1/2: Flock House virus RNA1/2. Positive controls for the viruses are indicated by their abbreviations followed by “+”.

SINV replication in Nup358-silenced flies

Although Nup358 mRNA silencing could not be confirmed for any of the F1 generations, the *in vivo* infections were still carried on as no other driver line was available at the time to produce an F1 generation with proper levels of silencing. To study the *in vivo* infection profile of SINV between the parental lines and the F1 generation of both crosses at different time points, flies were either injected with SINV-RLuc or a mock solution at day 0. Flies injected with virus were collected at one, two and three days post-infection (dpi), and mock-infected flies were only collected three days after infection. Similar to the *in vitro* testing, SINV-RLuc replication was determined by measuring *Renilla* luciferase luminescence. For the *Act5C-GAL4* × *UAS-shNup358* crosses (Fig. 7a), a significant decrease in luminescence was detected between the parental driver line and the F1 generation immediately at 1 dpi. Apart from this, no other significant differences in the luminescence fold change between each parental line and the F1 generation were observed for any time point. Interestingly, almost no luminescence values were detected for the *tubP-GAL80^{ts}/GAL4* line throughout the experiment (Fig. 7b). Therefore, significant differences between the luminescence levels of this parental line and the F1 generation were observed for all time points. Still, at 3 dpi a significant increase in luminescence could be seen for the F1 generation (Fig. 7b), compared to the *UAS-shNup358* line, indicating a higher SINV-RLuc replication. However, this experiment is not conclusive because of the abnormal response of the parental driver line to SINV-RLuc infection, probably due to the flies being infected by natural pathogens.

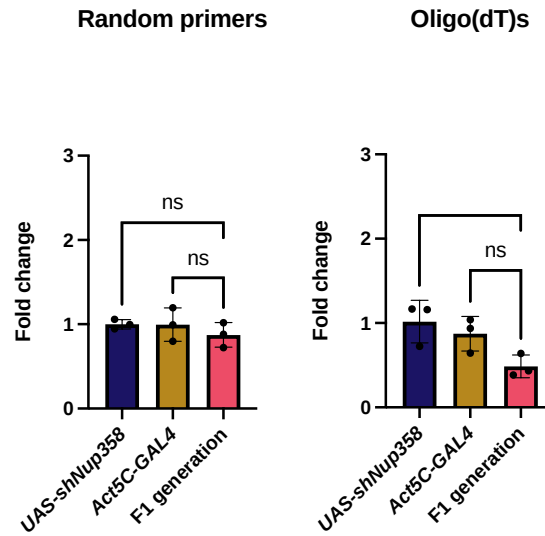
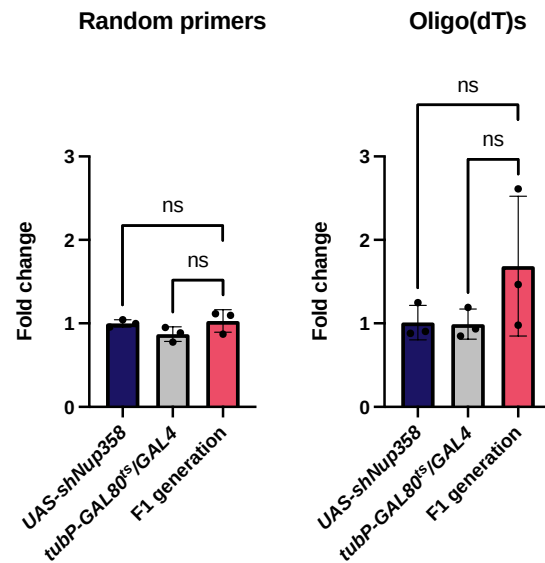
a*Act5C-GAL4* ' *UAS-shNup358***b***tubP-GAL80^{ts}/GAL4* ' *UAS-shNup358*

Figure 6 | Assessment of Nup358 silencing in the F1 generation. (a) *Act5C-GAL4* male flies were crossed with *UAS-shNup358* female virgin flies to obtain the F1 generation. Three pools of three female flies of each line (two parental and F1 generation) were lysed in TRIzol. (b) *tubP-GAL80^{ts}/GAL4* male flies were crossed with *UAS-shNup358* female virgin flies to obtain the F1 generation. Three individual male flies of each line (two parental and F1 generation) were lysed in TRIzol. Extracted RNA was reverse-transcribed to cDNA using either Random primers or Oligo(dT)s. RT negative samples were used as control for DNA contamination. CT values were obtained and ΔCT were calculated by subtracting the CT values obtained for the housekeeping gene *Rp49*. One experiment with three replicates was performed. Data are presented as mean $\pm$ s.d. of $2^{-\Delta\Delta\text{CT}}$ normalised to *UAS-shNup358*. A *P* value <0.05 was considered to be significant. ns: non-significant, **P* <0.05 .

Mortality in Nup358-silenced flies infected with DCV

The effect of DCV infection in flies of the parental lines and F1 generation of the two crosses was assessed by plotting a survival curve. Flies were infected either with DCV or a mock solution at day 0, and starting on day 1, mortality in each line was checked. No significant differences in the survival rate between each parental line and the F1 generation were detected for the *Act5C-GAL4* × *UAS-shNup358* crosses (Fig. 7c). For the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses, DCV-infected flies of the F1 generation died significantly sooner than those of the *UAS-shNup358* line (Fig. 7d). A weak significant difference was observed between the survival curves of infected flies of the *tubP-GAL80^{ts}/GAL4* driver line and the F1 generation. As stated above, the persistent infection of the driver line with *Wolbachia* and two other viruses does not allow to extract conclusions from this experiment.

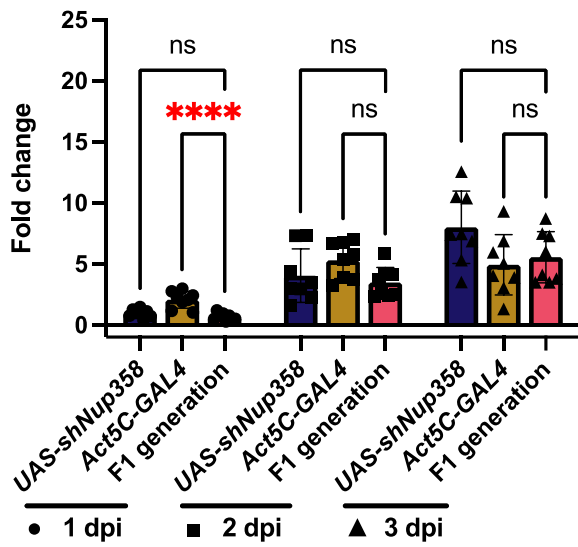
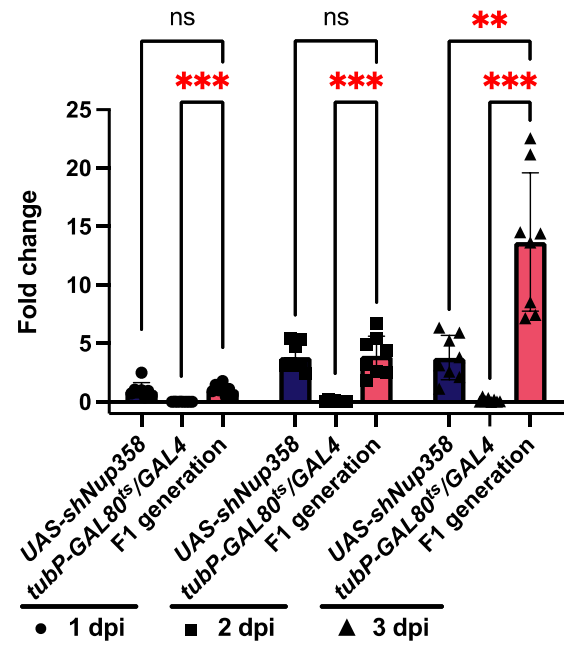
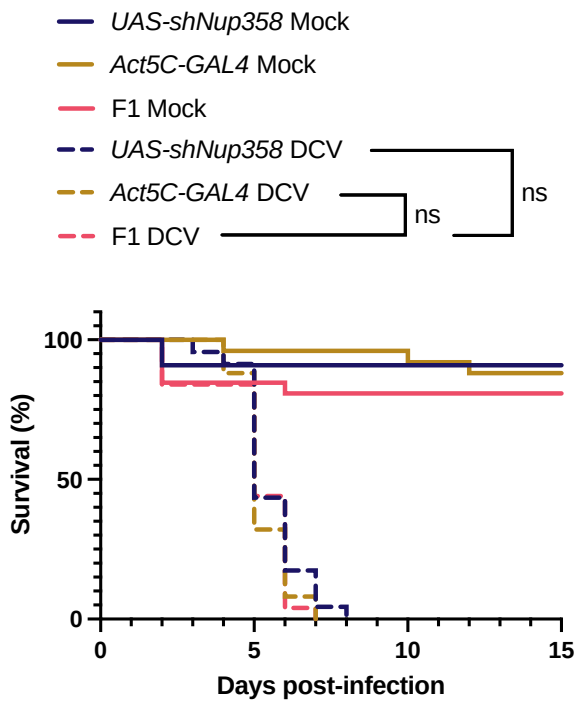
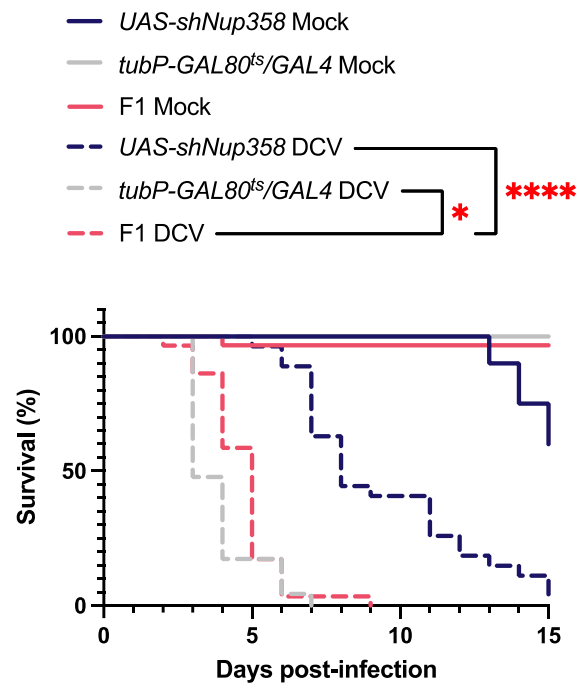
a*Act5-GAL4* × *UAS-shNup358***b***tubP-GAL80^{ts}/GAL4* × *UAS-shNup358***c***Act5-GAL4* × *UAS-shNup358***d***tubP-GAL80^{ts}/GAL4* × *UAS-shNup358*

Figure 7 | Profiles of viral infection in Nup358-silenced flies injected with SINV-RLuc or DCV. Flies of the parental lines and F1 generation of the *Act5C-GAL4* × *UAS-shNup358* and *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses were challenged with SINV-RLuc (**a,b**) or DCV (**c,d**). (**a,b**) 759 PFU/fly of SINV-RLuc or a mock solution were intrathoracically injected in flies, and infected flies were collected at 1 day post-injection (dpi), 2 dpi and 3 dpi. Mock-infected flies were collected at 3 dpi. Flies were lysed, *Renilla* luciferase luminescence was measured, and the values were used to estimate SINV-RLuc replication. One experiment with eight individual flies per line, at each time point, was performed. Fold changes in luminescence for each time point were compared using Welch's ANOVA followed by Dunnett's T3 multiple comparison post-hoc test (**b**). Data are presented as mean ± s.d. of luminescence values normalised to *UAS-shNup358* at 1 dpi. (**c,d**) 10 TCID₅₀/fly of DCV or a mock solution were intrathoracically injected in flies, and the mortality of flies daily monitored. One experiment with thirty flies per line and per condition was performed. Data are presented as variations of the survival rate through time. A *P* value <0.05 was considered to be significant. ns: non-significant, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

CHAPTER V: DISCUSSION

Cytosolic delivery of RNA molecules that can be processed by the siRNA or miRNA pathways (dsRNA or short hairpins) is a commonly used strategy to silence target mRNA and reduce protein levels. Here, *Drosophila* S2 cells were successfully transfected with dsRNA targeting Nup358 and Ago2, which led to a general decrease in the mRNA levels of these proteins (Fig. 1). Even though significant decreases were not detected for the Effectene-transfected cells, diminishing Nup358 mRNA levels to 60% (Fig. 1b) was considered enough to continue with the *in vitro* experiments. Ideally, the silencing should be repeated to obtain statistical significance (at least, three independent experiments) and should also be assessed at the protein level by Western Blot. However, there was no available antibody (Ab) against Nup358 either in the laboratory or commercially. This limitation could be fixed by asking an Ab-providing company to produce an Ab specifically against Nup358.

Silencing Nup358 and Ago2 mRNA did not alter the viability of the cells under the experimental conditions tested, suggesting that none of these proteins is involved in major processes indispensable for cell survival. This does not match previously presented data regarding the effect of silencing Nup358 on S2 cells proliferation (Forler et al., 2004). The authors had plotted the increase in cell number of non-treated cells and cells treated with dsRNA against Nup358 up to 8 days post-treatment. An arrest of cell proliferation was already noticeable after 4 days for Nup358-silenced cells; however, in our viability assay, cells were only transfected with dsNup358 for 2 days. At 2 days post-treatment with dsRNA, no difference in the number of cells was registered between the non-treated and Nup358 silencing conditions (Forler et al., 2004).

In my work, for the first time, Nup358-silenced cells are shown to increase SINV (Fig. 3) and DCV (Fig. 4b) replication comparatively to non-treated cells upon infection. These observations hint to the antiviral role of Nup358 *in vitro*, at least against these viruses. Curiously, upon SINV-WT infection, a significant increase in the viral titres was also detected for dsControl-treated cells (Fig. 3b). One possible explanation is that dsControl has a sequence-specific effect that is affecting the antiviral response. This could be assessed by using another control dsRNA, for example, against green fluorescent protein (GFP) mRNA (which is also not naturally produced by S2 cells) or a scramble dsRNA (does not target any specific sequence). The ELISA experiments, which aimed to study the impact of Nup358 on DCV loads, did not allow to obtain conclusive results (Fig. 4a). Even though cells were treated with dsRNA targeting Ago2, an antiviral protein of the siRNA pathway, no increased DCV loads were detected by ELISA for these cells. The reason behind this is most likely related to technical problems with the ELISA protocol since the experimental setup for S2 cells infection allowed to detect differences in DCV titres. Different dilutions of the primary Ab against DCV should be tested to try

to address these issues. Even if the ELISA experiments did not generate reliable results, the end-point dilution assay showed increased DCV titres in Nup358-silenced cells, confirming the antiviral effect for this protein also against DCV.

The observation of an antiviral effect of Nup358 *in vitro* justified testing if it had the same role *in vivo*. Here, two types of flies crosses were used: *Act5C-GAL4* × *UAS-shNup358* and *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358*. The *UAS-shNup358* and *Act5C-GAL4* parental lines were both free of *Wolbachia* and commonly persistent viruses (Fig. 5), so the F1 generation resulting from the crosses was not infected with any of the tested pathogens. Surprisingly, the F1 generation of the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses was also clean of pathogens (Fig. S3), although the *tubP-GAL80^{ts}/GAL4* line was infected with *Wolbachia*, DAV and Nora virus (Fig. 5). Flies from this parental line barely had any reproduction success due to premature death of the larvae. One possible explanation lies on the infection with *Wolbachia*, which are known to be vertically transmitted from females to progeny. These bacteria can manipulate their hosts' reproductive phenotypes with the aim of enhancing the production of infected progeny, namely females (Werren et al., 2008). Oddly, a bias towards male survival was observed for the *tubP-GAL80^{ts}/GAL4* flies but considering that they were also infected with two viruses, it is not possible to predict exactly the reason behind this phenotype. Even though Nora virus does not cause any obvious pathological effects (Habayeb et al., 2006) and DAV is only known to be less pathogenic than DCV (Ambrose et al., 2009), co-infection of these two viruses and *Wolbachia* in *D. melanogaster* has never been reported. Interestingly, the F1 generation produced by the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses did not exhibit the same reproduction profiles as its parental driver line, and female/male proportions were similar. This prompted to determine which pathogens were affecting the flies of the F1 generation and to confirm that these flies were not infected (Fig. S3). Since *Wolbachia* is maternally transmitted, by crossing non-infected female virgin flies of the *Act5C-GAL4* line with males of the *tubP-GAL80^{ts}/GAL4* line, their F1 generation was not infected with this bacterium. Nora virus mainly replicates in the intestines of flies, being horizontally transmitted after it is excreted in the feces (Habayeb et al., 2009). However, Nora virus was not detected in the flies of the F1 generation (Fig. S3). Though no Sigma virus positive control was available to validate the results for viruses detection, it is very unlikely that any of the parental lines (and subsequently the F1 generations) were persistently infected with this virus. Flies infected with Sigma virus are sensitive to CO₂ and, when challenged with high concentrations of this gas, fail to recover from paralysis (Rosen, 1980). Throughout the course of the experiments, in which flies had to be CO₂-anesthetised, no such event was ever observed, eliminating any doubts regarding the possibility of any of the used lines being infected with Sigma virus.

There is no apparent reason that allows to explain the non-detection of Nup358 mRNA silencing for the F1 generation of the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses. However, it must be taken into consideration the possibility that there was no Nup358 silencing in the F1 generation even if the *UAS/GAL80^{ts}/GAL4* system was complete under proper temperature conditions. Once again, the silencing should have also been tested at the protein level and the mRNA silencing should be replicated in at least three independent experiments.

Inadequate, or even non-existent, Nup358 silencing in the F1 generation of the *Act5C-GAL4* × *UAS-shNup358* crosses, and persistent infections of the *tubP-GAL80^{ts}/GAL4* line do not allow to draw any conclusions regarding the antiviral role of Nup358 *in vivo*. Nonetheless, some observations must still be pointed out and discussed. For the SINV-RLuc assays, the significant difference in viral replication between the *Act5C-GAL4* parental and the F1 generation at 1 dpi was attenuated at the other two time points (Fig. 7a). This may be due to differences between both lines in responding to viral infection at early stages but is unlikely due to effects of Nup358 silencing as there were no differences in luminescence between the F1 generation and the *UAS-shNup358* line for the same time point. The low luminescence levels obtained for the *tubP-GAL80^{ts}/GAL4* line throughout all the time points (Fig. 7b) indicate that there was barely any SINV-RLuc replication within these flies. As *Wolbachia*-containing flies have been shown to be resistant against infection by certain RNA viruses (Teixeira et al., 2008), it could be possible that SINV-RLuc replication was affected in a similar manner. An unsuccessful injection of the flies of the *tubP-GAL80^{ts}/GAL4* line might also have been responsible for the low viral replication observed. The high luminescence values of the F1 generation flies at 3 dpi in comparison to the *UAS-shNup358* parental (Fig. 7b) are interesting regarding the *in vivo* silencing of Nup358, as well as its antiviral role. However, the aim of this assay was to compare the F1 generations to both their parental lines, and since the *tubP-GAL80^{ts}/GAL4* line is not clean of pathogens, nothing can be assumed about either the silencing of Nup358 or its role. Also, these experiments were only performed once so they lack statistical significance.

DCV-infected flies from each parental line *Act5C-GAL4* or *UAS-shNup358* survived as well as the F1 generation (Fig. 7c), which it is in accordance with the SINV-RLuc results, showing no difference in the antiviral response for any of the lines. The survival curves of the *tubP-GAL80^{ts}/GAL4* × *UAS-shNup358* crosses were significantly different among each other (Fig. 7d). Flies from the F1 generation died sooner than those of the *UAS-shNup358* line, which is also consistent with SINV-RLuc replication for these lines (Fig. 7c, 3 dpi). The *tubP-GAL80^{ts}/GAL4* flies died soon after being challenged with DCV which might be due to the persistent co-infection of *Wolbachia* and the other two viruses. Even though it has been shown that *Wolbachia* induces resistance against DCV infection in *D. melanogaster* (Teixeira et al., 2008), it is also true that this protection

is dependent on the *Wolbachia* variants colonising the fruit flies (Chrostek et al., 2013). Therefore, no exact prediction can be made regarding the reason behind this observation, though it is probably related to the persistent infections of the *tubP-GAL80^{ts}/GAL4* line. Indeed, the driver lines used constitute the major limitation of the *in vivo* experiments coupled with the lack of time that would allow to either clean the infected driver line or to acquire a new driver line. For this reason, these experiments must be repeated using a proper driver line that is not infected and that ensures the development of an F1 generation with adequate silencing of Nup358.

Nup358 is a nucleoporin located at the most distal portion of the nuclear pore complex (NPC) cytoplasmic region. Very few information concerning this *Drosophila* protein is available and to date no antiviral role has been described for Nup358. However, considering its location within the NPC and its intracellular distribution, one can speculate about the possible mechanisms that Nup358 triggers which confer its antiviral properties. Stress granules (SGs) are membrane-less cytoplasmic structures which, among other macromolecules, mainly contain messenger ribonucleoproteins (mRNPs) stalled in translation initiation. SGs assemble under stress conditions and can affect antiviral responses, signalling pathways, and the localisation, translation and degradation of mRNA (Protter & Parker, 2016). Among other *HsNups*, *HsNup358* has been shown to localise to SGs (K. Zhang et al., 2018), and *HsAgo2* is also known to relocate to these structures (Pare et al., 2011). However, it has not yet been assessed whether Nup358 and/or Ago2 move to SGs in *D. melanogaster* cells. Viruses have been shown to interfere with the assembly of SGs, leading to opposite outcomes, as it seems that some viruses benefit from SGs assembly while others avoid it by interfering with the process (Q. Zhang et al., 2019). Specifically, infection of S2 cells with either DCV or cricket paralysis virus (CrPV), which belong to the *Dicistroviridae* family, inhibits the formation of SGs (Khong & Jan, 2011). These findings suggest that by inhibiting the formation of SGs, viruses avoid translation arrest in order to keep viral proteins available for replication and processing. Considering these concepts and the obtained results, different possibilities regarding the Nup358-mediated antiviral mechanism against SINV and DCV are proposed. One possibility is that Nup358 might control SG-related antiviral defence. When a virus hijacks the host's cytoplasmic machinery for its own replication, there is production of intermediate viral dsRNA forms, which eventually bind to Nup358. Upon viral dsRNA binding to Nup358, the nucleoporin promotes the assembly of SGs, by an unknown mechanism, simultaneously integrating the composition of the SGs. The SGs gather the mRNPs scattered around the cytoplasm, including those that are promoting viral proteins synthesis, arresting their activity. Meanwhile, viral siRNA-loaded Ago2/holo-RISC localises to the SGs, where viral RNA is accumulated, and cleaves the sequences against which it possesses specificity (Fig. A1). This ensures a tightly regulated and fast response against viral infection by limiting it to specific areas within the cytoplasm, though silencing could still take place in additional cytoplasmic locations.

The components of the NPC have different residence times within the complex, as the Nups that assemble the core are very stable while the most distal Nups are very dynamic, suggesting that they might have other functions unrelated to the NPC (Rabut et al., 2004). Other proposed mechanisms behind the antiviral role of Nup358 may lie on this putative ability to function independently of the NPC, since it locates to the periphery of the complex. Another possible mechanism that we propose is that upon binding of viral dsRNA to Nup358, this might disassemble from the NPC and translocate to the nucleus. Inside the nucleus, Nup358 could act as a transcription factor and promote the expression of genes related to the antiviral response or inhibit the transcription of proviral genes. It may also happen that, after dsRNA binding, Nup358 may be triggered to transmit a cytoplasmic signal to the nucleus that induces gene expression or repression (Fig. A2).

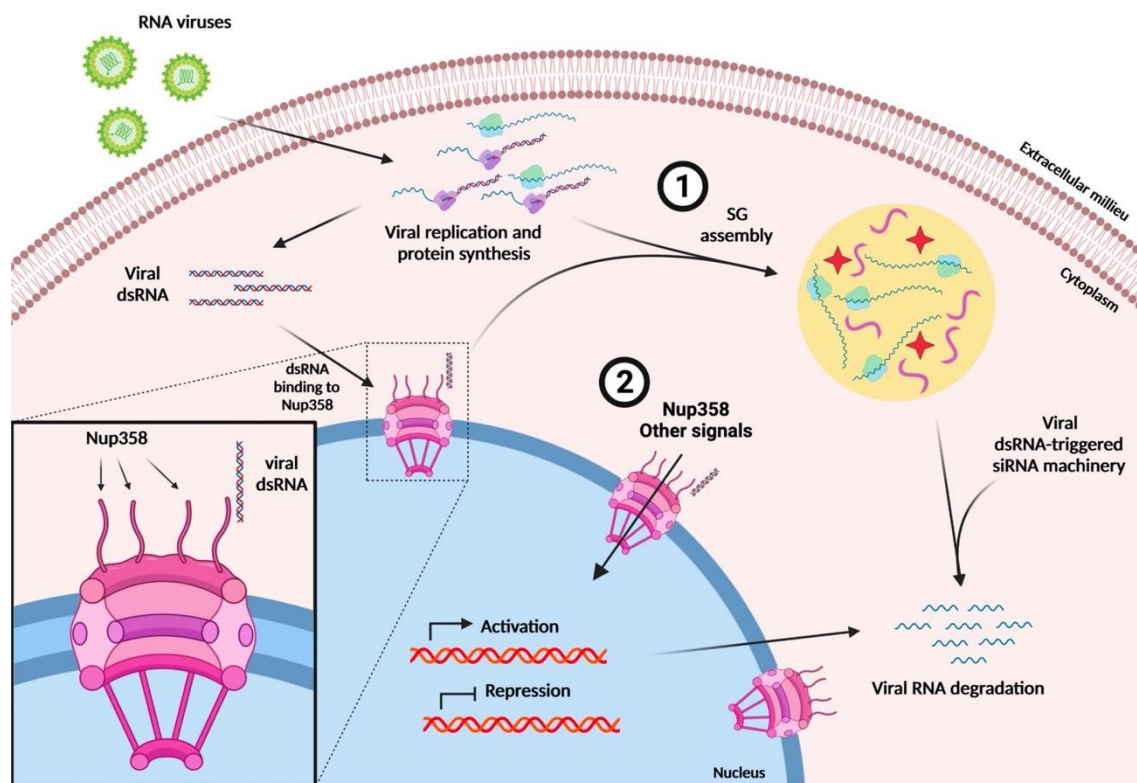


Figure A | Possible models for Nup358-mediated antiviral response in *Drosophila melanogaster*. During viral infection, there is replication of the viral genome and production of proteins required to generate the viral envelope. dsRNA of viral origin is formed as a consequence of genome replication and eventually bind Nup358. This may trigger Nup358 to dissociate from the nuclear pore complex (NPC) and induce the assembly of stress granules (SGs), where viral RNA-bound ribonucleoproteins (RBPs) may accumulate (1). Stress granules contain RBPs stalled in translation initiation, diverse copies of Nup358 (pink “S”) and other factors (red stars). Components of the siRNA pathway that were induced by viral infection, mainly Ago2/holo-RISC, localise to the SGs and promote the cleavage of viral RNA. It may also happen that when the dsRNA binds Nup358, it allows its translocation to the nucleus or the nuclear transport of other signal proteins (2). Both may induce patterns of gene activation/repression to promote an antiviral response that culminates in degradation of viral RNA.

CHAPTER VI: FUTURE PERSPECTIVES

Several speculations concerning the pathways through which Nup358 exerts its antiviral role could be pointed out but regardless of the exact mechanism, evidence must be gathered about the interaction between dsRNA and Nup358. After assessing whether Nup358 has indeed an antiviral role *in vivo* against SINV and DCV, other viruses can be tested to understand if this effect is specific or broadens, such as the natural *Drosophila* pathogen FHV. Considering that this project was based on evidence showing that Nup358 binds to dsRNA, this premise should be further confirmed *in vitro*. Upon treatment of S2 cells with dsRNA, it could be checked if Nup358 and dsRNA colocalise inside the cell by immunofluorescence, using antibodies against dsRNA and Nup358 or against the tag of a recombinant Nup358-tag protein. In case such protein was used, it should be tested whether the tag would affect the normal function of Nup358. An electrophoresis mobility shift assay (EMSA) would allow to check if Nup358 and dsRNA effectively bind *in vitro* since observing colocalisation does not necessarily mean that the molecules interact. It would also be interesting to test if dsRNA binding to Nup358 is affected by interactions of Nup358 with other proteins, for example, from the NPC.

To test the model proposing Nup358-induced and SG-mediated antiviral response, it would be interesting to check whether viral infection affects the colocalisation of both Nup358 and Ago2 to SGs relatively to non-infected cells, using the adequate SGs markers (Khong & Jan, 2011). Also, after treating the cells with dsRNA silencing Nup358, it could be checked if the SGs still maintain their structure or disintegrate.

To understand the impact of Nup358 during viral infection at the gene expression level, a transcriptomics experiment could be performed. For this study, four different conditions should be tested: non-infected and non-silenced cells, non-infected Nup358-silenced cells, virus-infected non-silenced cells and virus-infected Nup358-silenced cells. By comparing the results obtained for these conditions, genes whose expression was modified due to Nup358-silencing during viral infection could be identified.

One assumption inherent to this project is that Nup358 might have a role in viral infection since it binds dsRNA. However, it is possible that the antiviral role of Nup358 is independent of its dsRNA-binding property. To assess this, the domain responsible for the interaction of Nup358 to dsRNA should be determined. Repeating the EMSA using mutant Nup358 proteins that lack specific domains to see which mutations affect the binding to dsRNA could be an approach. Another approach consists of determining the structure of the dsRNA-bound Nup358 by cryo-electron microscopy. After determining the domain which interacts with dsRNA, a mutant Nup358 lacking this domain should be produced and its antiviral role tested *in vitro* and *in vivo*.

CHAPTER VII: CONCLUDING REMARKS

Upon comparison of SINV replication between non-transfected cells and Nup358-silenced cells, a significantly higher viral replication was detected for Nup358-silenced cells. The quantification of SINV and DCV titres in Nup358-silenced cells allowed to observe a significantly increase in viral titres relatively to non-transfected cells. These results demonstrate an antiviral property of Nup358 against SINV and DCV infections *in vitro*. The *in vivo* challenging of Nup358-silenced flies with SINV-RLuc and DCV was not conclusive. However, there is abundant evidence to propose that Nup358 has an antiviral effect in *Drosophila melanogaster*.

APPENDIX

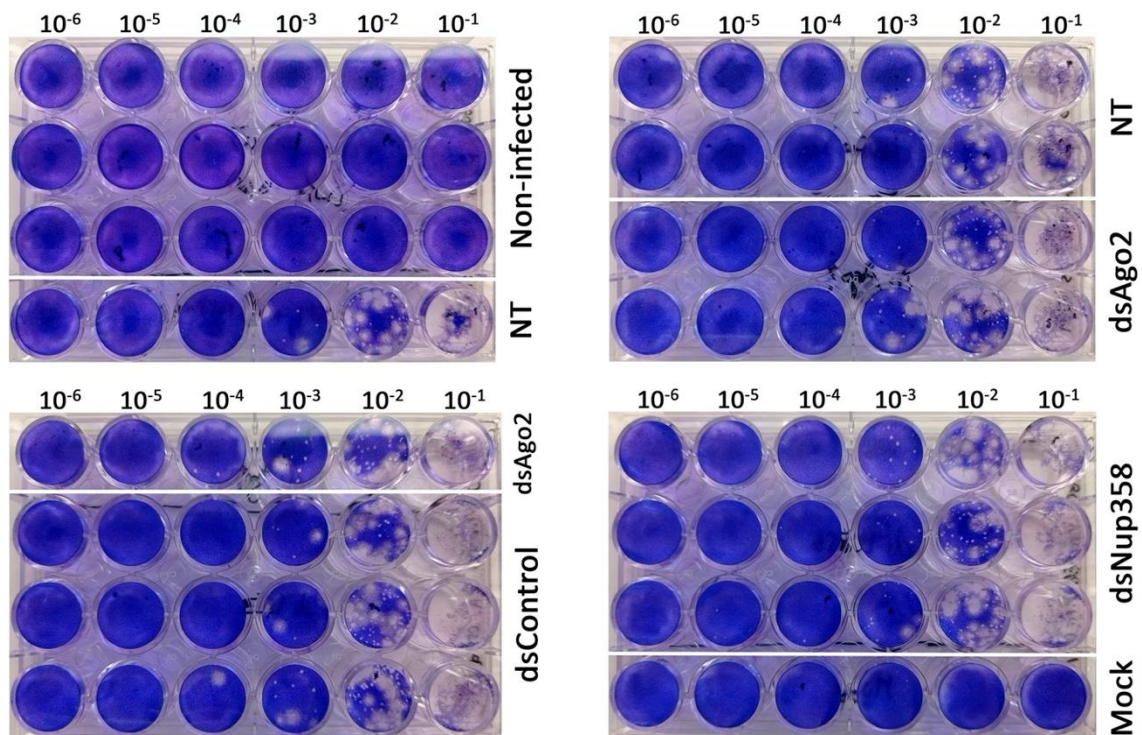


Figure S1 | Plaque assay for SINV-WT titration. Supernatants (SN) of non-infected and SINV-WT-infected S2 cells were collected and diluted from 10^{-1} to 10^{-6} in DMEM without FBS. Dilutions were added to wells (24 well-plate) with a monolayer of Vero-E6 cells and these were incubated at 37°C for 1 hour. An overlay of DMEM 2%FBS/0.8% CMC solution was poured to each well and the plates were incubated at 37°C for 72 hours. Cells were then fixed with 4% formaldehyde and stained with a 20% ethanol 0.23% crystal violet solution to count plaques (transparent areas) in each well. Plaques are sites of disruption of the Vero-E6 cells monolayer due to infection and cell death caused by a single plaque-forming unit (PFU) of SINV-WT. Upon counting plaques and calculating the PFU/mL for different dilutions from all the samples, the latter were averaged for each condition and SINV-WT titres were determined. Conditions: 'Non-infected' wells had SN of non-infected S2 cells added to them; 'NT' corresponds to infected S2 cells to which only transfection reagents were added; 'dsAgo2', 'dsControl' and 'dsNup358' wells were coated with SN from infected S2 cells silenced for the respective protein; 'Mock' wells only had DMEM without FBS added to them.

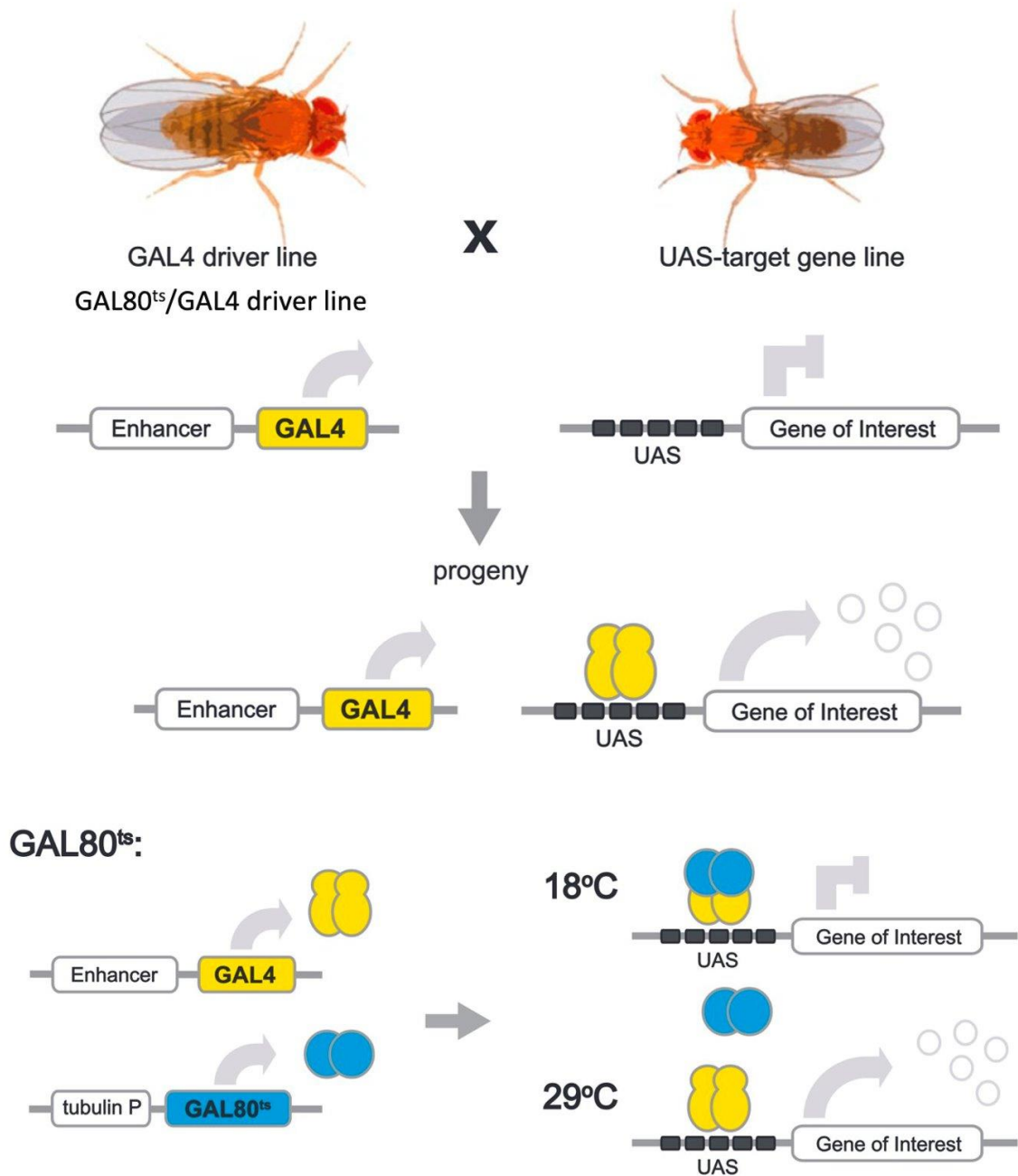


Figure S2 | The GAL4 system. GAL4 driver and upstream activation sequence (UAS)-target gene fly lines are generated and maintained as separate stocks. The target gene is not expressed in the absence of GAL4. Crosses of flies expressing GAL4 with those carrying the UAS-target gene results in targeted gene expression in the progeny of the cross (F1 generation). This system can be modified to modulate GAL4 expression both spatially and temporally. Upon presence of a ubiquitously expressed temperature-sensitive allele of GAL80 (GAL80^{ts}) at the permissive temperature of 18°C, GAL4 activity is inhibited. However, at the restrictive temperature of 29°C, GAL80^{ts} cannot bind GAL4, whose activity is not inhibited anymore, leading to the expression of GAL4-dependent transgenes. Both systems were used to generate flies expressing a short hairpin targeting Nup358 mRNA (shNup358) to induce Nup358 silencing. Figure modified from Caygill & Brand, 2016.

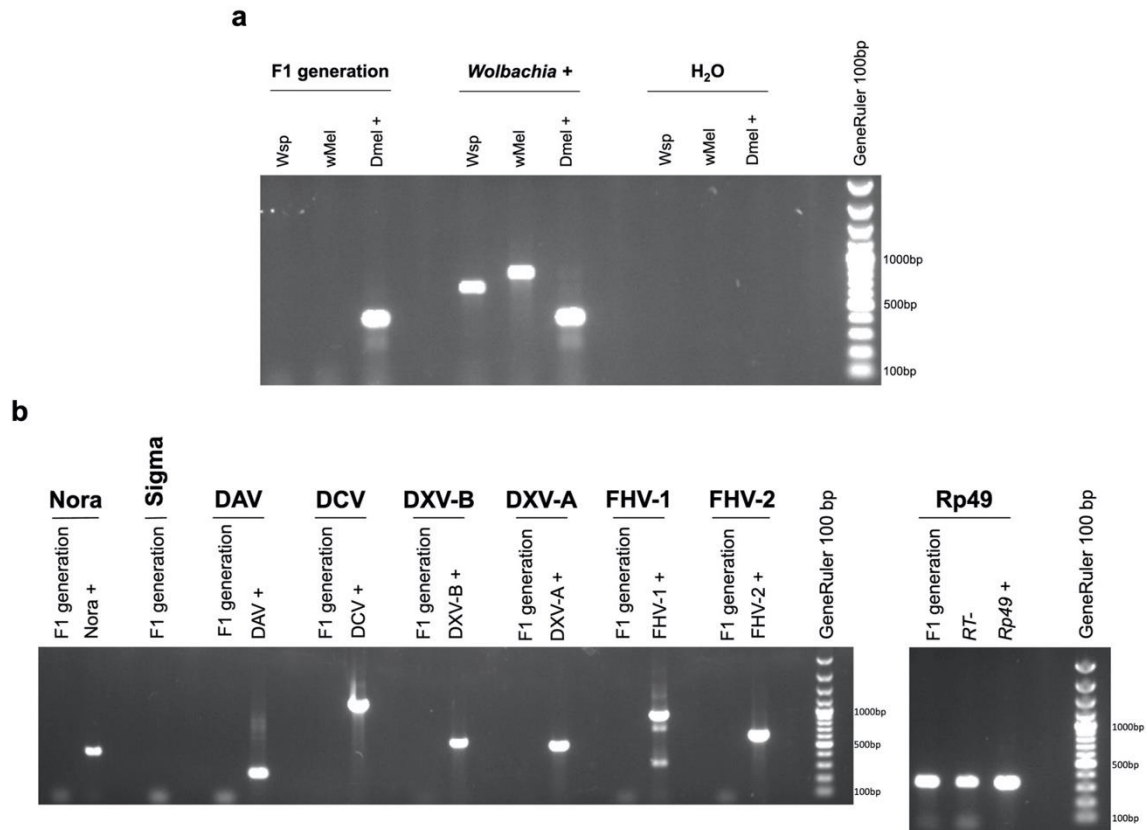


Figure S3 | *Wolbachia* spp. and viruses detection in F1 generation flies. One pool of three female and three male flies of the F1 generation resulting of *UAS-shNup358* and *tubP-GAL80^{ts}/GAL4* crosses was collected to test these lines both for the presence of both *Wolbachia* spp. (a) and *Drosophila*-specific viruses (b). Flies were lysed in Squishing Buffer supplemented with Proteinase K and the presence of *Wolbachia* DNA was assessed by PCR using pathogen-specific primers. DNA extracts from *Wolbachia*-infected flies were used as positive control and H₂O as negative control (a). Flies were lysed in TRIzol to extract RNA and reverse-transcribe it to cDNA. Virus-derived cDNA was detected by PCR with viruses-specific primers. cDNA samples amplified with the housekeeping gene Rp49 primers were used as an endogenous positive control for the RT-PCR and the RT negative samples were used to assess DNA contamination (b). Wsp and wMel primers bind to genes encoding for *Wolbachia* surface proteins; Dmel+ is an endogenous positive control for PCR; Nora: Nora virus; Sigma: Sigma virus; DAV: *Drosophila* A virus; DCV: *Drosophila* C virus; DXV-B/A: *Drosophila* X virus segment B/A; FHV-1/2: Flock House virus RNA1/2. Positive controls for the viruses are indicated by their abbreviations followed by "+". RT- sample shows a band due to a pipetting error and does not invalidate the results for viral detection.

Table S1 | SINV-WT titres (PFU/mL) of each replicate from three independent experiments determined by plaque assay.

	NT	dsControl	dsAgo2	dsNup358
Experiment 1	3.1×10^4	1.6×10^5	1.05×10^5	2.35×10^5
	6×10^4	1.1×10^5	1.65×10^5	2.75×10^5
	1.1×10^5	1.2×10^5	2.25×10^5	1.95×10^5
Experiment 2	4×10^4	1.35×10^5	1.8×10^5	2.1×10^5
	5×10^4	1.3×10^5	1.6×10^5	1.7×10^5
	7×10^4	-	2.35×10^5	1.05×10^5
Experiment 3	5×10^4	5×10^4	9×10^4	1.05×10^5
	3×10^4	4×10^4	1.05×10^5	7×10^4
	2.15×10^4	9×10^4	1.45×10^5	1.4×10^5

Table S2 | DCV titres (TCID₅₀/mL) of each replicate from two independent experiments determined by end-point dilution assay.

	NT	dsControl	dsAgo2	dsNup358
Experiment 1	8.89×10^6	5×10^6	5×10^7	2.81×10^7
	8.89×10^6	8.89×10^6	2.81×10^7	2.81×10^7
	1.58×10^7	1.58×10^7	2.81×10^7	5×10^7
Experiment 2	8.89×10^8	5×10^8	2.81×10^9	2.81×10^9
	1.58×10^9	1.58×10^9	2.81×10^9	2.81×10^9
	5×10^8	2.81×10^8	1.58×10^9	1.58×10^9

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