

# The role of p53 during chikungunya virus infection in mammal and insect

Lucie Cappuccio

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**THE ROLE OF P53 DURING CHIKUNGUNYA VIRUS INFECTION  
IN MAMMAL AND INSECT**

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## Abstract

The chikungunya virus (CHIKV) belongs to the *Alphavirus* genus which can be found in an ecological but not taxonomic group named arboviruses (for arthropod-borne viruses), indicating viruses transmitted by an arthropod vector hematophagous to a vertebrate host, during a blood meal. *Alphaviruses* are transmitted by bloodsucking arthropods, mainly mosquitoes, to humans and animals causing debilitating disease. While in humans, CHIKV-associated disease is characterized by fever, headache, and a typical acute infection, sometimes followed by persistent arthralgia or myalgia, the infection does not seem to cause significant pathology in mosquitos. Since the re-emergency of CHIKV at La Réunion in 2005-2006, the *Alphavirus*-host or vector interactions have been increasingly studied at the cellular and molecular level. Indeed, the identification of cellular and viral factors involved in human pathology and mosquito chronicity could lead to the development of antiviral treatments. Regulated apoptotic cell death and interferon Type-I immune response are strong antiviral defences participating in rapid viral elimination. Moreover, it has been demonstrated that p53 plays a central role in the regulation of cell death and the Type-I interferon signalling pathway during different viral infections. Finally, quite recently it has been suggested that in Dengue virus-infected insects, p53-induced cell death was associated with the permissiveness and resistance of vectors. Consequently, my work was to study the potential role of p53 and p53 isoforms on the cellular outcome and viral infection in mammals and insects infected with *Alphavirus*, mainly the chikungunya virus and to a lesser extent the Sindbis virus.

First, in order to investigate a potential function of p53 in viral infection in mammals and insects infected with chikungunya virus and Sindbis virus, we generated a p53 knockout human skeletal muscle cell line. In addition, thanks to a collaboration, we obtained the *Drosophila melanogaster* p53<sup>-/-</sup> mutant strain. We observed an opposite effect of p53 knockout on chikungunya virus infection in *in vitro* human cells and *in vivo* in *Drosophila melanogaster*. Indeed, we demonstrated an increase in CHIKV infection in p53 knockout human cells and a decrease in CHIKV and Sindbis virus replication in whole fly p53<sup>-/-</sup> mutants. Several analyses of the CHIKV-infected p53 knockout human cell line demonstrated an immune antiviral effect of p53 through the Type-I interferon production and signalling pathway and a negative impact on the protection of neighbouring cells. Moreover, it seems that infected-p53 knockout cells are not able to enhance IFN production and signalling. Further experiments are required to decipher the activity of p53 on immune signalling. In parallel, we have shown that CHIKV-induced cell death was p53-independent as no pro-apoptotic genes transcribed by p53 were regulated and the knockout of p53 did not decrease or delay virus-induced cell death.

Secondly, the p53 gene leads to several different protein isoforms which participate in p53 regulation through transcriptional and translational regulatory mechanisms. The biological functions of p53 protein isoforms are actively investigated in the field of cancerology. Δ133p53 is one of the most characterized isoforms although the biological significance of Δ40p53 is not fully understood. In the context of infection, it has been shown that Δ133p53 is modulated and impacts p53-dependent antiviral activity. Thus, for my project we investigated a possible function of Δ40p53 and Δ133p53 isoforms in CHIKV infection. We set up and produced (i) a human muscle cell line overexpressing endogenous Δ40p53 using CRISPR/Cas9 technology; and (ii) an inducible-system overexpressing Δ40p53 and Δ133p53 protein isoforms. We observed that endogenous overexpression of the Δ40p53 isoform led to a decrease in CHIKV infection, but the mechanism remains unclear. In parallel, the inducible system for overexpression of protein isoforms need to be improved in order to investigate the involvement of Δ40p53 and Δ133p53 protein isoforms in CHIKV infection.

**Key words:** alphavirus, chikungunya virus, p53, cell death, immune response, mammal, insect.

## 摘要

基孔肯雅病毒（chikungunya virus, CHIKV）属于甲病毒属，在生态学（而非分类学）上属于虫媒病毒（arboviruses，代表节肢动物传播病毒），该类病毒泛指节肢动物媒介在对脊椎动物宿主进行嗜血过程时传播的病毒。甲病毒由吸血节肢动物（主要是蚊子）传播给人和动物，引起衰弱性疾病。虽然在人类中基孔肯雅疾病的特点是发热、头痛和典型的急性感染，有时还伴有持续的关节痛或肌痛，但在蚊子中感染似乎不会引起明显的病理变化。自 2005-2006 年在留尼旺再次出现 CHIKV 感染事件以来，甲病毒与宿主或媒介的相互作用在细胞和分子水平上得到了越来越多的研究。事实上，对涉及人类病理和蚊子慢性疾病的细胞和病毒因素的鉴定，可以开发新型抗病毒治疗方法。调控的细胞凋亡和干扰素 I 型免疫反应可参与快速消灭病毒，是强力的抗病毒防御手段。此外，已经证明 p53 在不同的病毒感染过程中对细胞死亡和 I 型干扰素信号通路的调控起着核心作用。最后，最近有研究表明，在登革病毒感染的昆虫中，p53 诱导的细胞死亡与载体的允许性和抵抗性有关。基于以上研究背景，我的工作是以 CHIKV 及辛迪比斯病毒 (Sindbis virus) 为甲病毒代表，研究甲病毒感染哺乳动物和昆虫时，p53 及 p53 亚型对细胞命运和病毒感染的潜在影响。

首先，为了研究哺乳动物和昆虫感染 CHIKV 和辛迪比斯病毒后，p53 对病毒感染的潜在功能，我们构建了 p53 敲除的人骨骼肌细胞系。此外，得益于合作，我们获得了黑腹果蝇 p53-/- 突变株。我们观察到 p53 基因敲除对 CHIKV 在体外人细胞和体内黑腹果蝇感染的影响是相反的。事实上，我们已经证明了在 p53 敲除的人细胞中 CHIKV 感染增加，而在全部果蝇 p53-/- 突变体中 CHIKV 和辛迪比斯病毒复制减少。对 CHIKV 感染 p53 基因敲除人细胞系的一些分析表明，p53 通过 I 型干扰素的产生和信号通路具有免疫抗病毒作用，并对邻近细胞的保护产生负面影响。此外，感染 p53 基因敲除细胞似乎不能增强 IFN 的产生和信号传导。需要进一步的实验来解读 p53 对免疫信号通路活性的影响。同时，我们还已证明 CHIKV 诱导的细胞死亡不依赖于 p53，因为没有 p53 家族的靶基因受到调控，p53 的敲除并没有降低或延迟病毒诱导的细胞死亡。

其次，p53 基因导致多个不同的蛋白亚型，通过转录和翻译调控机制参与 p53 调控。p53 蛋白亚型的生物学功能在癌症领域有广泛的研究。Δ133p53 是最有特色的亚型之一，相反，Δ40p53 的生物学意义还没有被完全了解。在感染的背景下，已有研究表明了 Δ 133p53 被调控并影响 p53 依赖性的抗病毒活性。因此，在我的项目中，我们研究了 Δ 40p53 和 Δ 133p53 亚型对 CHIKV 生命周期的影响。我们建立并开展以下实验：(i) 利用 CRISPR/Cas9 技术过表达内源性 Δ 40p53 的人肌肉细胞系和(ii) 过表达 Δ 40p53 或 Δ 133p53 蛋白亚型的诱导系统。我们观察到内源性过表达 Δ 40p53 亚型导致 CHIKV 感染率下降，但具体机制仍不清楚。同时，为了研究 Δ 40p53 和 Δ 133p53 蛋白亚型在 CHIKV 感染中的参与情况，我们仍需改进蛋白亚型过表达的诱导系统。

**关键词：**甲病毒，基孔肯雅病毒，p53，细胞死亡，免疫反应，哺乳动物，昆虫。

## Résumé

Le virus du chikungunya (CHIKV) appartient au genre des *Alphavirus* qui peuvent faire partie d'un groupe écologique mais pas taxonomique, nommé les Arbovirus (pour « Arthropod-borne viruses ») signifiant des virus transmis par des arthropodes vecteurs hématophages à des hôtes vertébrés, au cours d'un repas sanguin. Les *alphavirus* sont transmis, principalement par des moustiques, à l'Homme et aux animaux causant des maladies invalidantes. Tandis que le CHIKV induit chez l'Homme une maladie caractérisée par de la fièvre, des maux de tête et une infection aigue typique, elle peut s'accompagner de douleurs articulaires et musculaires persistantes ; alors que chez le moustique l'infection ne semble pas causer de pathologie. A la suite de la réémergence du CHIKV au cours de l'épidémie de 2005-2006 à La Réunion, les interactions entre les *alphavirus* et leur hôte ou vecteur sont de plus en plus étudiées au niveau cellulaire et moléculaire. En effet, l'identification de facteurs cellulaires et viraux impliqués dans la pathologie de l'Homme et la chronicité du vecteur moustique permettrait de développer des traitements antiviraux. La mort cellulaire programmée (apoptose) et la réponse immunitaire de Type-I assurent une réponse antivirale rapide, participant à l'élimination du virus. De plus, il a été démontré que la protéine p53 joue un rôle central dans la régulation de l'apoptose et dans la voie de signalisation de la réponse interféron de Type-I. Pour finir, il a été suggéré récemment que l'infection d'un insecte par le virus de la Dengue, la mort cellulaire induite par p53 était associée à la permissivité et à la résistance du vecteur.

L'objectif de mon travail était d'étudier le rôle possible de la protéine p53 et des isoformes p53 sur la réponse cellulaire et l'infection virale chez le mammifère et l'insecte infectés par des *alphavirus*, en prenant le virus du chikungunya comme modèle principal et dans une moindre mesure le virus Sindbis.

Dans un premier temps, nous avons générée une lignée cellulaire de muscle squelettique humaine déleté de la protéine p53 et grâce à une collaboration, nous avons obtenu des souches *Drosophila melanogaster* p53-/- mutantes. Nous avons observé un effet opposé de la délétion de p53 sur l'infection du chikungunya entre la lignée cellulaire humaine et la drosophile. En effet, nous avons démontré que la délétion de p53 induisait une augmentation de l'infection du CHIKV dans les cellules humaines et une diminution de la réplication virale du CHIKV et du virus Sindbis chez les drosophiles. Plusieurs analyses ont montré que la protéine p53 était impliquée dans la réponse interféron de Type-I, indépendamment de son implication dans la régulation de la mort cellulaire au cours de l'infection du CHIKV dans les cellules humaines. En effet, la délétion de p53 ne permet pas la production d'interféron Bêta et par conséquence la production des gènes cibles de la voie de signalisation. L'activité de p53 sur la réponse immunitaire va être étudiée plus en détails dans des expériences futures. En parallèle, nous avons également montré que la mort cellulaire induite par le virus CHIKV était indépendante de p53 car aucun des gènes cibles de p53 étudiés n'était régulés et la délétion de p53 n'a pas diminué ou retardé l'induction de la mort cellulaire à la suite de l'infection virale.

Dans un deuxième temps, le gène p53 conduit à l'expression de nombreuses isoformes qui participent à la régulation de l'activité de p53. Les fonctions biologiques des isoformes p53 sont principalement étudiées en cancérologie. Parmi elles, l'isoforme Δ133p53 est l'isoforme la plus caractérisée et à l'inverse le rôle biologique de l'isoforme Δ40p53 n'est pas entièrement compris. Lors d'une infection par le virus Influenza A, il a été montré que l'isoforme Δ133p53 est modulée et impacte l'activité antivirale guidée par p53. Mon sujet de thèse a porté sur le rôle possible des isoformes Δ133p53 et Δ40p53 sur l'infection du CHIKV. Pour cela nous avons mis au point (i) des cellules humaines de muscle qui présentent une surexpression endogène de l'isoforme Δ40p53, générée par la technologie CRISPR/Cas9 et (ii) un système inducible pour surexprimer temporairement les protéines des deux isoformes. Nous avons observé que la surexpression endogène de l'isoforme Δ40p53 conduisait à une diminution de l'infection, cependant le mécanisme est inconnu. En parallèle, le système inducible de surexpression des isoformes doit être amélioré afin d'étudier le rôle possible des deux isoformes sur l'infection par le CHIKV.

**Mots clés :** *Alphavirus*, virus du chikungunya, p53, mort cellulaire, réponse immunitaire, mammifère, insecte.

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## ABBREVIATIONS

**(+)**ssRNA single-stranded positive-sense RNA

**A**DP Adenosine Diphosphate Ribose

A Alanine

*Ae Aedes*

Ago Argonaute

AMP antimicrobial peptide

AMPK adenosine monophosphate-activated protein kinase

Asp Aspartic acid

ATM ataxia-telangiectasia

ATP Adenosine TriPhosphate

ATR A-T and Rad-3 related

**B**cl-2 B-cell lymphoma 2

BHK baby hamster kidney

BL basal lamina

**C**DC Centers for Disease Control and Preventions

CDK cyclin-dependent kinase

CHIKV chikungunya virus

CIP1 cyclin-dependent kinase inhibitor protein 1

CPE cytopathic effects

CPV-I, -II cytoplasmic structure type-I, -II

*Cx Culex*

Cyt.c cytochrome c

**D**APK1 death-associated protein kinase 1

DBD DNA-binding domain

DC-SIGN Dendritic cell-specific ICAM-grabbing non-integrin

DENV dengue virus

DIAP *Drosophila* inhibitor apoptosis protein

DISC death-inducing signaling complex

DNA Deoxyribonucleic acid

**E**CSA East-Central-South African

EEEV Eastern Equine Encephalitis virus

eIF2 $\alpha$  eukaryotic translation initiator factor 2  $\alpha$

ER Endoplasmic reticulum

**F**ADD Fas-associated death domain

FL1 Four and a half LIM domains protein 1

FL full-length

**G**AG(s) Glycosaminoglycan(s)

**H**D Hinge domain

HDM2 human double minute 2

Hpi hours post-infection  
HPIV Human parainfluenza virus  
HS Heparan sulfate  
HSPG Heparan sulfate proteoglycan

**I**AP inhibitor apoptosis protein

IAV Influenza virus  
IBM IAP-binding motif  
ICE Interleukin 1 $\beta$  converting enzyme  
IFN Interferon  
IFN-I Interferon Type-I  
IKK Inhibitor of NF- $\kappa$ B factor Kinase  
IL Interleukin  
IMD immune deficiency  
IOL Indian Ocean lineage  
IRE1 $\alpha$  Inositol-requiring transmembrane endoribonuclease 1 $\alpha$   
IRF interferon regulatory factor  
ISGs IFN stimulates genes

**J**AK Janus kinase

JNK Jun NH<sub>2</sub>-terminal kinase

**k**<sub>Da</sub> kilo Dalton

**L**AMP1 Lysosomal-associated membrane protein 1

LTR long terminal repeat

**M**DA-5 melanoma differentiation gene 5

MDM2 mouse double minute 2  
miRNA micro RNA  
MMP mitochondrial membrane potential  
MMPs matrix metalloproteinases  
MOI Multiplicity of Infection  
MOMP mitochondrial outer membrane permeabilization  
mRNA messenger RNA

**N**<sub>C</sub> Nucleocapsid

NES Nuclear Export Signal  
NF- $\kappa$ B nuclear factor-kappa B  
NHPs Non-human primates  
NK Natural killer  
NLS Nuclear Localization Signal  
NRAMP2 Natural resistance-associated macrophage protein 2  
NS non-structural  
nsP non-structural protein  
NW New world

**O**<sub>D</sub> oligomerization domain

ONNV O'nyong nyong virus

OPG osteoprotegerin

ORF open reading frame

OW Old World

**P**AMP pathogen-associated molecular pattern

PARP poly(ADP)ribose polymerase

PGRP peptidoglycan recognition protein

PMCs Primary blood Mononuclear cells

pH potential of Hydrogen

piRNA piwi-interacting RNA

PKR protein kinase RNA-activated

PM Plasma membrane

pRb protein of Retinoblastoma

PRD proline-rich domain

PRR pattern recognition receptor

PS Phosphatidylserine

**R**ANKL receptor activator of nuclear NF\_κB ligand

RB1CC1 RB1-inducible coiled-coiled protein 1

RC replication complex

RdRp RNA-dependent RNA polymerase

RIG-I Retinoic acid-Inducible Gene product I

Rin Rasputin

RISC RNA induce silencing complex

RNA Ribonucleic acid

RNAi RNA interference

ROS reactive oxygen species

RRV Ross River virus

RVFV Rift Valley Fever virus

**S**FV Semliki Forest virus

SG salivary glands

SG Stress Granules

SINV Sindbis virus

siRNA silencing RNA

**T**AD Transactivation domain

TBK-1 TANK-binding protein 1

THOV Thogoto virus

TIMP tissue inhibitor of metalloproteinases

TIR Toll-interleukin-1 resistance

TLR Toll-like receptor

TNF Tumor necrosis factor

TRAF TNF receptor-associated factor

TRAIL-(R) TNF-related apoptosis-inducing ligand (receptor)

TRIF TIR domain-containing adaptor inducing IFNβ

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

**U**PR Unfolded protein response

UTR untranslated region

**V**aline

VC vector competence

VEEV Venezuelan Equine Encephalitis virus

VOPBA virus overlay protein binding assay

VSV vesicular stomatitis virus

**W**EEV Western Equine Encephalitis virus

WNV West Nile virus

**X**BP1 X-box binding protein 1

**Y**FV Yellow fever virus

**Z**IKV Zika virus

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## BIBLIOGRAPHIC SYNTHESIS

The bibliographic synthesis is divided into four chapters. The first one describes the family of *Alphavirus* where it is introduced in the classification and affiliation of the arbovirus group. Also, the viral replication of some *alphaviruses* is presented, comparing mammal and mosquito cells. Chapter two describes in more detail the chikungunya virus: its discovery and transmission in mammal hosts and the mosquito vector. Then, chapter three describes the comparative immune and apoptotic cell response during *alphavirus* infection in mammals and insects. Finally, chapter four explains the activity of the transcription factor p53 on cellular outcome during non-arboviral and arboviral infections.

## 1 Alphaviruses

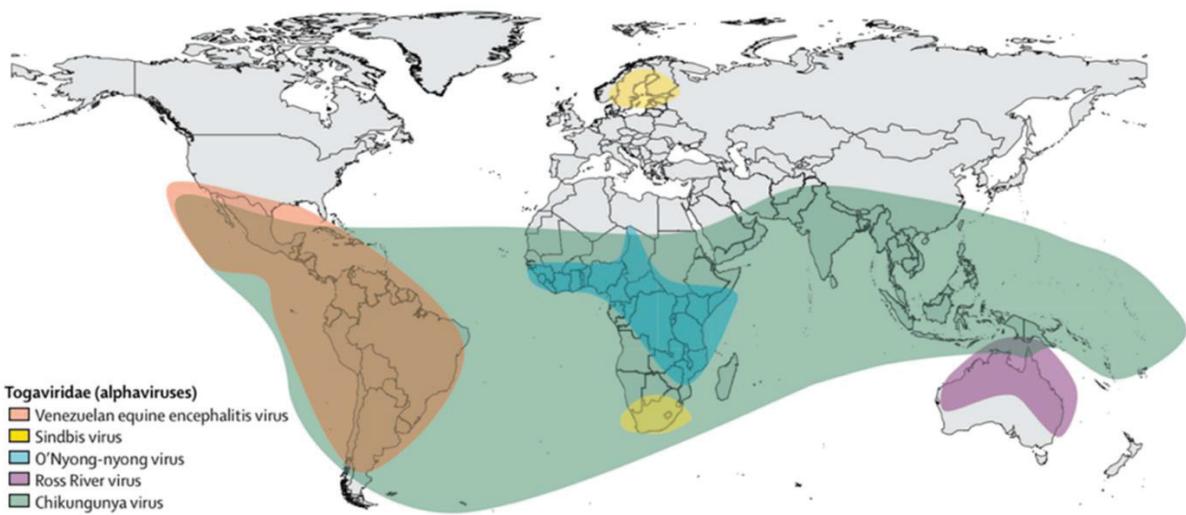
### 1.1 Classifications

#### 1.1.1 Taxonomy of *Alphavirus*

Regarding the Baltimore classification, the genus *alphavirus* belongs to the group IV with single-stranded positive-sense RNA viruses ((+)ssRNA).

*Alphaviruses* (family *Togaviridae*, genus *Alphavirus*) include approximately 30 members. Among the different species, some alphaviruses are pathogenic for humans with diseases ranging from mild to severe. Historically, *Alphaviruses* were divided into New World (NW) and Old World (OW) alphaviruses regarding their global distribution, evolution, pathogenicity, tissue and cellular tropism or interactions with respective hosts. Old World alphaviruses (chikungunya virus, Sindbis virus, Semliki Forest virus, Ross River virus, etc.) are mainly found in Asia, Africa and Europe and infections in vertebrate are characterized by rash, fever, arthralgia, and myalgia. New World alphaviruses (Eastern-, Western-, Venezuelan-, Equine encephalitis viruses, etc.) are found in North and South America and infections are characterized by debilitating febrile disease and encephalitis (Figure 1).

Moreover, the genus *Alphavirus* was classified antigenically into seven complexes. Table 1 presents the seven complexes which are: Barmah Forest Complex, Ndumu Complex, Middelburg Complex, Semliki Forest Complex, Western Equine Encephalitis Complex, Eastern Equine Encephalitis Complex and Venezuelan Equine Encephalitis Complex. Finally, thanks to a phylogenetic approach, the evolutionary history and mechanisms of emergence of alphaviruses was conducted using part of the envelope glycoprotein (E1) sequence (Powers *et al.* 2001).



**Figure 1: Worldwide distribution of Old-World alphaviruses (Sindbis virus, O'nyong nyong, Ross River virus and chikungunya virus) and New-World alphavirus (Venezuelan equine encephalitis virus), from (Charlier *et al.* 2017).**

Seven Complexes	Viruses
Unclassified	Salmon Pancreatic Disease Virus (SPDV) Sleeping Disease Virus (SDV) Southern Elephant Seal Virus (SESV)
Barmah Forest Complex	Barmah Forest Virus (BFV)
Ndumu Complex	Ndumu complex Ndumu Virus (NDUV)
Middleburg Complex	Middleburg Virus complex Middleburg Virus (MIDV)
Semliki Forest Complex	Chikungunya Virus (CHIKV) O'Nyong Nyong Virus (ONNV) Mayaro Virus (MAYV) Una Virus (UNAV) Bebaru Virus (BEBV) Semliki Forest Virus (SFV) Ross River Virus (RRV) Getah Virus (GETV)
Western Equine Encephalitis Complex	Aura Virus (AURA) Sindbis (Ockelbo Virus) (OCKV) Sindbis Virus (SINV) Western Equine Encephalitis Virus (WEEV) Highlands J Virus (HJV) Fort Morgan Virus (FMV)
Eastern Equine Encephalitis Complex	Eastern Equine Encephalitis Virus (EEEV)
Venezuelan Equine Encephalitis Complex	Venezuelan Equine Encephalitis Virus (VEEV)

Table 1: The seven antigenic complexes (based on Powers *et al.* 2001).

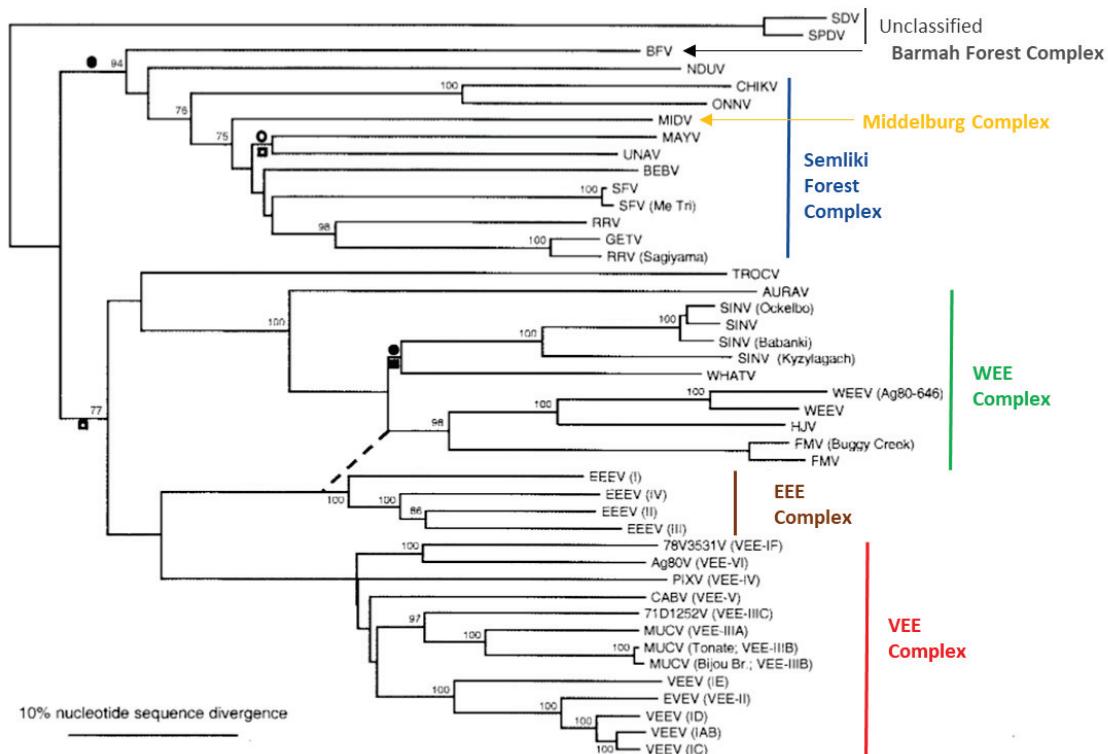


Figure 2: Phylogenetic tree of the genus *alphaviruses*, generated from a partial E1 glycoprotein sequence using the neighbor-joining program (based on Powers *et al.* 2001).

### 1.1.2 Arbovirus: an ecological group

Viruses belonging to *Alphavirus* genus can also be grouped in an ecological but not taxonomic group called Arboviruses (an acronym for “arthropod-borne viruses”) indicated viruses transmitted by an arthropod hematophagous vector to a vertebrate host during a blood meal. Arboviruses comprise viruses of nine families, and nearly 600 arboviruses are known at present and registered in this catalog <https://www.cdc.gov/arbocat/default.aspx> by Centers for Disease Control and Prevention (CDC). Around 50 to 100 of the arboviruses identified can cause disease in vertebrates (humans, domestic animals, wild animals, and birds) and other arboviruses can be exclusively pathogenic for human beings. The majority of arboviruses causing severe morbidity and mortality in human beings around the world belong to *Togaviridae* (*alphavirus* genus), *Flaviviridae* (*flavivirus* genus) families and to some *Bunyavirales* families. They are mainly transmitted by Culicine mosquitoes, genera *Aedes* and *Culex*, with the exception of the O’nyong-nyong virus (*alphavirus*) transmitted by anopheline mosquitoes, genus *Anopheles*. Only the female mosquito acquires an arbovirus during a blood meal, which is necessary for egg development.

Hematophagous arthropods include species of the class Insecta with numerous viruses transmitted by the well-studied *Aedes* mosquito (class Insecta, order Diptera, family Culicidae, tribe Aedini, genus *Aedes*) but also less well-studied insects like culicoids (class Insecta, order Diptera, family Ceratopogonidae, subfamily Ceratopogoninae, tribe Culicoidini, genus *Culicoides*) or biting midges and sandflies (class Insecta, order Diptera, family Psychodidae, subfamily Phlebotominae, genus *Phlebotomus*) and also arthropods belonging to Arachnida class like ticks (order Ixodida) and mites. Table 2 presents a non-exhaustive list of examples of arboviruses and their principal vector and vertebrate host(s) (Table 2).

Order/Family	Genome	Genus	Viruses		Major vector(s)	Vertebrate host(s)
<i>Togaviridae</i>	Single-stranded positive-sens RNA	<i>Alphavirus</i>	Chikungunya virus (CHIKV)		Mosquito <i>Aedes</i>	bird, rodent, non-human primates (NHPs)
			Semliki Forest virus (SFV)			
			Sindbis virus (SINV)		Mosquitoes <i>Culex</i> and <i>Aedes</i>	bird, rodent
			East, West and Venezuelan - Equine Encephalitis virus (EEEV, WEEV and VEEV)			bird, horse, rodent
<i>Flaviviridae</i>		<i>Flavivirus</i>	Dengue virus (DENV)		<i>Aedes</i>	Non-human primates
			Yellow fever virus (YFV)			
			Zika virus (ZIKV)		<i>Culex</i>	
			West Nile virus (WNV)			bird, horse
<i>Bunyavirales order</i>	Single-stranded negative-sense RNA	<i>Orthobunyavirus</i>	Schmallenberg virus	Biting midges <i>Culicoides</i>	ruminants (sheep, goat)	
		<i>Phlebovirus</i>	Rift Valley fever virus (RVFV)	Mosquitoes <i>Aedes</i> and <i>Culex</i>		
		<i>Orthonairovirus</i>	Crimean-Congo hemorrhagic fever (CCHFV)	Ticks		
<i>Rhabdoviridae</i>		<i>Vesiculovirus</i>	Cocal virus	probably mites, sandflies	rodent, possibly bats	
<i>Orthomyxoviridae</i>		<i>Thogotovirus</i>	Thogoto virus	Ticks	camel, cattle	
<i>Reoviridae</i>	Double-stranded RNA	<i>Orbivirus</i>	Bluetongue virus (BTV)	Biting midges <i>Culicoides</i>	ruminants (sheep)	
<i>Asfarviridae</i>	DNA	<i>Asfarivirus</i>	African swine fever virus (ASFV)	Ticks	warthog, forest hog	

**Table 2: Non-exhaustive list of arboviruses accompanied by arthropod vectors and vertebrate hosts (the human host is not presented in this table), (based on Hubálek, Rudolf, and Nowotny 2014)**

## 1.2 Viral particle structure and genome organization

Alphaviruses are small, enveloped viruses about 70 nm in diameter, with an icosahedral shape. The genome is approximately 11,800 nucleotides, constituting a positive-sense, single-strand RNA ((+ssRNA) with a 5' 7-methylguanosine cap and a 3' poly-A tail as well as 5'- and 3'-untranslated regions (UTRs).

The (+)ssRNA contains two open reading frames (ORFs). The first one is flanked by the 5' cap and a UTR region for non-structural polyproteins and the non-coding junction region between both ORFs contains the C-terminus region of the non-structural polyprotein (Figure 3). The promoter of the 26S subgenomic mRNA along with the start site and untranslated region allowing the transcription of the second ORF is present in the junction region. The second ORF encodes structural proteins and present a 3' UTR and a poly(A) tail (Strauss and Strauss 1994). The 5' and 3' UTRs non-coding regions are involved in the regulation of viral gene expression but also virus-host interactions that are associated with viral evolution, host range and pathogenesis (Hyde *et al.* 2015).

The non-structural ORF encodes four essential nonstructural proteins (nsP1, 2, 3 and 4) and the second one, the 3' ORF, translated from the 26S subgenomic mRNA encodes five proteins (capsid, E3, E2, 6K and E1), with three major structural proteins (capsid, E1 and E2).

The viral particle is composed of the lipid bilayer envelope associated with 240 copies of nucleocapsid forming a solid shell that encapsidates genomic RNA. Inside the lipid bilayer membrane, the E1 and E2 glycoproteins are arranged in heterodimer subunits which are then combined into trimers forming the 80 spikes at the surface of the virion (Figure 3).

### **1.2.1 Non-structural proteins**

The non-structural protein nsP1 (~60 kDa) presents a methyltransferase and guanylytransferase activity necessary in the capping of virus mRNA. NsP1 is involved in the synthesis of 49S minus-strand RNA. It can also interact with phospholipids of cellular membranes thanks to its amphipathic loop and palmitoylation sites, which is important for the replication of alphaviruses in a specific structure called replication complex (RC) anchored to the plasma membrane (Bakar and Ng 2018).

The nsP2 (~90 kDa) is a multifunctional enzyme and regulatory protein. It presents an N-terminal RNA helicase domain which can unwind double-strand RNA and an RNA 5'-Triphosphatase activity essential for mRNA capping. Finally, a C-terminal protease activity is responsible for non-structural polyprotein processing. nsP2 also contains a Nuclear localization signal (NLS) and a nuclear export signal (NES). During viral replication, nsP2 is involved in the transcriptional shut-off of mammalian cells and in virus-associated cytotoxicity but not in the vector (Akhrymuk, Kulemzin, and Frolova, 2012). These activities of nsP2 in mammals and insects will be discussed in detail in chapter 3.

The nsP3 (~60 kDa) presents an ADP-ribose 1-phosphate phosphatase activity allowing protein regulation and is involved in minus-strand and 26S subgenomic RNA synthesis. The protein comprises three domains: an N-terminal macro-domain, a central zinc-binding domain and a C-terminal hypervariable domain.

NsP4 (~ 70 kDa) is the core of the RNA-dependent RNA polymerase allowing viral RNA transcription, however all the nsPs are required and essential for RNA synthesis. NsP4 presents a C-terminal Adenylyltransferase activity, which may allow the generation of the poly(A) tail at the 3' end (Pietilä, Hellström, and Ahola 2017).

### 1.2.2 Structural proteins

Thanks to four non-structural proteins, the five structural proteins are synthesized: the capsid, E3, E2, 6K and E1. They are first synthesized as a structural polyprotein and then separated by viral and cellular factors. The structural proteins are involved in virion binding and entry, nucleocapsid assembly and virion budding from the plasma membrane.

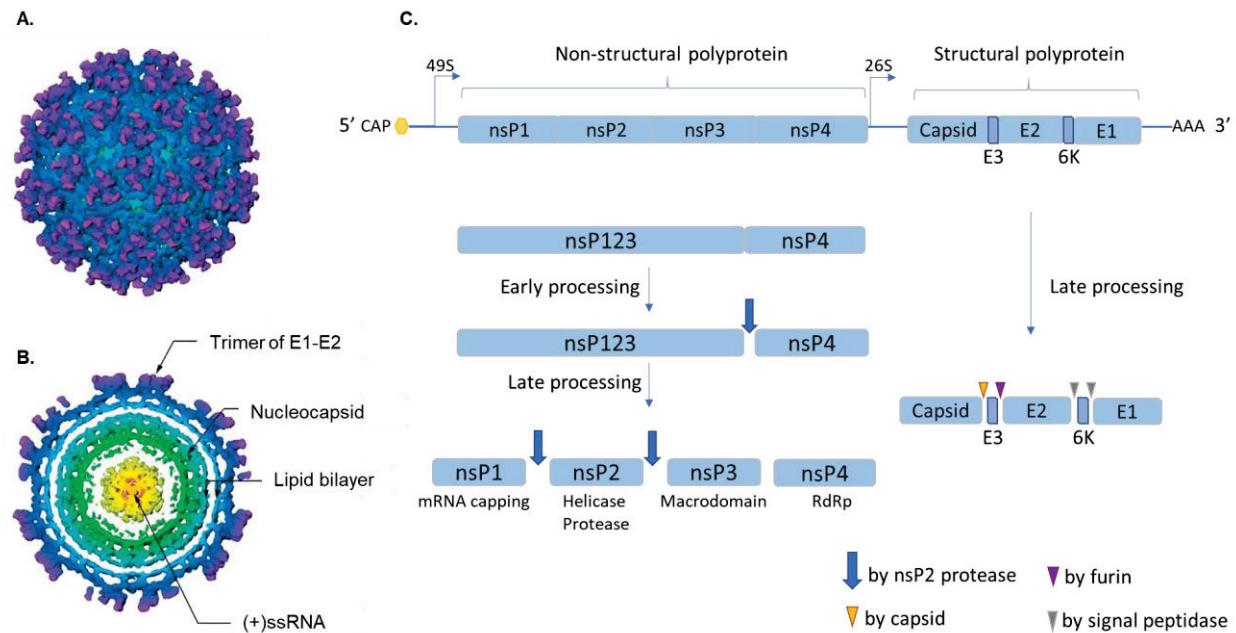
The capsid (~30 kDa) presents an N-terminal RNA binding region allowing the encapsidation of the viral genome and a C-terminal serine protease activity region which can cleave itself out of the structural polyprotein (Hahn and Strauss 1990). The capsid also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) at the end of the N-terminal region and the beginning of C-terminal region, respectively (Thomas *et al.* 2013). Lastly, the monomeric capsid will assemble to form the nucleocapsid of the viral particle by interaction with viral RNA and glycoprotein E2.

The E2 glycoprotein (~ 40 kDa) presents glycosylation sites responsible for binding to the cell and the E1 glycoprotein (~45 kDa) presents a fusion peptide involved in membrane fusion during the entry of the viral particle. It is also responsible for the formation of the icosahedral shell.

6K is a small structural protein (6 kDa) presenting cysteine residues which seem important for the formation of infectious particles and up to 30 copies of 6K are incorporated in the virus particle (Melton *et al.* 2002). It has been shown that in mammalian cells, 6K can be associated with membrane modification by forming a cation-selective channel leading to the permeability of the membrane (Melton *et al.* 2002).

The E3 protein is also a small protein (~10 kDa) whose exact role is not yet well understood but it has been hypothesized that E3 is involved in virus assembly and in infectious particle

production. Recently, it has been shown that E3 can mediate a pH protection of E1 during virus assembly (Uchime, Fields, and Kielian 2013).



**Figure 3: Viral particle and genome organization of Alphavirus.**

**A.** Structure of a VEEV particle generated by electron cryomicroscope and **B.** Slice of a VEEV particle showing a trimer of E1-E2, the lipid bilayer from the host membrane, the nucleocapsid and the genomic positive-sense, single-strand RNA (+)ssRNA (based on Paredes et al. 2005). **C.** The Alphavirus genome (+)ssRNA possesses a 5' cap and a 3' poly(A) tail and 5' and 3' untranslated regions (UTRs) surrounded by two open reading frames. The non-structural proteins (nsP1-4) are translated from the 49S genomic RNA while the structural proteins (Capsid, E3, E2, 6K and E1) are translated from subgenomic 26S RNA. In the early and late process of non-structural protein synthesis, the polyprotein is cleaved by nsP2 and in the late process of structural protein synthesis the polyprotein is cleaved by the viral capsid and host proteases (furin, signal peptidase).

### 1.3 Replicative cycle in mammals and mosquitos

This part will present the replicative cycle of alphavirus in mammal and mosquito cells. The replicative cycle has been studied more in mammal cells. Indeed, only a few works present the replicative cycle in mosquito cells and this part is mainly based on findings using the Sindbis virus as an alphavirus species.

### 1.3.1 Binding and entry by endocytosis

Enveloped viruses use specific receptors and co-receptors to enter specific cells but also attachment factors to promote binding at the cell surface. Alphaviruses then enter the cell through the receptor-mediated endocytosis entry process.

Among the attachment factors, it has been shown that several alphaviruses can use glycosaminoglycans (GAGs) to enter mammalian cells. GAGs are a family of highly sulfated linear polysaccharides, ubiquitous at the cell surface and categorized into four groups: heparan sulfate (HS), chondroitin sulfate, keratan sulfate and hyaluronan. Mammalian cell surface binding of SINV, RRV, SFV, VEEV and EEEV has been shown to be dependent on heparan sulfate (Byrnes and Griffin 1998; Gardner *et al.* 2013; Smit *et al.* 2002). DC-SIGN and L-SIGN, two transmembrane C-type lectins, calcium-dependent carbohydrate-binding proteins, have been shown to be important in cell-adhesion and pathogen-recognition factors for the entry of alphaviruses into mammalian cells (Klimstra *et al.* 2003).

Human T-cell Immunoglobulin and Mucin-domain (TIM) and TAM (Tyro3, Axl and Mer) transmembrane receptors participate in the clearance of apoptotic cells by recognising phosphatidylserine (PS) at the surface of dead cells. PS can be present at the surface of the viral particle after budding at the PS positive-membrane and some viruses have evolved to exploit and bind to TIM and TAM receptors to enter targets cells. Indeed, it has been shown that the Dengue virus exploits TIM and TAM receptors for entry into cells, as do SINV and RRV and many other enveloped viruses (Jemielity *et al.* 2013; Meertens *et al.* 2012). The role of PS in the viral particle membrane will be discussed in more detail in chapter 3.

Compared to the entry process into mammalian cells, very few studies have been performed to understand this process in the mosquito. In 2014, using virus overlay protein binding assays (VOPBA) and mass spectroscopy, a study showed a 50 kDa protein attached to CHIKV and identified as an ATP synthase beta subunit (ATPS $\beta$ ). Using antibody inhibition and siRNA targeting the ATPS $\beta$ , the authors showed a significant reduction of viral entry in *Aedes albopictus* and *aegypti* cell lines (Fongsaran *et al.* 2014). Moreover, the SINV infection of mosquito salivary glands by feeding of *Aedes aegypti*, previously infected with a heparan sulfate proteoglycan (HSPG), led to more cytopathic effects, indicating an association between SINV and HSPG in the mosquito (Ciano, Saredy, and Bowers 2014).

Concerning specific receptors of alphaviruses, using a whole genome screen in *Drosophila* cells with siRNA technology, the natural-resistance associated macrophage protein (NRAMP2/Malvolio/DMT1) was described as the receptor of SINV in *Drosophila* as well as in mosquito and mammalian cells (Rose *et al.* 2011). Recently, Mxra8 has been demonstrated to be the receptor of several arthritogenic alphaviruses like CHIKV, RRV, Mayaro and O'nyong nyong viruses in mammalian cells and mouse (Zhang *et al.* 2018), and involved in the pathogenesis of CHIKV in mouse and transgenic *Drosophila* (Zhang *et al.* 2019). Concerning the glycoprotein involved in the binding, it seems that E2 is the candidate for attachment to GAGs (Silva *et al.* 2014) and to the Mxra8 receptor (Zhang *et al.* 2018).

Following binding, alphaviruses are internalized into the cell *via* the formation of clathrin-dependent endocytosis vesicles, then the clathrin-coated vesicles are uncoated and form endosomes (Bernard *et al.* 2010), leading to the release of nucleocapsids and viral genomes via fusion between the membranes of endosomes and viral particles. Alphaviruses were shown to enter mainly via clathrin-mediated endocytosis even though in some cell lines another pathway can be used for alphavirus-entry. Indeed, using siRNAs against the clathrin chain, CHIKV infection in 293T was not decreased, while in two other cell lines (HUVEC and U2OS) CHIKV infection decreased drastically (Bernard *et al.* 2010).

Thus, it seems that the entry pathway in mammalian cells could be cell-specific, at least *in vitro*. Moreover, it has been shown that the fusion process occurs in early endosomal compartments for SINV, SFV and CHIKV (van Duijl-Richter *et al.* 2015; Marsh, Bolzau, and Helenius 1983) but in late endosomes for VEEV (Colpitts *et al.* 2007). Finally, in mosquito cells, it has been shown that CHIKV and VEEV entry also use the clathrin-mediated endocytosis pathway; however, this entry seems dependent on Rab5 and Rab7-positive endosomes, suggesting a fusion process in early and late endosomes (Colpitts *et al.* 2007; Nuckols *et al.* 2014).

### 1.3.2 Fusion and Viral genome release

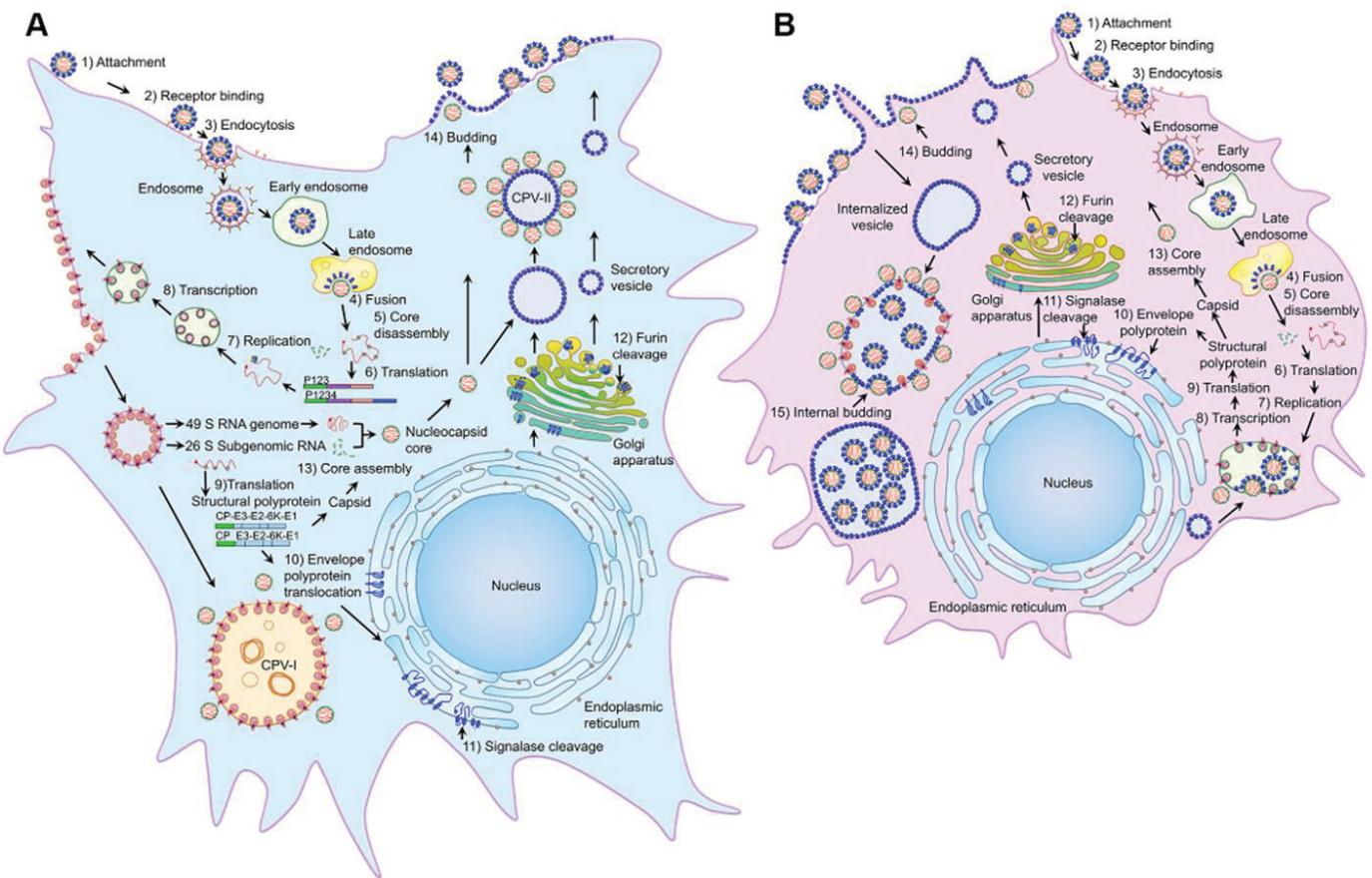
The molecular mechanisms involved in membrane fusion are highly conserved between alphaviruses in mammalian cells and seem similar in mosquito cells. Indeed, this process is low pH and cholesterol dependent. The fusion process requires the destabilization of the E1-E2

heterodimer leading to the exposure of the E1 fusion loop, the integration and trimerization of E1 glycoprotein inside the endosome target membrane, and the formation of fusion pores. First, the mildly acidic pH in endosomes triggers the destabilization of E1-E2 and a domain of the E2 glycoprotein moves away, exposing the E1 hydrophobic fusion loop. The presence of cholesterol in the target membrane and specific amino acids in E1, important for lipid and pH sensing, enhances the fusion potential. Then, as the pH decreases, E2 is completely dissociated from E1 which can trimerize. This step brings the viral membrane closer to the endosomal membrane, forcing them to merge in a process called hemifusion (van Duijl-Richter *et al.* 2015). Finally, the fusion pore is formed and can expand, allowing the nucleocapsid access to the cytoplasm where the replication process will occur.

### **1.3.3 Replication complex and assembly**

The RNA synthesis of alphaviruses is an associated-membrane process. However, it seems that different membranes and vesicles are involved in the replication of viral RNA in mammalian and mosquito cells (Figure 4).

In mammalian cells, replication is initiated in the cytoplasm by the expression of the nsP1234 precursor from the 49S of viral RNA and followed by the cleavage of nsP123 and nsP4 by nsP2 protease activity. The polyprotein P123 and nsP4 associate with the viral RNA and host proteins form the membrane-bound replication complex (RC) used for the generation of full-length minus-strand synthesis. The proteolytic process on P123 causes the release of nsP1, nsP2 and nsP3. It has been shown recently that the hypervariable domain of nsP3 interacts with the mammalian cellular factor FHL1, promoting the replication of viral RNA (Meertens *et al.* 2019). Moreover, nsP2 and nsP3 have been reported to induce a transcriptional and translational shut-off of the mouse 3T3 cell line infected by SINV, participating in the efficient viral replication and inhibition of host response (Akhrymuk, Frolov, and Frolova 2018).



**Figure 4: Replicative cycle of the Sindbis virus (Alphavirus) in mammalian (A) and mosquito (B) cell lines.** SINV is internalized into the cell by clathrin-mediated endocytosis. Fusion between the endosome and the viral particle is pH dependent and releases the viral genome into the cytoplasm (steps 1 to 5). Then, non-structural proteins are translated and the association of viral non-structural proteins with cellular proteins forms the replication complex RC and induces spherule structures on the endosome and plasma membranes in mammals and only in large internal vesicles in the mosquito, where the nucleocapsid is still detectable (steps 6 to 8). The formation of spherules allows the translation of structural proteins from subgenomic 26S RNA and the translocation of glycoproteins but not capsid into the endoplasmic reticulum and Golgi apparatus for glycosylation, maturation, and plasma membrane translocation via the secretory pathway (steps 9 to 12). In parallel, in the cytoplasm viral capsid associates with new viral RNA produced from the intermediate negative strand, forming the nucleocapsid NC (step 13). The NC binds the glycoprotein E2-E1-E3 complex and buds at the plasma membrane in mammals. In the mosquito a second internal budding site seems to involve large vesicles (step 14 in mammal cells and steps 14 and 15 in mosquito cells). CPV (-I and -II): cytoplasmic structure type I or II (Jose et al., 2017).

Then, the maturation of RC induces bulb-shaped membrane invaginations called “spherules” in type I cytoplasmic structures (CPV-I) where RNA synthesis occurs. The CPV-I originate from endosomes and are maybe guided by nsP1 to the plasma membrane (PM) thanks to the capacity of nsP1 to interact with phospholipids of cellular membranes in mammalian cells, as shown in SFV-infected BHK cells (Kujala *et al.* 2001). Next, the structural proteins Capsid-E3-E2-6K-E1 are translated from the 26S subgenomic RNA and then autocatalytically processed at the N terminus by the serine protease activity of the capsid. The capsid is released into the cytoplasm and the remaining structural polyprotein E3-E2-6K-E1 translocates to the Endoplasmic Reticulum (ER) and the Golgi apparatus thanks to a signal peptide present at the N-terminal region.

Once in the RE, the E1 and E2 precursor (pE2) assemble in heterodimers, and during Golgi maturation a furin cleavage releases E3 to the E1-E2 heterodimers. In the cytoplasm, the capsid encapsidates new single genomic RNA to form the nucleocapsid (NC), associated with type II cytopathic vacuoles that contain E1-E2 glycoproteins and originate from the Golgi network. The CPV-II is presumably involved in the transport of glycoproteins and NC to the plasma membrane which is the site of virus budding.

In mosquito cells, it has been shown that the RC of SINV is not found at the PM and does not form in CPV-I but in acidic vesicles that contain the viral glycoproteins, which is not the case in mammalian cells. In addition to different morphologies in these cytopathic vacuoles in mosquito and mammalian cells, additional internal budding of particles has been shown in mosquito vesicles (Jose *et al.* 2017).

Authors have hypothesized that in the mosquito, alphavirus replication and assembly processes have evolved to a state that favors persistent infection, maybe by preventing antiviral cellular response. Interestingly, the replication of alphaviruses in CPV-I and -II in mammal cells is similar to that of mosquito vacuoles and suggests that even in the mammal system, alphaviruses have evolved to escape the immune response during replication. However, in the study of Jose *et al.*, the *Aedes albopictus* C6/36 cell line was used. Although these cells are known to present a dysfunctional RNA interference (RNAi) antiviral response and thus may not be the best cellular model, this article is one of the only complete *in vitro* studies on alphavirus infection in mammals and mosquitos.

The assembly of new particles takes place at the plasma membrane where one new virion is composed of 240 copies of capsid associated with the viral genome and 80 spikes of E1-E2 glycoproteins in the plasma membrane. The capsid and E2 interact and drive the budding process where the virions acquire a bilayer membrane from the mammalian or mosquito cell. Figure 4 present the replicative cycle of the Sindbis virus in mammalian and mosquito cell lines.

The next chapter will present in more detail the chikungunya virus, including the transmission between vertebrates and invertebrates, the epidemiology, the dissemination in humans and the route of infection in the mosquito. Lastly, the vaccine and treatments for humans and anti-viral strategies in the mosquito vector will be presented.

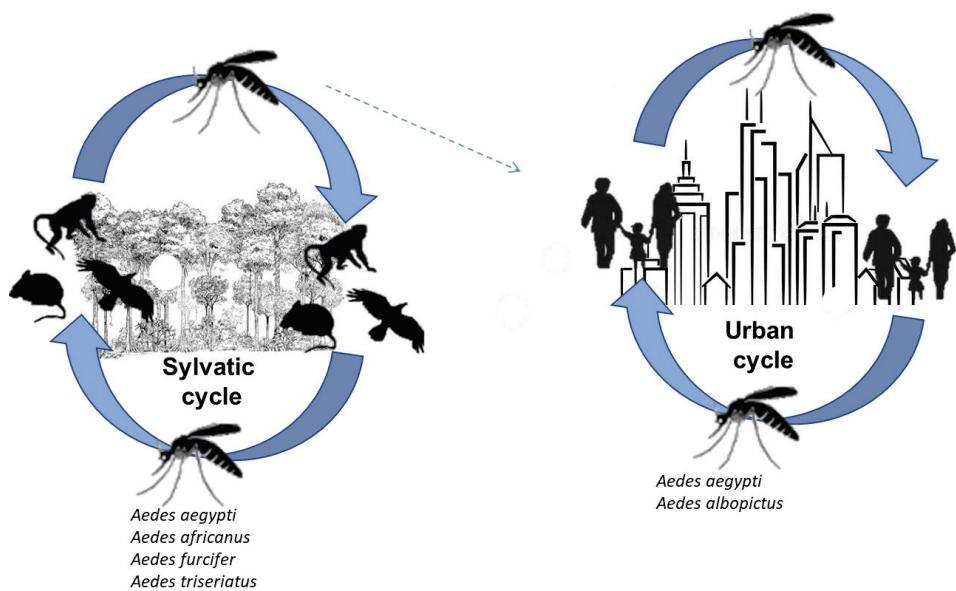
## 2 Chikungunya virus – an Old-World *alphavirus*

### 2.1 Discovery

The Chikungunya virus (CHIKV) was first described as a human pathogen after isolation in the serum of infected patient suffering from arthritic disease in 1952 in Tanzania. The name chikungunya refers to this characteristic posture of infected patients and means “that which bends up” in the African Kimakonde language (Lumsden *et al.* 1955; Robinson *et al.* 1955; Ross *et al.* 1956). However, some research suggests that CHIKV outbreaks occurred around 1780 but were attributed to Dengue virus infections. Indeed, the signs and symptoms are very close, and these viruses circulate in the same areas. Most infected people develop an acute illness characterized by fever, rash, headache, incapacitating polyarthralgia, arthritis and myalgia. However, unlike Dengue, a characteristic of CHIKV disease is a recurring musculoskeletal disease that can persist for several months to years after acute infection. CHIKV disease in the human host has a low fatality rate (less than 0.1%), but the acute and chronic illnesses have a considerable impact on the quality of life.

## 2.2 Transmission cycles and vector distribution

CHIKV was originally transmitted in natural forest habitats between *Aedes* (Ae.) arboreal mosquitoes and nonhuman primates (monkeys) and other vertebrates (birds, rodents), which do not seem to show symptoms of infection and can help to maintain the virus in nature due to high viremia. This type of transmission is termed an enzootic sylvatic transmission cycle. Humans can become infected if they encroach into the forest habitat, through deforestation, agriculture, urbanization or hunting, and are bitten by infected mosquitoes or if infected mosquitoes move into areas of human habitation. When an infected human enters an urban area, the arbovirus infection can spread amongst other humans via anthropophilic urban mosquitoes, i.e. *Ae. aegypti* and *Ae. albopictus*. At this moment, the sylvatic transmission cycle can switch to an urban transmission cycle. In densely populated tropical regions, the urban transmission cycle can cause an explosive epidemic in which the virus can be maintained in the population (Figure 5).



**Figure 5: Sylvatic and urban transmission cycles**

Regarding the vectors involved in the sylvatic transmission cycle of CHIKV, in Africa, this virus has been detected in forest *Aedes* species like *Ae. aegypti*, *Ae. furcifer*, *Ae. africanus*, and *Ae. taylori* (Diallo *et al.* 1999). *Aedes aegypti* has become very well adapted to urban environments in Africa and has progressively spread from tropical Africa to North Africa and also in the New World and Asia (Powell and Tabachnick 2013). In Asia, CHIKV is mainly maintained in urban

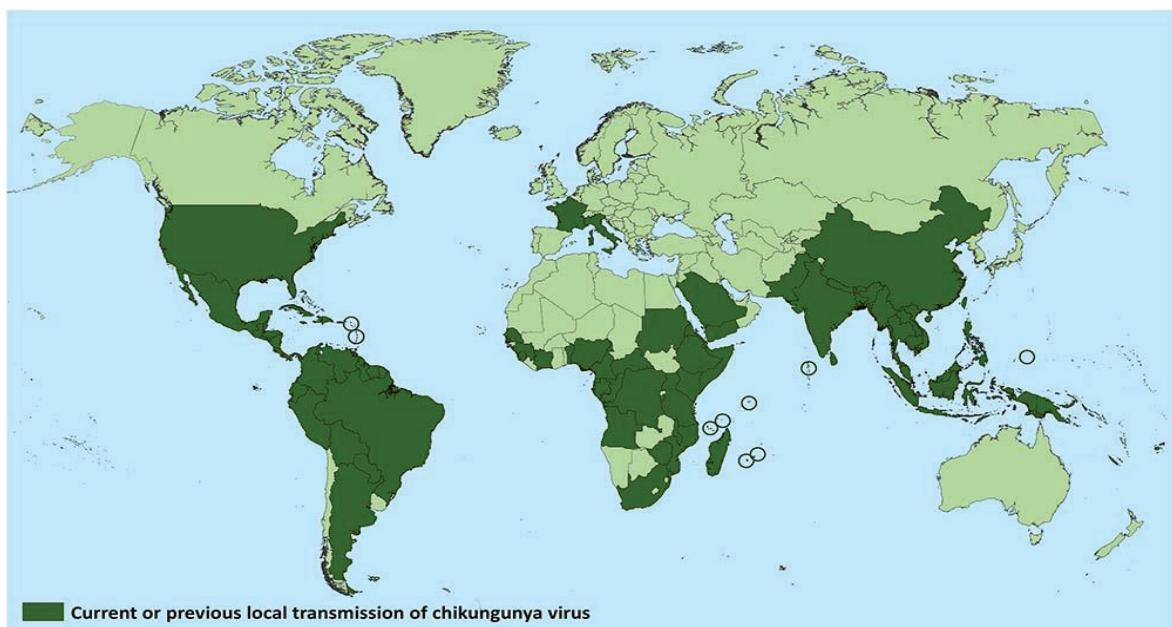
transmission cycles between humans and *Aedes aegypti* and *albopictus*. *Aedes albopictus* was originally found in forests of Southeast Asia but is now also present in rural areas and can colonize tropical and temperate regions. In Europe, it is now established in almost 20 countries and Europe is considered to be vulnerable to the transmission of CHIKV and other “tropical” arboviruses like DENV.

Vertical transmission *via* the eggs of an infected female mosquito has been shown to occur experimentally at a very low rate in *Aedes aegypti* infected by CHIKV (Agarwal *et al.* 2014). During the CHIKV epidemic in La Réunion in 2005-2006, maternal-fetal transmission of the virus was reported for 10 newborns among 84 infected pregnant women (Robillard *et al.* 2006).

### 2.3 Epidemiology

Since the first reports of CHIKV infected patients, several epidemics occurred in sub-Saharan Africa and Asia throughout latter half of 20<sup>th</sup> century. Thanks to phylogenetic analyses on CHIKV sequences, three distinct lineages have been identified, two from Africa and one from Asia: the West African (WA) lineage, the East-Central-South African (ECSA) lineage and the Asian lineage. In Africa, the epidemic emergence of CHIKV is induced by transition from the sylvatic cycle involving the mosquito vector and non-human primates into the urban cycle involving the mosquito vector and human hosts, while in Asia, CHIKV seems to circulate principally in the urban cycle. The re-emergence of an ECSA strain occurred during an outbreak in Kenya in 2004 and spread to a number of Indian Ocean islands including Comoros, Seychelles, Mayotte, Mauritius, Madagascar and La Réunion (Coffey, Failloux, and Weaver 2014). During the epidemic of La Réunion, in 2005-2006, the magnitude of the outbreak was unexpected with approximately one third of the population infected (approximately 260 000 infected individuals) and 300 deaths. The strain at the origin of the epidemic in the Indian Ocean islands has been named Indian Ocean lineage (IOL) and presents an Alanine (A) amino acid at position 226 of the E1 protein. However, a new strain during the outbreak in La Réunion has been identified and presents a mutation in the E1 protein and gene: with a Valine (V) in the place of the Alanine (A) at position 226 (E1-A226V). S. Higgs and colleagues proposed that this mutation has influenced the fitness of the virus for the new vector *Aedes albopictus*, but also viral infectivity and tropism (Tsetsarkin *et al.* 2007). Indeed, this hypothesis provides a plausible

explanation of how the virus strain causes an epidemic in a region lacking the principal vector *Aedes aegypti*. First, they proposed that the E1-A226V mutation could modify the cholesterol dependence of the virus (Tsetsarkin *et al.* 2007). However, later they advanced that adaptation to *Aedes albopictus* does not correlate with this cholesterol dependence (Tsetsarkin, McGee, and Higgs 2011). In addition to the Indian Ocean islands, the virus spread in India and parts of Southeast Asia with six million estimated cases of CHIKV disease. Air travelers have largely facilitated the expansion of the virus, even in more temperate regions like Europe and the United States (Gibney *et al.* 2011). For example, the first autochthonous transmission was reported in Italy in 2007 and in France in 2010 (Grandadam *et al.* 2011; Rezza *et al.* 2007). The global expansion of the chikungunya virus occurred from 2011 to 2014, with outbreaks in the Western Pacific, South Pacific and Caribbean and the spread of the Asian lineage strain in the Americas and the ECSA lineage in Brazil. Because of globalization, climate change, areas of dense population and the presence of relevant vectors, the risk of explosive outbreaks in the Western Hemisphere is high (Figure 6).



**Figure 6: Countries and territories where chikungunya cases were reported in 2019.**  
(\*Does not include countries or territories where only imported cases have been documented),  
(map from Centers for Disease Control and Prevention, as of September 2019).

## 2.4 Infectious route in competent vectors

Among the many species of mosquitoes, only a small number can acquire arbovirus infection and transmit it to an uninfected vertebrate. It seems that there is a specificity in the vector-arbovirus interaction, combining vector intrinsic factors such as mosquito physiology, genetic or antiviral cellular responses and consequently viral replication and propagation.

In comparison to the vertebrate host, arboviruses do not seem to cause significant pathology in the mosquito vector. From the oral acquisition of a viremic bloodmeal to the transmission to a new uninfected vertebrate host, the arbovirus replicates in the cells of the arthropod and must cope with antiviral pathways such as cell death and innate immune responses and several tissue barriers from the midgut until the saliva. Indeed, among the many species of mosquito, some are permissive (competent) and other are resistant to the infection and part of this competence is linked to the several tissue barriers that can be crossed or not during viral dissemination. Figure 7 shows a schematic presentation of the typical route in a permissive mosquito and the four principal tissue barriers (Midgut Infection Barrier **MIB**; Midgut Escape Barrier **MEB**; Salivary Gland Infection Barrier **SGIB** and Salivary Gland Escape Barrier **SGEB**).

In this part we describe the typical route of alphavirus infection in competent and permissive mosquitos, defined by 1) the acquisition of the virus; 2) passage through different barriers, and 3) replication and dissemination to salivary glands to successful transmission to another vertebrate host. The role of vector immune and cell death responses in determining vector competence (VC) in overcoming tissue barriers will be discussed in the following part.

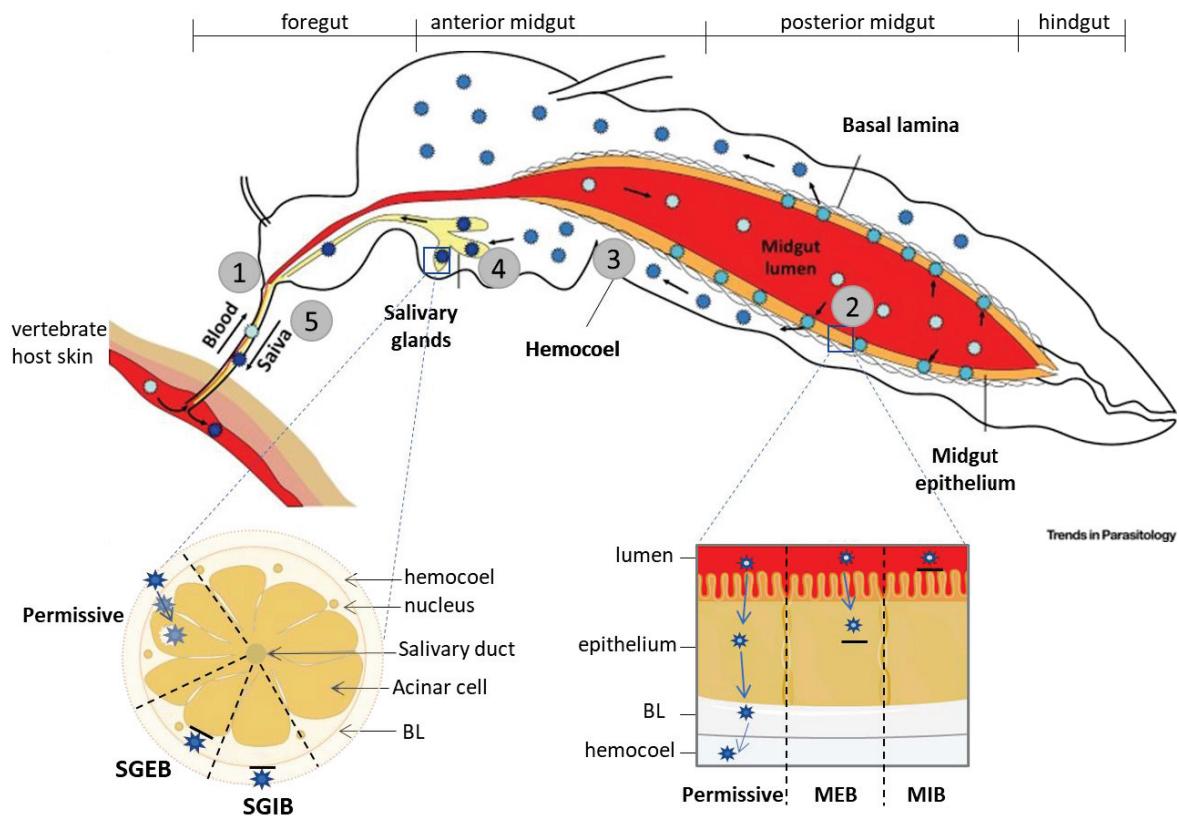
### 2.4.1 The initial site of infection: the midgut

The digestive tube of the mosquito is divided into three major regions: the foregut, the midgut, and the hindgut. The foregut is a region extending from the mouth to the midgut and the foregut extending to the anus. The midgut epithelium is surrounded by an extracellular matrix, called basal lamina (BL) on the internal side of the mosquito. After a viremic bloodmeal, viruses enter the midgut lumen and are close to the single layer of epithelial cells (Figure 7). It has been shown that a small number of cells can be infected during this initial infection. For example, VEEV infects an average of 28 midgut cells in the *Aedes* mosquito and WNV less than 15 midgut

cells in *Culex* (Scholle and Mason, 2004; Smith and Weaver, 2008). Moreover, specific regions of the midgut (posterior, and/or anterior regions) are preferentially infected. From this step, the virus is confronted with the midgut tissue barrier composed of an epithelial layer separating the lumen of the midgut from the hemocoel (blood-containing body cavity and function as part of the circulatory system). Once the virus enters a cell, viral replication starts, and the place of viral maturation and accumulation can also vary, according to the virus-mosquito combination. The endoplasmic reticulum (ER) has been shown to be involved in EEEV synthesis in *Ae. triseriatus* but, in contrast, it seems that CHIKV does not enter the cisternae of the ER in *Ae. aegypti* cells (Franz *et al.* 2015). These differences could be linked to a different temporal analysis of infection or a virus-vector specific interaction. New virions are accumulated at the plasma membrane facing the basal surface and, after budding, viruses are in contact with the basal lamina of the epithelium and need to pass through it to enter the hemocoel and disseminate. The basal lamina predominantly consists of laminin and collagen IV and presents small pores with a size exclusion of 9-12 nm. The BL acts as a very strong mechanical barrier for viruses and the pores are too small for alphaviruses to pass through (60-80 nm).

However, during bloodmeal digestion, it has been shown that the basal lamina is degraded by matrix metalloproteinases (MMPs) and becomes permissive. This change in basal lamina integrity was observed in *Culex pipiens* infected by WNV. However, in *Aedes aegypti* infected by CHIKV it has been shown that, curiously, overexpression of the *Ae.aegypti* tissue inhibitor of metalloproteinases (AeTIMP) induces the efficient dissemination of CHIKV (Dong, Balaraman, *et al.* 2017). As basal lamina degradation/remodeling involves activities of metalloproteinases and inhibitors, they hypothesized that the expression of inhibitors could be accompanied by the transient restructuring of basal lamina allowing the passage of CHIKV.

Following dissemination from the midgut, the virus disseminates up to the salivary glands and saliva. To disseminate to secondary organs, the hemocytes (insect blood cells) have been shown to be an important vehicle in the case of SINV infection of *Ae.aegypti* (Parikh, Oliver, and Bartholomay 2009). Moreover, it has been proposed that the infection of hemocytes leads to a high rate of infection which is required before the infection of salivary glands. Indeed, since only a few epithelial cells of the midgut are infected, the quantity of viral particles after the passage through basal lamina is small. It has been proposed that hemocytes are a vehicle for the virus to the salivary glands and allow the replication of the virus to produce enough particles for efficient infection of the mosquito's head.



**Figure 7: Schematic representation of typical infection of a permissive mosquito by alphavirus and the four tissue barriers.** The infection of a competent vector: 1) acquisition of the virus, 2) infection of the midgut epithelium and passage through the midgut barrier; 3) replication and dissemination through the hemocoel, 4) infection of the salivary glands and escape barriers, and 5) dissemination to saliva and release to a new vertebrate host with saliva components. MIB (Midgut infection barrier) and MEB (Midgut escape barrier). SGIB (Salivary gland infection barrier) and SGEB (Salivary gland escape barrier). BL (Basal Lamina). (based on Rückert and Ebel 2018).

#### 2.4.2 The final site of infection: the salivary glands

The salivary glands of female mosquitoes are paired organs located in the thorax and each gland is composed of three lobes (two lateral and one median). Each lobe presents a central internal duct containing a cavity for saliva storage and surrounded by a monolayer of epithelial and acinar cells that connect the lobes. The monolayer is bounded externally by a BL. Once again, the salivary glands consist of a tissue barrier that must be crossed by the virus to reach the saliva. The CHIKV, present in the hemocoel must penetrate the BL to join the acinar cells and replicate inside to then be disseminated into the saliva cavity and released during blood feeding. It seems that CHIKV can infect both the lateral and median lobes of *Ae. aegypti* (Janzen,

Rhodes, and Doane 1970), whereas SINV can infect only the lateral lobe in *Ae. albopictus* (Bowers, Abell, and Brown 1995). In *Ae. albopictus*, CHIKV particles were observed in acinar cells and in the apical cavity where the virus can be stored in saliva. Interestingly, Vega-Rua and colleagues showed that the nucleocapsid could bud at the plasma membrane of acinar cells and at the membrane of vesicles located in the apical cavity (Vega-Rúa *et al.* 2015). Once in the cavities of acinar cells during new blood feeding, the virus is released with saliva into the vertebrate host.

The saliva of the mosquito contains several proteins such as degrading enzymes, antimicrobials, and anti-inflammatory peptides. The proteins in the salivary glands of *Ae. aegypti* infected by CHIKV were triggered at 3- and 6-days post-infection (dpi) and showed the regulation of about ten secreted proteins at both times. Proteins involved in the anti-inflammatory effect, blood feeding and some protease inhibitors were increased while anti-oxidant related proteins like protein disulfide isomerase (PDI) involved in re-establishment of redox homeostasis were downregulated (Tchankouo-Nguetcheu *et al.* 2012). The proteins found in the saliva of infected mosquitoes could participate in the primo infection in the skin of host vertebrates. Indeed, the presence of anti-inflammatory protein and protease inhibitors could lower the host anti-viral immune response and subsequently create an efficient micro-environment for viral infection. However, it has been shown that the host vertebrate immune response of a murine model can induce specific antibody IgG response against the salivary protein called 34k2 from *Aedes albopictus* and *Aedes aegypti*. Interestingly, as the proteins showed very a low level of immune cross-reactivity, they proposed to use this protein as species-specific markers of host exposure (Montero *et al.* 2019).

## 2.5 Pathogenesis and cellular tropism in vertebrate hosts

### 2.5.1 Human infection: clinical signs

CHIKV disease in humans is symptomatic in 72-95 % of cases with an incubation period of 2 to 4 days. The infection is characterized by an acute phase with febrile illness: fever in 90% of patients ( $>39^{\circ}\text{C}$ ) and frequently accompanied by muscle (myalgia) and joint (arthralgia) pains in about 85% of patients. Other symptoms include headache, cutaneous manifestations, and

digestive troubles. The acute phase generally disappears after one or two weeks. However, for around 35% of patients, joint pain can persist for several weeks to months and present similarity with rheumatoid arthritis. A systematic review has shown that around 25% of CHIKV cases would develop post-CHIKV chronic inflammatory rheumatism for more than 15 months and 14% would present chronic arthritis (Rodríguez-Morales *et al.* 2016). In addition to typical symptoms, during the CHIKV outbreak on La Réunion, some neurological complications were described for the first time, such as encephalitis and encephalopathy, showing a new CHIKV tropism for the central nervous system (Matusali *et al.* 2019).

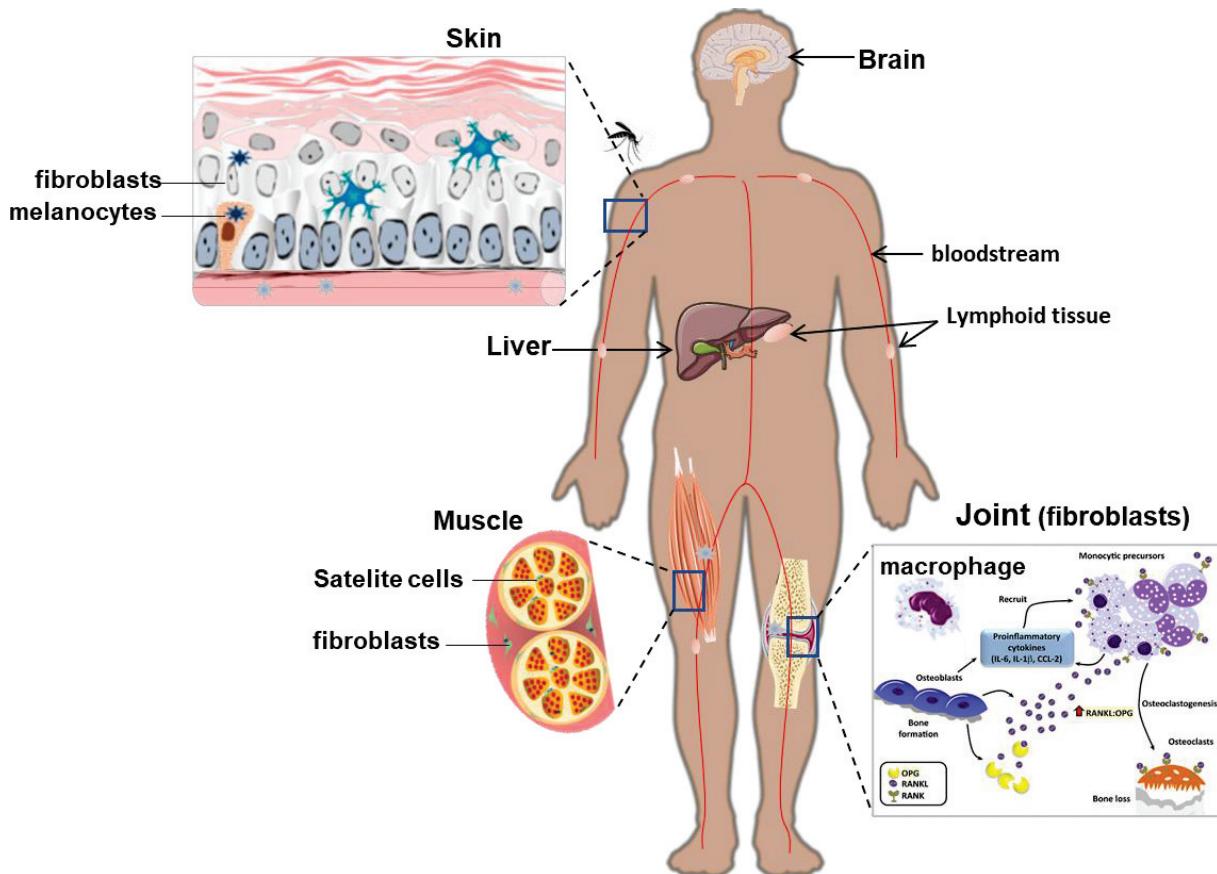
### **2.5.2 Acute and chronic phases**

In this part we present the dissemination of CHIKV from the skin to secondary organs and cellular tropism. Moreover, knowledge of characteristic-associated alphavirus chronicity will be introduced.

Acute CHIKV infection is generally associated with high fever, rashes, and severe muscle and joint pains for 7 to 10 days. It follows the chronic CHIKV disease typically defined by musculoskeletal disease for several months and sometimes for years, beyond the onset of acute disease. Chronic CHIKV disease has been reported principally after the outbreak in La Réunion in 2006-2007. While the majority of individuals fully recovered, a large proportion of individuals experienced chronic CHIKV disease and long-term impaired quality of life has been reported for some individuals (McCarthy, Davenport, and Morrison 2018).

Following CHIKV inoculation through mosquito bite, viral particles enter the subcutaneous capillaries, where they are in contact with susceptible cells in the skin (Figure 8). The main infected cells are fibroblasts, endothelial cells, and macrophages (Dupuis-Maguiraga *et al.* 2012). Local replication in the skin is associated with a very early Type-I interferon response. Indeed,  $\text{IFN}\alpha/\beta$  was detected very early in the infection and its concentration is correlated with viral load in plasma. On the one hand, the production of  $\text{IFN}\alpha$  for ten days was associated with viral clearance without detection of adaptative immunity through  $\text{IFN}\gamma$  in serum (Chow *et al.* 2011).

On the other hand, Hoarau *et al.* observed a high level of IFNy and cytokines IL-12 in patients from La Réunion (Dupuis-Maguiraga *et al.* 2012) and Wauquier *et al.* showed an overproduction of IFN $\alpha$  and IFNy during the acute phase in infected individuals in Gabon (Wauquier *et al.* 2011) (Figure 9). The skin represents the site of local viral replication following a mosquito bite, and the new particles produced are probably transported to secondary lymphoid organs not far from the site of inoculation. Moreover, blood transports viral particles either in free virion form or in the form of infected monocytes, to the target organs like liver, muscle, joints, and the central nervous system (Schwartz and Albert 2010). While the evidence of production of viral particles from monocytes has not been demonstrated, Her *et al.* have detected CHIKV antigens in monocytes exposed to MOI 10 to 50 and this result was supported by the detection of antigen-positive monocytes from acutely infected patients (Her *et al.* 2010).

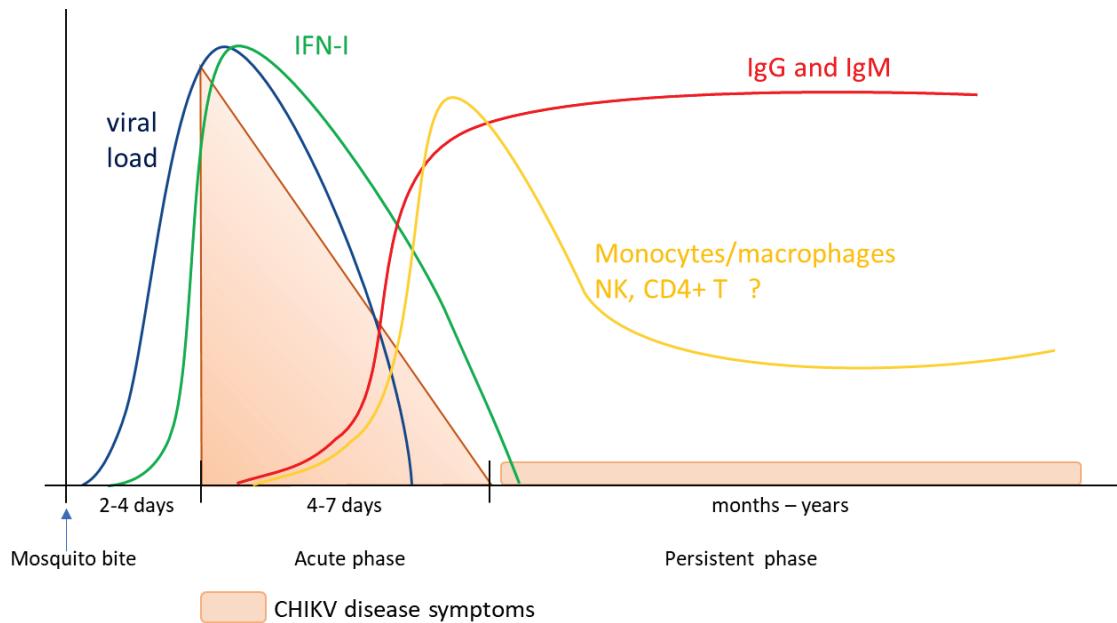


**Figure 8: CHIKV dissemination and targets in the human.** Transmission of CHIKV to humans occurs following a female Ae.aegypti or Ae.albopictus mosquito bite. The virus can replicate in the skin (fibroblasts and melanocytes cells) and disseminate to the liver (endothelial cells), lymphoid tissue, muscles (fibroblasts and satellite cells) and joints (fibroblasts) and the brain through the bloodstream. The persistent phase is characterized by persistent muscle and joint pains. The persistent phase seems to involve macrophage and monocyte inflammatory response (based on Matusali *et al.* 2019; Schwartz and Albert 2010).

The Chikungunya virus reaches muscles and joints where myalgia and/or arthralgia symptoms may persist for several weeks, months or even years. Following the outbreak at La Réunion, it was described in muscle biopsies from two patients that differentiated myotubes that were not permissive to CHIKV, whereas satellite cells (skeletal muscle progenitor cells) were positive for CHIKV (Ozden *et al.* 2007). The other characteristic disease symptom of CHIKV is arthralgia. Viral RNA has been detected in the synovial tissues and fluids during acute and chronic CHIKV infection, where synovial fibroblasts and macrophages were sensitive to CHIKV (Young *et al.* 2019).

Chronic CHIKV disease characterized by persistent arthralgia seems to be an immune and cell death mediated response also associated with risk factors. Nevertheless, CHIKV antigens have been detected only during the persistent phase. The osteoblasts are infected, leading to the secretion of proinflammatory and pro-osteoclastic factors (IL-6, IL-1  $\beta$ , CCL-2 and RANKL). In normal joints, the cytokine RANKL (receptor activator of the nuclear factor NF- $\kappa$ B ligand) can induce the differentiation of osteoblasts into osteoclasts and OPG (osteoprotegerin) can inhibit the action of RANKL. The RANKL:OPG ratio controls this differentiation. However, during CHIKV infection the RANKL:OPG ratio increases, favoring osteoclast differentiation and leading to bone loss and promoting inflammation (Chen *et al.* 2015). Viral persistence suggests that cells can be chronically infected and are perhaps able to control or block cell death. However, it has been observed that in chronic CHIKV individuals, the damaged synovial tissues present an intense immune response on the one hand and a strong programmed cell death characteristic on the other (Hoarau *et al.* 2010). Interestingly, it has been shown that the human monocyte MM6 cell line was infected with a very low rate of RRV replication and the infection led to a late apoptotic cell death feature without immune response control, suggesting that monocytes could be a viral reservoir during chronic RRV disease (Krejbich-Trotot *et al.* 2016). In parallel, the infection of macrophages in joints was associated with the production of cytokines and chemokines such as interleukins (IL)-6, IL-8, CCL-2/MCP-1 and, moreover, it has been proposed that the phagocytosis of apoptotic bodies from infected fibroblasts could also participate in viral persistence and the inflammation of musculoskeletal tissues in chronic CHIKV disease. Finally, Natural Killer NK cells and T cells together with monocytes were found activated and attracted to joint tissues (Dupuis-Maguiraga *et al.* 2012), (Figure 9). Nevertheless, the mechanisms

involved in viral persistence are still poorly understood as are the beneficial and deleterious effect of local inflammation and cell death on viral persistence.



**Figure 9: Schematic representation of CHIKV pathogenesis and immune response in humans.** Following a mosquito bite, the virus infects different cells of the skin and disseminates to secondary organs through blood vessels. After the incubation period of around 4 days, the symptoms of the acute phase are triggered and accompanied by IFN-I response in nonhematopoietic cells. One week after infection, the viral load decreases and ~70% of infected patient recover and present evidence of CHIKV-specific adaptative immunity, whereas ~30% of patients experience long-term sequela with persistent infection for several months or years.

## 2.6 Vaccine, antiviral treatments, and strategies in humans and mosquitos

Two strategies against CHIKV and other arboviruses have emerged: the first is the development of a vaccine for humans and the second the inhibition of viral infection in mosquito.

### 2.6.1 Vaccine and antiviral treatments in humans

To date, no specific treatments or licensed vaccines are available. Up to now, patients have been treated with symptomatic treatments like anti-inflammatory drugs to treat chronic arthralgia and myalgia. Chloroquine, a known drug against malaria, initially showed promising results in patients, however, several years later it was documented that after 200 days of treatment, patients complained more about joint pain than control group (Brighton 1984; De

Lamballerie *et al.* 2008). Another anti-inflammatory molecule, named bindarit, had been shown useful in the treatment of CHIKV-induced arthritis in a mouse model (Rulli *et al.* 2011).

Regarding vaccines, a recent publication reported the success of an experimental CHIKV vaccine (phase 2) which confers durable immune response and is safe for healthy volunteers aged 18 to 60 (Chen *et al.* 2020). The volunteers received two intramuscular doses of the vaccine and showed strong immune response to CHIKV after 72 weeks of the study (Chang *et al.* 2014; Chen *et al.* 2020). This vaccine approach is based on a virus-like particle (VLP) presenting structural proteins recognized by the immune system, suggesting a protective immune response to the CHIKV.

Other vaccines are under development such as a chimeric vaccine with a VSV backbone and CHIKV structural proteins (Chattopadhyay *et al.* 2013), and a recombinant measles-virus-based chikungunya vaccine (Ramsauer *et al.* 2015).

### **2.6.2 Antiviral strategies in the mosquito**

In response to insecticide resistance, alternative strategies are needed for controlling the vector and preventing epidemics. Previously, the primary strategy was the use of synthetic chemicals to kill adult vectors using aerial spraying. In parallel, the control of larvae has been developed by chemical application, microbial larvicides and biological agents (predator fishes that eat mosquito larvae). The obstacle to larval control is the ability of detect, access, and eliminate the breeding zone, which is a costly task. Thus, new strategies have been developed thanks to entomological and epidemiological approaches such as attractive targeted sugar baits, the bacteria *Wolbachia*, the sterile insect technique and genetic manipulation (Achee *et al.* 2019). The impact of *Wolbachia* in the inhibition of arbovirus in the mosquito will be discussed below (Chapter 3).

### 3 Cellular responses in *Alphaviruses*-infected mammals and insects

Soon after viral detection, different cellular pathways are activated to control viral replication such as innate immune response and apoptosis. In vertebrates, the innate immune response seems essential to limit viral replication, whereas in the mosquito vector competence seems to be linked to a basal immune response. In parallel, it is not clear if the apoptosis induced in infected mammals and mosquitos is an anti-viral or pro-viral response. These two pathways linked to others and the viral strategies to counteract cell defense will be discussed in this chapter.

#### 3.1 Innate immune response

##### 3.1.1 In mammals

Following the bite of an infected mosquito, the infection of fibroblasts and other cells by alphaviruses leads to the production of Type-I IFN, as described in the previous chapter. The production of IFN-I is triggered by the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). The PAMPs include surface glycoproteins, single-strand RNA, and double-strand RNA.

###### - The Toll-like receptors

The Toll-like receptors (TLRs) are membrane-bound glycoproteins found at the PM and in endosomes. They are composed of an extracellular leucine-rich domain that mediates the recognition of PAMPs and an intracellular domain, Toll-Interleukin-1 resistance (TIR) that mediates the recruitment of downstream proteins. The dimerization of TLRs is induced by the binding of PAMP to the TLR, which induces change receptor conformation and allows recruiting adaptor proteins and initiating the signaling pathway. The dsRNA produced during alphavirus replication and internalized in the endosome can be detected by TLR. TLR3 was identified as being essential against viral dsRNA (Lai *et al.* 2011; Priya, I. K. Patro, and Parida 2014) and is expressed in the endosome and phagosome and at the surface of fibroblasts. Following TLR3

stimulation, the expression of IFN-I is promoted by IRF3 and IRF7 (Interferon regulatory factor) and Nuclear factor NF-κB transcription factors through the activation of adaptor protein TRIF (TIR domain-containing adaptor inducing IFNβ, also called TICAM1). Indeed, TRIF binds to TLR3 and recruits TRAF3 or TRAF6 (TNF receptor-associated factor). The association with TRAF3 allows recruiting TBK-1 (TANK-binding protein 1) and IKKε (Inhibitor of nuclear factor Kappa-B factor Kinase subunit epsilon), which catalyze IRF3 and IRF7 phosphorylation to promote their nuclear translocation and binding to the IFN-I promoter (Pfeffer 2011). The TRAF6 association leads to receptor-interacting protein RIP1 recruitment, the transforming growth factor β-associated kinase TAK1 and IKK α and β (Figure 10 “Infected cell”). The inactive NF-κB is retained by its inhibitor IκB in the cytoplasm. IκB phosphorylation by IKK leads to the degradation of IκB, which allows NF-κB nuclear translocation and the transcription of target genes comprising IFN-I and pro-inflammatory cytokines such as IL-6, IL-1β and TNFα (Takeda and Akira 2015).

Her *et al.* have shown an increase in CHIKV susceptibility in human and mouse fibroblasts with defective TLR3 signaling. Moreover, antibodies against CHIKV generated in Tlr3 *-/-* mice present a lower neutralizing capacity *in vitro* (Her *et al.* 2015). Moreover, the immune response has been characterized in the brain of CHIKV-infected mice with up-regulation of antiviral genes and cytokines such as TLR3, TRAF6, TRIF, IFNβ, IL-6, Mx-2, and OAS. They confirmed the role of TLR3 by pretreatment with Poly I:C (a TLR3 agonist) accompanied by the reduction of CHIKV titer in the brain and the protection of mice (Priya, I K Patro, and Parida 2014). Interestingly, differences have been identified in immune response intensity and viral replication by comparing the ECSA CHIKV genotype without mutation at position E1 226 (A226) and with mutation (A226V). The 226V mutant showed relatively lower induction of TLR3 and 7, IFNβ, OAS-3, Mx in a mice neuroblastoma N2a cell line. Knowing that the CHIKV 226V strain is more virulent, the associated downstream antiviral response could participate in higher pathogenesis (Priya *et al.* 2013).

The ssRNA of alphaviruses can be recognized by TLR7 and TLR8 present only in the endosome compartment and activate a signaling pathway through the adaptor myeloid differentiation primary-response protein 88 (MyD88). TLR8 leads to the activation of NF-κB through the TRAF6 and IKK complex, whereas TRL7 activates IFN-I production through TRAF6/3 and IRF3/7 factors.

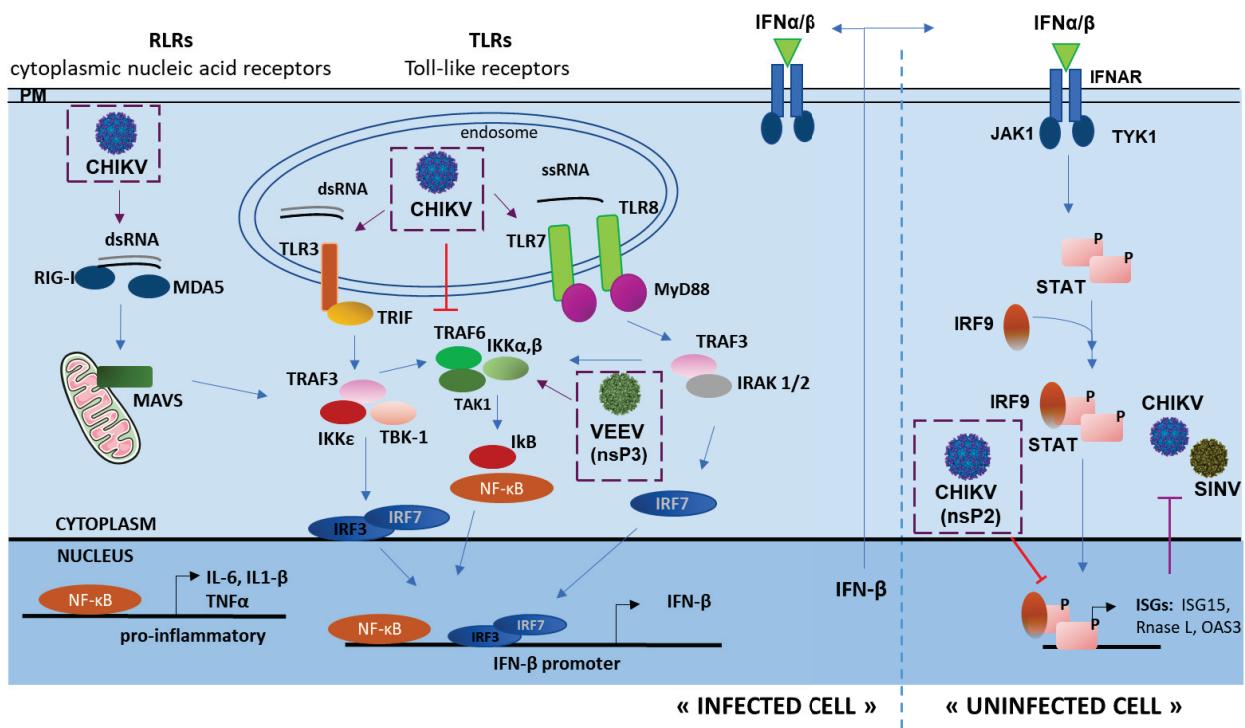
To conclude, IRF3/7 and NF-κB participate in the activation of IFN $\beta$  and pro-inflammatory cytokines and are essential against alphaviruses infections. NF-κB is involved in an antiviral, protective response and in the induction of cell death, which will be discussed in the following section.

- *Detection of cytoplasmic viral RNA by cytoplasmic nucleic acid receptors*

In addition to the Toll-like receptors, another mechanism can detect viral RNA and control infection: the TLR-independent mechanism. IFN-inducible RNA helicase RIG-I (retinoic acid-inducible gene product I) and melanoma differentiation-associated gene-5 MDA-5 can detect cytoplasmic RNA and induce IFN expression (Priya *et al.* 2013). These proteins present a C-terminal helicase domain, accompanied with ATPase activity and a caspase-recruitment domain (CARD) in the N-terminal region. The activation of RIG-I and MDA5 induces a conformational change that allows exposing the CARD domain and mediating several interactions. For example, the CARD domain can bind to MAVS (mitochondrial antiviral signaling protein), which is anchored in the membrane of the mitochondrion and acts as an adaptor protein leading to the activation of IRF3 and NF-κB. Moreover, by this interaction, the MAVS can participate in the activation of caspases involved in the apoptotic cell death pathway. The association of MAVS with TRADD (TNFR1- Associated death domain protein) in a complex involving TRAF6, FADD and IKK complex leads to the activation of NF-κB, while the complex involving TRAF3 leads to the activation of IRF3/7 and the induction of IFN $\beta$  production (Figure 10 “Infected cell”).

Interestingly, it has been shown that VEEV and RVFV infections lead to a macromolecular reorganization of IKK $\beta$  that subsequently produces a lower molecular weight complex, and IKK $\beta$  seems to be involved in viral replication and the production of VEEV. Indeed, a decrease in RNA copies and infectious particles has been observed with IKK $\beta$  inhibitor treatment and by using mammalian cell IKK $\beta$ -/-; the infection of *in vivo* mice pretreated with this inhibitor increased their survival. Finally, based on proteomics analysis, it has been suggested that nsP3 could interact with IKK $\beta$ , suggesting that the IKK $\beta$  function is required for VEEV replication (Amaya *et al.* 2014).

The infection of human primary synovial fibroblasts with CHIKV has shown an increased level of miRNA-146a expression, previously reported in synovial tissues affected by rheumatoid arthritis. The miRNA are small non-coding RNAs that bind to target messengers and play an important role in the regulation of infection, immune response, and inflammation. miRNA-146 expression is controlled by NF- $\kappa$ B and can directly downregulate the level of TRAF6 and IRAK1 (IL-1 receptor associated kinase 1), leading to the suppression of NF- $\kappa$ B signaling. During CHIKV infection, it has been shown that the increase of cellular miRNA-146a results in the downregulation of TRAF6 and IRAK1, suggesting a mechanism that exploits the RNA silencing pathway by CHIKV to modulate immune response in fibroblasts (Selvamani, Mishra, and Singh 2014).



**Figure 10: Type-I interferon production and JAK-STAT signaling pathway activation after Alphavirus infection.** The detection of ssRNA and intermediate dsRNA structures involve different pathogen recognition receptors (PRRs). Toll-like receptors TLR3, TLR7 and 8 and retinoic acid-inducible gene I (RIG-I – like receptors (RLRs)) like MDA5 and RIG-I activate a signalling cascade involving adaptor protein MyD88, TRIF and Interferon response factors (IRF) 3 and 7. The pathways lead to the expression of IFN-I (IFN  $\alpha$  and  $\beta$ ), which is secreted in the extracellular compartment to activate the Jak-STAT pathway by activation of IFNAR. Binding to IFNAR leads to the activation of associated-receptors Janus kinase (JAK1) and Tyrosine kinase (TYK1), which activate STAT by phosphorylation and dimerization and bind to IRF9 (forming the ISGF3 complex). The transcription factor STAT-IRF9 translocates to the nucleus and induces interferon stimulated gene (ISGs) transcription. The ISGs will protect infected and uninfected cells, leading to the control of viral dissemination.

- Pathway activated by IFN-I: The JAK-STAT signaling pathway

The production of IFN $\alpha$  and  $\beta$  activates the antiviral signaling pathway in the infected cell and in uninfected neighboring cells to prevent their infection. IFN $\alpha/\beta$  is secreted into the extracellular environment and recognized by the interferon-alpha receptor IFNAR (composed of IFNAR1 and 2 subunits). The activation of IFNAR activates the receptor-associated Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which trigger the activation of transcription factors STAT1 and STAT2 by phosphorylation and dimerization. Active STAT with IFN-regulatory factor 9 (IRF9) form the interferon-stimulated gene factor 3 (ISGF3) complex, which translocates to the nucleus and binds to IFN-stimulated response element (ISRE) sequences to activate antiviral genes (Ivashkiv and Donlin 2014) (Figure 10 “Uninfected cell”).

An extensive study of SINV identified several interferon-stimulated genes (ISGs) such as ISG15 and RNase L (Lenschow *et al.* 2007). For CHIKV, an antiviral role of OAS3 in infected HeLa cells has been reported (Bréhin *et al.* 2009). It has been also shown that IFN-I controls CHIKV infection in non-immune cells in the mouse and the production of IFN by fibroblasts is controlled by MAVS downstream of RIG-I and MDA5 (Schilte *et al.* 2010). Moreover, it has been shown that IRF1 protects *in vivo* mouse against CHIKV infection in non-hematopoietic muscle cells after 20 days, suggesting a protective role of IRF1 in the post-acute phase (Nair *et al.* 2017).

The OAS 2'-5'-oligoadenylate synthase proteins catalyze ATP (Adenosine Triphosphate) into 2'-5'-linked adenosine oligomers and these oligomers activate RNaseL that degrades both viral and cellular RNAs into short fragments. This process inhibits virus replication of diverse RNA viruses like vesicular stomatitis virus (VSV, family *Rhabdoviridae*), human parainfluenza virus-3 (HPIV, family *Paramyxovirus*). Moreover, OAS/RNaseL has been shown to induce apoptosis (A. Zhou *et al.* 1998).

The Mx GTPases is a group of guanine-hydrolyzing enzymes. The human genome encodes MxA and MxB but only MxA demonstrates an antiviral activity. The MxA protein can bind virus structures and it has been shown that recognition of the Thogoto virus (THOV, family *Orthomyxoviridae*) nucleocapsid leads to the inhibition of viral replication (Kochs and Haller 1999). Moreover, during SFV infection of human HEp-2 cells the antiviral activity of MxA has been shown through the inhibition of viral replication and non-structural proteins synthesis (Landis *et al.* 1998).

Old-World *Alphaviruses* evolved to escape the immune response of vertebrates and it has been discovered that the nsP2 of Old-World alphaviruses can affect the expression of ISGs by action of nsP2 on STAT1 and by action on general transcriptional shut-off in vertebrate infected cells. It has been shown that the C-terminal domain of CHIKV nsP2 promotes the nuclear export of STAT1, avoiding STAT1 binding and consequently preventing the induction of ISGs (Göertz *et al.* 2018). Moreover, it has been shown that nsP2 of SFV, SINV and CHIKV can translocate to the nucleus and induce the rapid degradation of RPB1, a subunit of the RNA polymerase II complex (Ivan Akhrymuk, Kulemzin, and Frolova 2012). However, the degradation of RPB1 is independent of the protease activity of nsP2, suggesting that nsP2 utilizes a cellular proteolytic process for the degradation of RNA pol II. Rapid (around 6-8 hours post-infection) general transcriptional shut-off can finally prevent all the different antiviral responses, not only immune response but also cell death. Moreover, the non-structural protein NSs of Bunyamwera virus (*Bunyavirus*) has been shown to interfere with mRNA transcription by targeting RNA polymerase II and a mutant NSs is accompanied by a higher induction of IFN than WT (Thomas *et al.* 2004; Weber *et al.* 2001). The non-structural protein NS1 of non-arbovirus Influenza A virus (IAV, family *Orthomyxoviridae*) presents multiple functions of which a major one is the deregulation of RNA Polymerase II, leading to a global transcriptional downregulation and affecting antiviral response and virulence (Zhao *et al.* 2018).

New-World alphaviruses have also evolved to inhibit host transcription but, interestingly, it is the capsid, and not nsP2 that is responsible for transcriptional inhibition, by forming a complex with cellular factors inside the nucleus that consequently obstruct trafficking and lead to decreased mRNA transcription (Lundberg, Carey, and Kehn-Hall 2017).

### **3.1.2 In the mosquito**

The immune pathway in insects has been extensively studied in *Drosophila melanogaster* (Hoffmann 2003), after which global transcriptome analysis of the vectors Anopheles, Aedes aegypti and Culex gave abundant genetic information about vector-borne interactions and the involvement of immune response and cell death in vector competence. In this first section, we will describe the immune pathways in the mosquito activated during arbovirus infection.

- RNA interference (RNAi) pathway

RNAi in insects plays an important role in controlling and limiting virus infection. The RNAi pathway was first discovered in the fruit fly *Drosophila* (Galiana-Arnoux *et al.* 2006). This insect's response utilizes virus-generated double strand RNA (dsRNA) to induce small interfering RNA (siRNA) that allows targeting viral RNA for degrading and blocking viral replication. Up to now, three different RNA interference pathways have been characterized: silencing RNA (siRNA), micro RNA (miRNA) and PIWI-interacting RNA (piRNA). siRNAs are generated from dsRNA either derived from virus for example or encoded by the insect genome. miRNAs are generated from cell transcripts and allow gene regulation at translational level. piRNAs are transcribed from the cellular genome and occur in the germ line.

In this part, only the siRNA pathway will be described as predominantly responsible for antiviral activity. The intermediate viral dsRNA is recognized by Dicer-2 (member of the RNase endoribonuclease family) in complex with R2D2. The dsRNA is cleaved into 21 nucleotide siRNA by Dicer-2 and loaded into the pre-RISC (pre-RNA-induced silencing complex) complex where it is unwound, and the guide strand generated is used to target viral RNA by base pairing. The recognition is ensured in RISC and the RNase activity of the Argonaute-2 (Ago-2) protein cleaves the recognized viral RNA genome (Figure 11). In flies, the loss-of-function by mutations of the Dicer-2 gene (*dcr-2*) or *ago-2* fails to control viral replication. In *Aedes* and *Anopheles* species infected by arbovirus, virus-derived siRNA have also been detected. In SINV-infected *Ae. aegypti*, the RNAi pathway was activated by the production of virus-specific siRNA and the silencing of RNAi components Dicer-2 and Ago-2 resulted in a transient increase of SINV replication (Campbell *et al.* 2008). Keene and colleagues have shown that co-injection of the O'nyong-nyong virus (ONNV)-GFP virus and dsRNA silencing Ago2 or Ago3 in *Anopheles gambiae* result at 6 days post-injection (dpi) in an increase of viral replication and production. Interestingly, they also silenced ONNV-nsP3 and showed a significant decrease in infection at 3 and 6 dpi, revealing an important role of nsP3 in viral replication and maybe in the control of antiviral vector response (Keene *et al.* 2004). Additionally, an RNAi inhibitor B2 (viral suppressor of RNAi (Sullivan and Ganem 2005)) gene adding into SINV genome has been shown to increase the mortality rate of the mosquito and viral replication, indicating that an immune response is involved in the control of viral replication and virus-induced pathogenic effects on the vector (Cirimotich *et al.* 2009). Additional evidence for the importance of RNAi in limiting

arboviral infection in a permissive vector was obtained in studies using DENV-2 in the *Ae. aegypti* mosquito (Sánchez-Vargas *et al.* 2009).

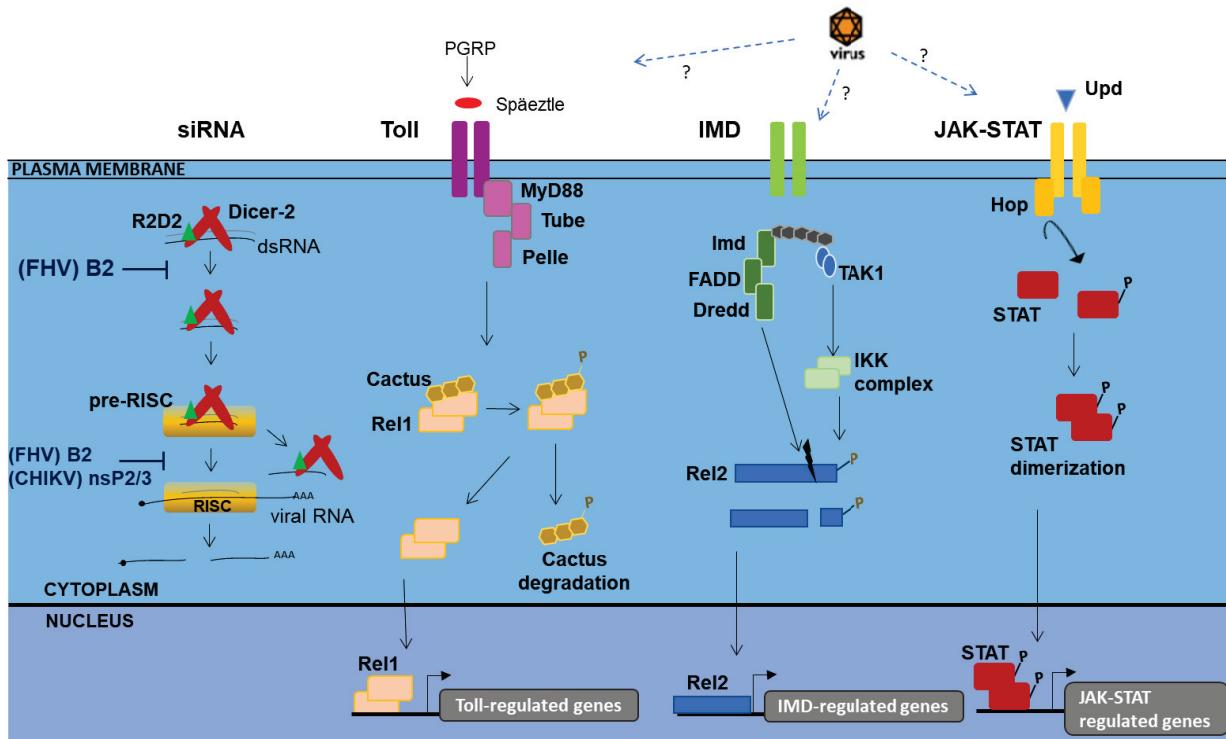
More recently, Mathur *et al.* described that CHIKV nsP2 and nsP3 exhibit an RNAi suppressor activity in the *Ae. aegypti* Aag2 cell line, involving the helicase domain and the macrodomain, respectively (Mathur *et al.* 2016).

- *The Toll pathway*

The Toll pathway has been characterized as playing an essential role in the fly's defense against Gram-positive bacteria, fungi, and viruses by the expression of antimicrobial peptides (AMPs). The expression of AMPs in the conserved signaling pathways Toll and IMD is regulated by the activity of transcription factors of the NF-κB family (Mussabekova, Daeffler, and Imler 2017). The Toll and IMD pathways share similarities with the inflammatory pathways regulated by Toll-like receptors in mammals (interleukin-1 receptor and the TNF receptor). First, the recognition of pathogen-derived ligands by pattern recognition receptors (PRRs) such as peptidoglycan recognition proteins (PGRP) triggers the cleavage of the neurotrophin-like cytokine Späetze, which circulates in the hemolymph as an inactive precursor and is cleaved by proteolytic processing, allowing its activation and binding to the transmembrane receptor Toll (Weber *et al.* 2003). Then, the associated adaptor proteins MyD88, Tube and the kinase Pelle induce the phosphorylation and subsequent proteasomal degradation of the negative regulator Cactus, allowing the release and translocation of NF-κB protein Rel1 (Dorsal in *Drosophila*) to the nucleus and activation of Toll-pathway related genes like antimicrobial peptides (AMPs) (Figure 11).

The activation of the Toll pathway in the mosquito midgut has been reported (Sanders *et al.* 2005) at the early-stage of *Ae.aegypti* infection by SINV. The activation of the Toll pathway has been also demonstrated in the midgut of *Ae.aegypti* infected by DENV at the early stage of infection (3 dpi) (Ramirez and Dimopoulos 2010). Finally, silencing of MyD88 leads to increased DENV infection and the transient activation of Rel1 reduces DENV infection in the midgut (Xi, Ramirez, and Dimopoulos 2008). The activation of the Toll immune response in the midgut of a major vector of DENV seems important for the control and reduction of the viral load which

could limit the infection. Also, it seems to be linked to efficient infection, possibly participating in the permissiveness of the midgut.



**Figure 11: Innate immune pathways in the mosquito: siRNA, Toll, IMD and JAK-STAT pathways.** The siRNA pathway is initiated when a virus-derived double-strand RNA (dsRNA) in the cytoplasm is detected by Dicer-2 and R2D2 and cleaved into 21 base pairs (bp) siRNA in length by Dicer-2. The siRNA is loaded in pre-RNA-induced silencing complex (RISC), which unwinds and degrades one of the siRNA strands and uses it to target and degrade a single-strand viral RNA. Protein B2 of the flock house virus (FHV-insect virus only) can block siRNA signaling. It was proposed that nsP2 and 3 of CHIKV also present an RNAi suppressor activity. In the Toll pathway, the detection of PRRs triggers the cleavage of the cytokine Späetze which allows signaling through MyD88, Tube and Pelle and the degradation of Cactus. The degradation of the negative regulator Cactus which was bound to Rel1, allows the nuclear translocation of Rel1. The IMD pathway is activated by ligand binding to receptor triggering through Imd, FADD and Dredd, a caspase and kinase activation and the nuclear translocation of the Rel2 transcription factor. The JAK-STAT pathway is activated when Upd binds to the receptor and activates the kinase receptor-associated Hop, which phosphorylates STAT. The phosphorylation of STAT allows dimerization and nuclear translocation.

#### - The IMD pathway

The immune deficiency (IMD) pathway is, like the Toll pathway, initiated by ligands binding to PGRP and followed by signaling through adaptor protein IMD (Kleino and Silverman 2014). Several caspase-like proteins and kinases then lead to splitting the pathway into two branches for the activation of Relish (in *Drosophila*)/Rel2 (in mosquito). IMD activation recruits dFADD that recruits the caspase-8 homolog Death-related ced-3/Nedd2-like protein DREDD, leading to

the polyubiquitination of IMD. TAK1 binds to the polyubiquitin chain and is responsible of the JNK pathway by assembly and activation of the IKK complex (IKK $\beta$  and IKK $\gamma$ ). The IKK complex mediates the phosphorylation of Rel2, which is cleaved by DREDD and can be translocated to the nucleus and regulate the transcription of target-genes (Figure 11).

- The JAK-STAT pathway

The JAK-STAT pathway is also a well conserved pathway in insects which was first discovered in *Drosophila* and orthologs of STAT. Components have been predicted in several *Anopheles* species and in *Ae. aegypti* and *Cx. quinquefasciatus*. In the fly, the pathway is triggered by the binding of Unpaired (Upd) to the cell surface receptor Dome, leading to its dimerization, activation, and phosphorylation of receptor-associated Hop Janus kinases. This is followed by the recruitment and phosphorylation of the STAT transcription factor, which can dimerize and translocate to the nucleus to activate the expression of effector genes (figure 11).

The activation of Toll, IMD and JAK-STAT pathways has been described in response to SINV and DENV in *Ae. aegypti* and WNV in *Culex pipiens* (Luplertlop *et al.* 2011; Zink *et al.* 2015).

A transcriptomic analysis of *Aedes aegypti* permissive and resistant strains infected with DENV showed an enrichment of several immune-related transcripts of Toll, IMD and JAK-STAT pathways in resistant but not in permissive mosquito strains. Moreover, many immune transcripts were more abundant in the carcass (without midgut) of the resistant strain, but in the midgut these transcripts were less abundant than in the permissive strain (Sim *et al.* 2013). These results could suggest that the immune response in the midgut of the permissive strain is necessary for efficient infection by Dengue virus, perhaps to lower the viral load at a specific level allowing the passage through the midgut barrier and then dissemination. On the contrary, the results obtained from resistant mosquitoes suggest that the infection of the midgut did not induce a significant immune response leading to a higher viral load which could be detected after the escape of from the midgut barrier, probably through the hemocytes. In the first case, the mosquito can become chronically infected and in the second case the viral infection and propagation were stopped. Was the cell antiviral immune response of a permissive vector controlled by the virus through the evolution of the interaction?

- Wolbachia

In addition to antiviral immune signaling pathways, other extrinsic factors can be involved in the control of viral infection, such as the bacteria *Wolbachia*.

*Wolbachia* is an intracellular bacterium that can manipulate the reproduction of the insect host. The transfer of the bacteria in laboratory *Aedes* mosquito strains inhibits viral dissemination. Indeed, the artificial administration of the *Wolbachia* wMel strain (from *Drosophila*) in *Aedes aegypti* and *albopictus* vectors increases resistance to DENV and CHIKV. The same result was obtained in *Drosophila melanogaster* injected with CHIKV and *Culex quinquefasciatus* infected with West Nile Virus (Glaser and Meola 2010). However, most of the results were obtained with a high density of *Wolbachia*, higher than that detected naturally in the mosquito. The presence of *Wolbachia* seems to impact arbovirus infection by the upregulation of innate immune pathways like Toll, IMD and JAK-STAT, suggesting a first line that rapidly blocks virus infection. Moreover, the competition for lipid metabolism and autophagy modulation have also been suggested (Sinkins 2013).

To conclude this part, it is suggested that a specific level of immune response is required for vector competence and in a compartment-dependent manner. It also seems obvious that other intrinsic, extrinsic factors and signaling pathways are involved in efficient replication such as control of cell death during viral replication. Indeed, apoptotic cell death is another efficient antiviral response that can completely suppress viral replication. However, until now, it has not been clear whether apoptosis plays a role in determining the outcome of arbovirus infection.

### **3.2 Apoptosis**

Apoptosis is a very well conserved pathway in vertebrate and invertebrate organisms, and it is a tightly controlled process where the cell, in response to a wide range of stimuli like DNA damage, oncogenic factors, virus infection and signals from immune cells activates different self-destruction pathways. An apoptotic cell presents a characteristic morphology including chromatin condensation, DNA fragmentation and loss of phospholipid asymmetry in the membrane, in contrast with necrosis. Apoptosis culminates with the engulfment of the apoptotic cell by phagocytic and non-phagocytic cells, preventing the leakage of cytoplasm and

the activation of inflammatory response. The induction of apoptosis by different stimuli will ultimately activate protease effectors responsible for the collapse of the cell.

### 3.2.1 In mammals

#### 3.2.1.1 The apoptosis signaling pathway

- Caspases: the proteases effectors of apoptosis

In 1993, Yuan and colleagues showed the involvement in apoptosis of a cysteine protease CED-3 in *Caenorhabditis elegans* (Yuan *et al.* 1993). Since then several studies have identified at least 14 distinct mammalian caspases with their orthologs present in species ranging from nematodes to dipteran *Drosophila* and mosquitoes. The first mammalian caspase, called caspase-1 or interleukin 1 $\beta$ -converting enzyme (ICE), was involved in inflammatory response and at least eight caspases can be activated during apoptosis (Salvesen and Dixit 1997). Caspases cleave many cellular proteins during apoptosis. They are synthesized as inactive precursors (procaspases) and their activation is guided by proteolytic cleavage. They are classified into two categories, the “initiator” and “effector” caspases which include caspases 2, -4, -5 -8, -9, -10 and -3, -6, -7, respectively. Once activated by autocleavage, an initiator caspase will activate an effector caspase through cleavage at specific internal aspartic acid (Asp) residues that separate large and small subunits (Shi 2002). Once activated, the effector caspases cleave cellular substrates such as actin, nuclear lamin or DNA-dependent protein kinase.

Apoptosis can be triggered by two distinct pathways: one activated by the binding of ligands of death receptors and generally called “extrinsic” pathway and the second one involving mitochondria and B-cell lymphoma 2 (Bcl-2) family of proteins and called “intrinsic” pathway (Figure 12). However, the two pathways are interconnected by the caspases and share the same proteins regulated in apoptosis.

- Death receptors and mitochondrial signaling pathway

Death receptor-mediated apoptosis is activated by stress stimulation sensed by receptors of the tumor necrosis factor (TNF) family including the TNF receptor (TNF-R), TNF-related

apoptosis-inducing ligand receptor (TRAIL-R) and Fas (also called CD95 or Apo-1). The death receptor ligands are secreted by immune cells and involved in the control of immune response, while the receptors can be expressed at the plasma membrane of different cell types. Among the soluble ligands, the best characterized are members of TNF $\alpha$ , Fas ligand FasL and TNF-related apoptosis inducing ligand TRAIL. The death receptors are transmembrane proteins, composed of an extracellular ligand-binding region, a membrane-integrated region, and a cytoplasmic death domain. The binding of FasL or TRAIL to the extracellular region of death receptors Fas or TRAIL leads to the oligomerization of the receptor and subsequently to a signal transduction involving the formation of the multi protein Death-inducing signaling complex (DISC). DISC includes Fas-associated death domain FADD recruited by the cytoplasmic death domain region of the receptor, and procaspase-8/10. Caspase-8 is activated by association to DISC and can activate caspase-3/7. The binding of TNF to TNF-R requires the TRADD intermediate for the association of FADD and procaspase-8/10. TRADD can also be associated with RIP1 and TRAF2, leading to the activation of the NF- $\kappa$ B signaling pathway (Muppudi, Tschopp, and Siegel 2004).

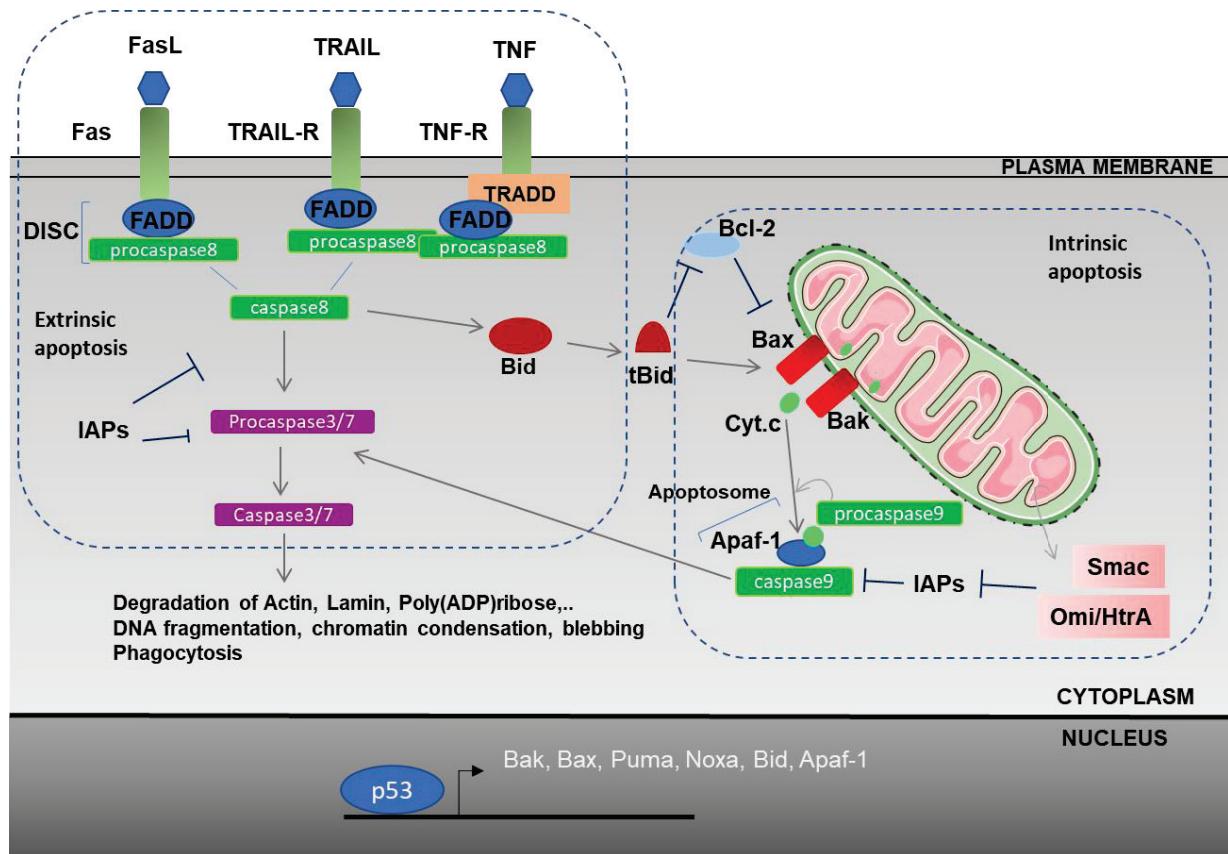
- Regulation of caspase-mediated apoptosis by the IAP family

The apoptotic pathway is always ready to be activated by the continual expression of procaspases and the IAP (inhibitor of apoptosis proteins) family of proteins is essential for inhibiting apoptosis in several distinct ways. The IAP proteins contain several BIR domains (baculovirus IAP-repeat) containing the anti-apoptotic function and one RING domain that presents a ubiquitin ligase activity. Their function is neutralized by inhibitor molecules, referred to as IAP-antagonists. The IAP family includes XIAP (X-linked IAP), c-IAP1 and c-IAP2 in mammals. A high level of IAPs binds to caspase and inhibits caspase-mediated apoptosis: for example, c-IAP2 and XIAP promote the ubiquitination and degradation of caspase-3/7 (Huang *et al.* 2000). The IAP-antagonists can participate in the induction of apoptosis. In mammals, IAP-antagonists group Smac and HtrA2/Omi and are localized at the mitochondria. They contain an IAP-binding motif (IBM) conferring the ability to bind physically to IAPs (Figure 12) (Vasudevan and Ryoo 2015).

In parallel, mitochondrial-mediated apoptosis can be activated by caspase-8 in the Fas death receptor-dependent pathway. It was shown that Bid, localized in the cytoplasm, can be cleaved by caspase-8 and the truncated Bid (tBid) translocates to the mitochondria, leading to mitochondrial damage (Li *et al.* 1998). The damage of the mitochondria is defined by mitochondrial outer membrane permeabilization (MOMP) which promotes the release of cytochrome c, triggering the formation of the multiprotein complex called the apoptosome. Cytochrome c binds to the apoptosis protease-activating factor 1 (Apaf-1) which oligomerizes into the functional complex apoptosome and activates procaspase-9 which in turn activates caspase-3 (Figure 13), (Xu *et al.* 2014; Zou *et al.* 1999).

- Regulation of mitochondria-mediated apoptosis by the Bcl-2 family proteins

The Bcl-2 proteins present either pro- or anti-apoptotic properties and all contain four conserved bcl-2 homology (BH) motifs (BH-1-BH4) and a TM motif for anchoring to membranes including mitochondrial membranes, the nuclear envelope, ER, and lysosomes. Understanding of Bcl-2 proteins is derived from massive investigation in humans, the mouse, the nematode *C.elegans* and the fly *Drosophila melanogaster*. In mammals, the anti-apoptotic proteins include six proteins, including Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, Bfl1/A1 and Mcl1, while the pro-apoptotic proteins can be divided into the multi-domain Bax family (Bax, Bak and Bok), and the eight BH3-only proteins including Bim, Bad, Bid, Noxa and Puma. The Bcl-2 proteins regulate intrinsic apoptosis through specific binding to the mitochondrial membrane and the final step is the oligomerization of Bax or Bak (and Bok) at the mitochondrial membrane, resulting in the formation of pores and the release of cytochrome c (Banjara *et al.* 2020). The anti-apoptotic, pro-survival, Bcl-2 proteins are associated with the intracellular membrane and their anti-apoptotic action is antagonized by pro-apoptotic proteins. For example, in *C. elegans*, the orthologue of Bcl-2, CED-9, sequesters the Apaf-1 orthologue CED-4 at the mitochondrial membrane and prevents the activation of caspase CED-3 (Spector *et al.* 1997). The role of pro-survival bcl-2 is not limited to apoptosis; indeed, it has been proposed in processes like autophagy or homeostasis. It has been discovered for instance that the Bcl-2 protein family can be involved beyond apoptosis in calcium trafficking and metabolite transport across the mitochondria, which participate in mitochondrial energy metabolism (Danial, Gimenez-Cassina, and Tonnerre 2010).



**Figure 12: Death receptors- and mitochondria-mediated apoptosis in mammals.** The death receptors Fas, TRAIL and TNF form the DISC after binding to their ligands and trigger the activation of initiator caspase-8. Activated caspase-8 can activate effector caspase-3 and/or cleave the BH3-only protein Bid. The truncated Bid (tBid) is linked to mitochondria-mediated apoptosis by the inhibition of anti-apoptotic Bcl-2 and association with pro-apoptotic Bax and Bak. The formation of pores in the mitochondria membrane triggers the release of cytochrome c into the cytoplasm and the formation of the apoptosome through the recruitment of Apaf-1, dATP and procaspase-9. The initiator caspase-9 is activated by its association with the apoptosome and upstream by IAP-antagonists released from the mitochondria. Active Caspase-9 cleaves caspase-3 leading to the degradation of nuclear components like lamin, DNA fragmentation and cellular skeleton disruption. The apoptotic bodies subsequently formed are recognized and phagocytosed by professional and non-professional cells.

#### - p53-mediated apoptosis

Transcriptional factor p53, thoroughly studied in cancerology, is increasingly analyzed in the fields of virology and immunology for its central role in the transcriptional regulation of apoptotic and immune response-related genes. Chapter 4 provides a description of p53 and its interplay with viruses. In this part, the induction of the apoptosis dependent p53 signaling response will be described. Transcription factor p53 can induce the transcription of apoptotic genes involved in the intrinsic pathway as well as the extrinsic pathway. For example, after DNA damage recognition by p53, *fas* is upregulated transcriptionally as well as *trail*. Several members of the Bcl-2 family are targets of p53: *bax* was the first gene identified and a Bax-

deficient mouse exhibited a decrease in p53-mediated apoptosis (Yin *et al.* 1997). Other pro-apoptotic targets like Puma, Noxa Bid or Apaf-1 can be p53-upregulated (Moroni *et al.* 2001; Oda *et al.* 2000). Interestingly, the p53 induced transcription of Bid promoted the convergence of extrinsic and intrinsic pathways.

### 3.2.1.2 Regulation of apoptosis during alphavirus infection

During a viral infection, apoptosis is generally described as a defense mechanism induced to limit virus replication and production and prevent the infection of neighboring cells. On the one hand, many viruses have developed the capability to delay or inhibit apoptosis either by the induction of cellular anti-apoptotic proteins or by the expression of anti-apoptotic proteins of their own (Figure 13). On the other hand, it has been shown that apoptosis can be a pro-viral pathway for replication and dissemination.

The first cell death analysis was documented using SINV and SFV. The infection of baby hamster kidney (BHK) cells, rat prostatic adenocarcinoma (AT-3) cells and mouse neuroblastoma (N18) cells with SINV results in apoptosis clearly observable at 24 hpi, resulting in nuclear condensation and membrane blebbing (Levine *et al.* 1993). In this same study, they transfected AT-3 cells with the *bcl-2* gene and showed a conversion of lytic SINV infection to persistent infection, examining the viability and production of viral particles in the supernatant, indicating the involvement of the mitochondria in SINV-induced apoptosis. Another study demonstrated that SFV infection of AT-3 cells induces apoptosis and that the overexpression of Bcl-2 led to cell survival and drastically reduced the percentage of infected cells. They suggested that the anti-apoptotic Bcl-2 function worked at an early stage of SFV infection, participating in the inhibition of virus replication, however the double positive cells (viral glycoproteins plus *bcl-2*) underwent apoptosis later. These results might signify that the restriction of viral replication and delaying cell death could efficiently infect AT-3 cells and produce new viral particles (Scallan, Allsopp, and Fazakerley 1997). Lundstrom and colleagues showed that the overexpression of *bcl-2* in rat RIN cells protected the SFV-infected cell from undergoing apoptosis until 72 hours post-infection (Lundstrom, Pralong, and Martinou 1997). Another study confirmed the synthesis of *bcl-2* during the early stages of SFV infection and that no apoptotic cells were detected at those time points. Moreover, *bcl-2* expression in BHK-21 and

AT-3 cells did not confer protection at later time points and the infection did not lead to cytochrome c release from the mitochondria. They concluded that cell death during SFV infection in BHK-21 and AT-3 cells was not triggered by the 'intrinsic' pathway of apoptosis (Kiiver, Merits, and Sarand 2008). Finally, more recently, Urban and colleagues demonstrated that SFV infection led to apoptosis that did not require TRAIL or TNF-mediated signaling, but mitochondrial Bak leading to cytochrome c release and the activation of caspase-9 and caspase-3 (Urban *et al.* 2008). Interestingly, they demonstrated that among BH3-only proteins, only Bid was associated with SFV-induced cell death, given that caspase-8 and Bid cleavage occurred downstream of Bak. They suggested that Bid cleavage served to amplify the induction of apoptosis during SFV infection.

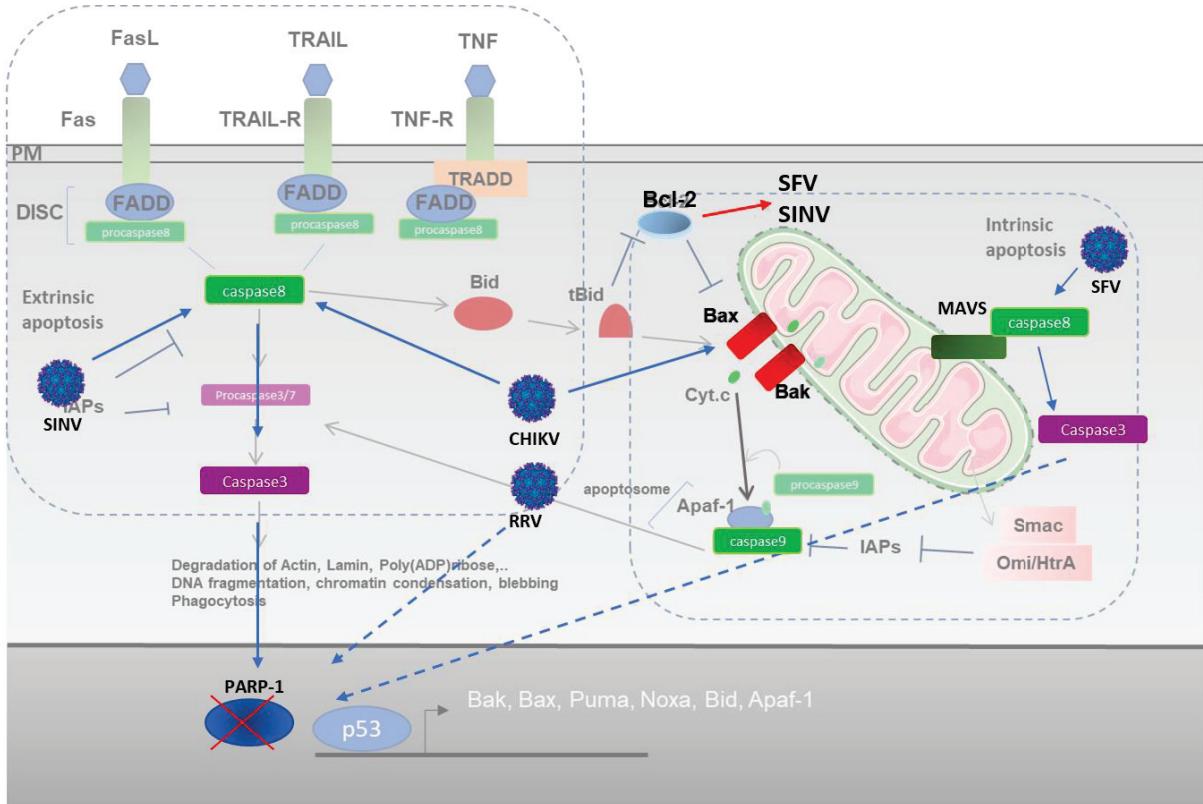
Taken together, these studies using SFV revealed that cells infected by SFV undergo apoptosis, however it remains controversial as to which signaling pathways lead to host cell death. We suggest that these differences in conclusions could come from the mammal cell lines and the viral molecular approaches used. Moreover, we propose that finally both intrinsic and extrinsic pathways are involved during SFV infection.

The *in vivo* mouse infection of VEEV is associated with cell death in the brain, demonstrated by the TUNEL assay (DNA fragmentation) and morphological changes (Jackson and Rossiter 1997). Finally, when mice were infected with SINV, the apoptotic cells were detected principally in the brain and contained viral antigens, suggesting that apoptosis was triggered in virus-infected cells (Lewis *et al.* 1996).

The role of caspases in alphaviruse-induced apoptosis has been investigated. Nava and colleagues showed in BHK cells infected by SINV, that the activation of caspases results in the cleavage of the intracellular target PARP (Poly (ADP)-ribose polymerase) at 24 hpi, which is involved in DNA repair. Then, using zVAD-FMK pan-caspase inhibitor, they showed a decrease in SINV-induced cell death in a dose-dependent manner. Using a more specific inhibitor, CrmA, which inhibits more specifically caspase-8 and caspase-1 (involved in IL-1 $\beta$  production) they obtained the same result, suggesting the involvement of caspase-1 or caspase-8 in SINV-induced apoptosis maybe through the activation of caspase-3 and cleavage of PARP (Nava *et al.* 1998). Another team showed that the cleavage of PARP-1 was detected at 20-24 hpi and the cleavage of caspase-3 at 16-20 hpi in mouse primary fibroblasts infected by SINV. These results

demonstrate that PARP-1 activity ceased immediately after caspase-3 activation, reinforcing the previous result (Nargi-Aizenman *et al.* 2002). Finally, the human neuroblastoma cell line SH-SY5Y is permissive to CHIKV (Solignat *et al.* 2009) and presents several apoptotic features starting between 24-36 hpi (Dhanwani *et al.* 2012): the detection of CHIKV glycoprotein E1 and cytochrome c translocation to cytoplasm at 16hpi followed by the detection of cleaved caspase-3 and cleaved-PARP at 24 and 36hpi. In parallel, a decrease of Bcl-2 from 36 to 48hpi was detected. The morphological changes and viability of CHIKV infected cells are associated with apoptotic protein activation with complete cell death between 36 and 48hpi. The infection is also accompanied by a significant alteration in mitochondrial membrane potential (MMP) and an elevation of Reactive oxygen species (ROS) evaluated by an increase of Glutathione (GSH) level at 36 and 48 hpi. GSH is a mitochondrial transmembrane protein with one part anchored in the mitochondria while the other is cytoplasmic. GSH is involved in mitochondrial cell death and, interestingly, it has been shown that the origin of the cytotoxicity and kinetics of GSH depletion and S-glutathionylation (formation of protein-GSH mixed disulfides) could determine whether cells undergo apoptosis or necrosis (Di Stefano *et al.* 2006). This result suggested that the involvement of ROS through an elevation of GSH could determine if a cell undergoes apoptosis or necrosis during CHIKV infection and maybe by other alphaviruses. The other cell death pathways such as necroptosis and pyroptosis will not be presented and discussed in this manuscript by choice, however some studies have shown their induction in the context of certain RNA virus infections and have raised many new and fascinating questions (Kaczmarek, Vandenabeele, and Krysko 2013).

To conclude this section, the infection of different cell lines with different alphaviruses seems to lead to the late induction of apoptosis involving the mitochondria earlier and the caspases of extrinsic and/or intrinsic apoptosis afterwards.



**Figure 13: Apoptosis induction during alphavirus infections in mammals.**

The dynamics of the mitochondria is considerably altered during apoptosis, and the infection of human astrocytoma cells U87MG by VEEV results in the loss of mitochondrial membrane potential at 6 hpi accompanied by an increase of Reactive Oxygen Species (ROS) inducing a cytopathicity impact of VEEV infection. In parallel, the infection of C6/36 mosquito cells reveals a less susceptible mitochondrial dysfunction at 6hpi. The viral capsid of VEEV in U87MG cells is localized at the mitochondria which starts to become fragmented and presents a perinuclear accumulation (Keck *et al.* 2017). Several virulent factors have been described as associated with the mitochondria and inducing apoptosis. For example, the NSs of the Rift Valley Fever virus (RVFV) was detected in the mitochondria of infected hepatic HepG2 cells, contributing to ROS accumulation, and the ROS levels were correlated with the activation of NF- $\kappa$ B and p53 responses. ROS accumulation contributes to innate immune response and apoptosis through the activation of the host signaling response such as NF- $\kappa$ B and AP-1. (Narayanan *et al.* 2014). In this study, NF- $\kappa$ B /p65 activation occurred earlier than p53 and an increase of the pro-apoptotic Noxa p53-target gene has been shown.

Interestingly, the mitochondrial induction of caspase-8 and caspase-3 activation was demonstrated in mouse embryonic fibroblasts (MEFs) and human HeLa cells infected with SFV. Previously, it has been shown that Bax/Bak were efficient for apoptosis induction during SFV infection, however cells lacking Bax/Bak continue to die in a caspase-dependent manner. It has also been demonstrated that caspase-8 with MAVS anchors directly on the mitochondria in a FADD-dependent manner, without the activation of TNF-associated proteins and or an effect on MOMP. In this context, the activation of caspase-8 and 3 was very fast (detectable at 8 hpi). Moreover, caspase-8 and MAVS-deficient MEFs cells delayed cell death but were not protected from caspase-3 activation, suggesting another SFV-induced caspase-3 activation. It was only when Bax/Bak were knocked out that the depletion of caspase-8 and MAVs induced a protective effect against SFV-induced cell death (El Maadidi *et al.* 2014). Thus, these results showed that the mitochondrion was involved in SFV-induced cell death and the absence of MOMP could be explained by the timing of the analysis.

Macrophages and monocytes are believed to play an important role in the pathogenesis of alphavirus-mediated arthritis. Krejbich-Trotot and colleagues evaluated the permissiveness and cellular response of a human monocyte acute leukemia MM6 cell line infected by Ross River virus (RRV). After the infection of MM6 cells with MOI 1, growth kinetics was detected every day until 7 days of infection and the virus titer increased slightly between 1 to 3 dpi with a maximum of  $10^3$  PFU/ml (compared to  $10^8$ PFU/ml in VeroE6 or HEK-293 cell lines) and decreased to 100 PFU/ml after 3dpi. Surprisingly, the very low replication and production rate of RRV was not associated with the control of viral infection by Type-I interferon production and cytokine expression. At day 5, MM6 infected cells underwent apoptosis with Bax mitochondrial localization and cleavage of PARP. Moreover, the few cells rescued from apoptosis presented a viral genome analyzed by RT-qPCR for up to 45 days (Krejbich-Trotot *et al.* 2016). These results indicated that RRV can persistently infect monocytes. Moreover, the very late apoptosis induction suggests that in the absence of apoptosis the cells were still infected without inflammatory response. This cellular condition could participate in the chronic form of RRV and maybe other arthritogenic-*alphaviruses*. For CHIKV, it is still poorly understood what promotes the persistent infection of CHIKV in muscles. Recently, it has been shown that CHIKV infection of mouse muscle induced arthritis during the chronic phase and presented a

weak level of viral RNA in myofibers, dermal and muscle fibroblasts for at least 16 weeks after infection. However, after 7 dpi, viral RNA drastically decreased and the subgenomic promoter was active only during the first week (acute and post-acute phases). The chronic phase presented several characteristics of arthritis and these results seem to indicate that genomic RNA coding for non-structural proteins participates in the induction of the chronic phase. However, the signals that switch off non-structural protein translation on the genomic RNA are still unknown. During the chronic phase it has been demonstrated that the cellular populations positive to CHIKV detection are the skin fibroblasts, skeletal muscle cells and synovial tissues (Young *et al.* 2019). These results indicate that a subset of infected cells survive CHIKV infection, i.e. they were not killed or did not succumb to lytic infection. However, the mechanism that protects these infected cells from cell death, and how they can become chronically infected, is still unknown. The non-translation of subgenomic RNA coding for nucleocapsid and structural proteins could participate in the persistence of viral RNA and subsequently in chronicity. To conclude, we propose that the non-structural proteins of CHIKV are involved in the control of cell death during the chronic phase.

In addition, it has been shown that CHIKV RNA were found adjacent to the periosteum (membrane that covers the outer surface of bones) and it has been suggested that fibroblasts and osteoclasts could be the positive population of CHIKV RNA. As has been suggested, the infection of osteoclasts can perturbate the function of these cells which could also explain the histological damage observed. However, it is still unknown what promotes these symptoms during the chronic phase. Whereas chronic inflammation has been clearly identified in this tissue, viral replication has not been detected, but the presence of CHIKV RNA in *in vivo* mice has been demonstrated. Additional studies on apoptotic features in these survival populations of cells could contribute to better understanding of the mechanism in long-term pathogenesis and could contribute to the development of treatments. However, it seems that using only an *in vitro* system will not be sufficient and rodent and non-human primate (NHPs) models are necessary to consider the complex environment of joints and muscles. The study of CHIKV pathogenesis in NHP hosts could be very interesting (Broeckel *et al.* 2015; Higgs and Ziegler 2010).

The induction of apoptosis seems to occur principally at the beginning of infection in *in vitro* systems. However, the first cycle of alphavirus replication occurs around six hours after entry,

indicating that apoptosis is not induced between 6 and 20 hours of infection. This observation could indicate that apoptosis must be inhibited or delayed in different ways to allow viral replication and escape from antiviral response. Several associated pathways can delay and crosstalk with apoptosis, like autophagy, cell death inducers (RE stress, oxidative stress) and the NF- $\kappa$ B signaling pathway.

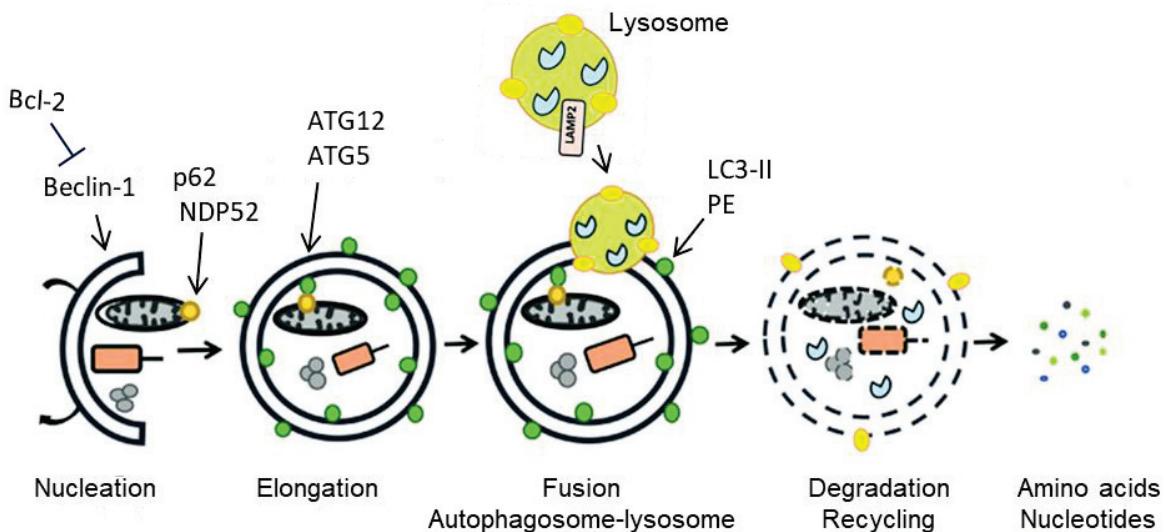
Moreover, it seems important to discuss the activity of nsP2 presented previously, regarding the general transcriptional shut-off of infected cells from 6-8 hours post infection. Indeed, it has been shown that the transfection of CHIKV nsP2 in VeroE6 led to significant cytopathic effects (CPE) and the mutation of nsP2 NLS abrogated the inhibition of the antiviral interferon JAK-STAT pathway, whereas combined mutation in NLS and a conserved proline P718S allows a non-cytopathic RNA replicon (Fros *et al.* 2013). The nsP2 of alphaviruses presents multiple activities involved in RNA replication, the inhibition of innate immune response through the JAK-STAT signaling pathway, and it participates to a large extent in the cytopathic effects.

In conclusion, *Alphavirus* infection induces extrinsic and intrinsic apoptotic cell death. Moreover, viral proteins may control apoptosis and connect signaling pathways such as endoplasmic reticulum stress, the transcription pathway, and oxidative stress in order to produce new virions. Lastly, the control of cell death and immune response could influence the mechanism involved in alphaviral persistence.

### **3.2.1.3 Apoptosis-autophagy balance and crosstalk with other pathways**

Joubert and colleagues have shown a cross talk between apoptosis and autophagy pathways where autophagy can play a pro- or anti-viral role during CHIKV infection.

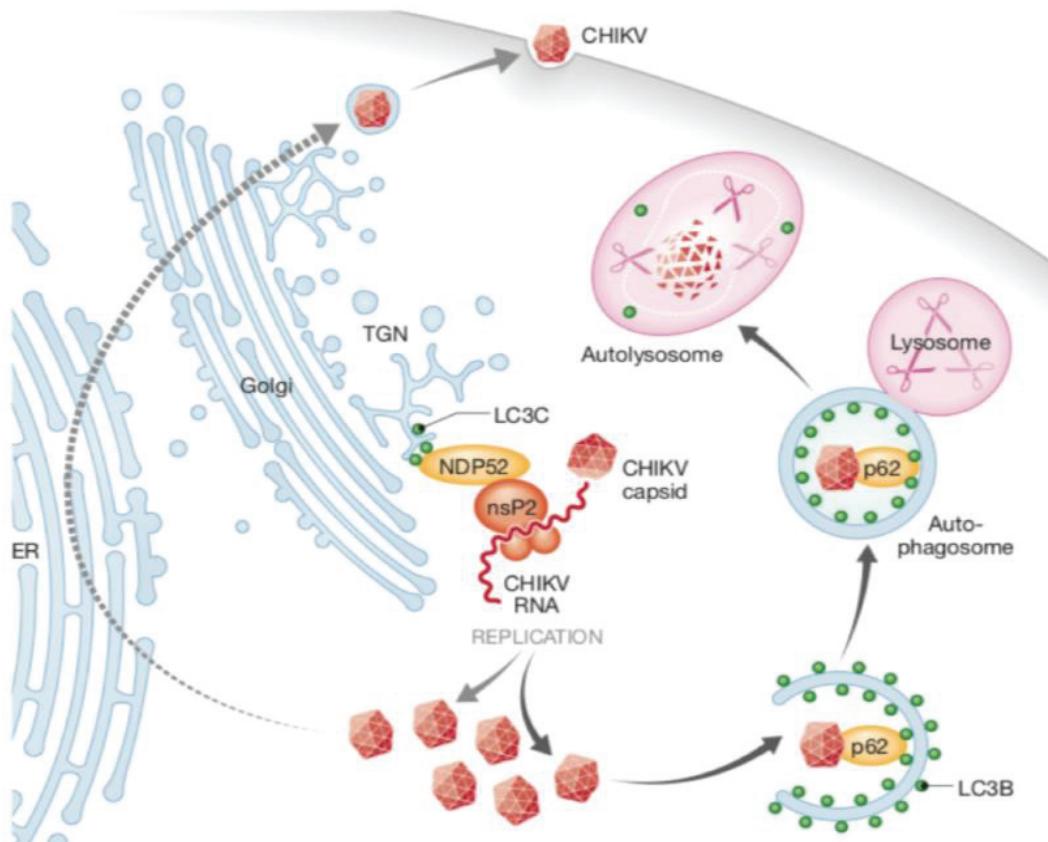
Briefly, autophagy is a degradative system that controls the clearance and recycling of cellular constituents. It is initiated by the formation of a double-membrane (phagophore) that requires elongation and maturation in vesicles called autophagosome. The autophagosomes sequester cytoplasmic constituents and organelles, which are degraded by hydrolytic enzymes after fusion between autophagosomes and lysosomes (forming the autophagolysosome). Figure 14 presents an overview of the autophagy pathway and proteins involved in the mechanism.



**Figure 14: An overview of the autophagy pathway in mammals.** Autophagy (also called macroautophagy) is initiated by the recognition of cargo molecules in the cell thanks to target-specific autophagy receptors such as p62 and NDP52 with PE LC3-II, leading to the formation of membranes from the Endoplasmic reticulum which involves Beclin-1. The formation, elongation, and maturation of the autophagosome require several Atg proteins (Atg5, Atg12, etc.). Following closure, the autophagosome is transported by the microtubule, leading to its fusion with lysosomes, forming the autolysosome. Fusion in mammal cells requires LAMP-2 and Rab proteins such as Rab5 and Rab7 (not presented in the figure). Next, the acidification of the autolysosome allows the action of hydrolytic enzymes, the hydrolases. The sequestered materials are degraded into amino acids or fatty acids and transported back to the cytoplasm for reuse in cellular metabolic processes. PE: phosphatidylethanolamine; LAMP-2:Lysosomal-associated membrane glycoprotein 2 (Adapted from Kocaturk and Gozuacik 2018)

The induction of autophagy has been demonstrated during several alphavirus infections such as SINV, SFV and CHIKV. During the CHIKV infection of the HEK293 cell line, which stably expressed LC3-GFP, an accumulation of GFP-LC3-positive puncta from 4 to 48 hpi was shown. The induction of autophagic flux by Rapamycin pretreatment increased the number of infected cells and viral RNA in the supernatant of cells. However, the inhibition of autophagic flux by treatment with 3-MA or by transfection of siRNA beclin-1 led to a significant decrease of CHIKV RNA production in the supernatant and number of infected cells (Krejbič-Trotot, Gay, *et al.* 2011). These results indicate that autophagy is induced early in viral infection (4hpi) and could be required for effective CHIK replication. Another study, following the CHIKV infection of HeLa cells showed an increase in LC3-II expression and a decrease of p62 receptors from 15 to 24hpi, indicating an enhancement of autophagic flux. In parallel, the depletion of beclin-1, Atg6 and 7 and p62 is associated with an increase of virus-induced cell death. Moreover, in CHIKV infected HeLa and primary human labial fibroblasts (HLFs) it was shown that p62 colocalized with

ubiquitinated viral capsid and LAMP1 (receptors of lysosomes) and resulted in an autophagy-mediated antiviral response, whereas CHIKV nsP2 can interact with NDP52 next to the replication complex and could promote viral replication (Judith et al. 2013), (Figure 15).



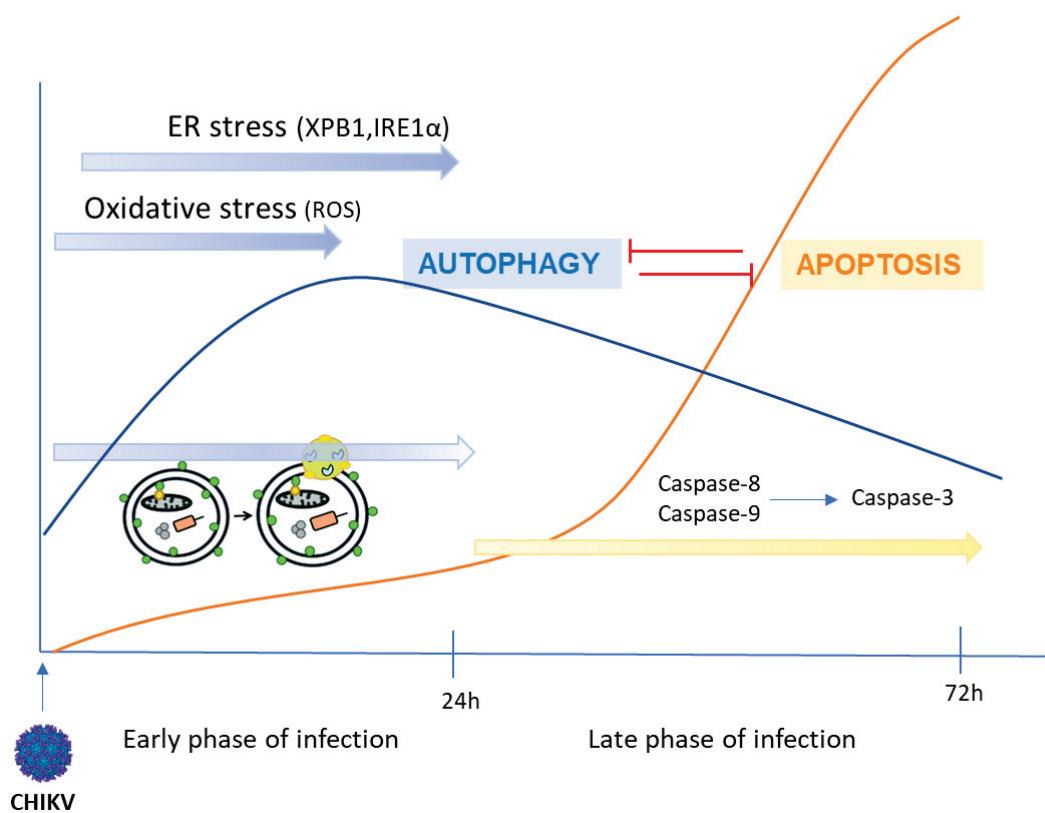
**Figure 15: Dual effect of p62 and NDP52 on CHIKV capsid and nsP2 during autophagic flux.** NDP52 can interact with nsP2 and participate in viral replication while p62 interacts with ubiquitinated capsid, allowing its degradation by autophagic flux. LC3B, LC3C: light chain protein 3B and 3C; ER: Endoplasmic Reticulum; TGN: Trans Golgi network; NDP52: nuclear dot protein 52 (Münz 2013).

Additional results following SINV infection in mice have shown that the inhibition of neuronal Atg5 resulted in increased mortality. It has been suggested that p62 can interact with the viral capsid, promoting the clearance of the latter through the autophagy pathway, which could be important for the survival of infected cells (Orvedahl *et al.* 2010).

There is evidence that points either to an anti-viral or a pro-viral role of autophagy during alphavirus infection. Therefore, apoptosis and autophagy are two crosstalk pathways that also communicate with other signaling pathways. The hypothesis that apoptosis could be delayed at the early stage of CHIKV infection by anti-apoptotic pathways has been put forward by some teams. Abraham and colleagues investigated the crosstalk of pathways in U-87 MG cells

infected by CHIKV. From 48 to 96hpi, they showed apoptotic induction by the loss of MMP, PARP-1 cleavage and DNA fragmentation in dose-dependent CHIKV. Before apoptosis, at 24hpi autophagy was triggered and earlier, at 12hpi, the activation of ER stress was identified by the detection of unconventional splicing of the X-box binding protein-1 (XBP1). During ER stress, the global shut-off of the cell can be regulated by the phosphorylation level of initiation factor eIF2 $\alpha$  from 24 to 72hpi. The eukaryotic initiation factor eIF2 $\alpha$  can in turn regulate mRNA translation and it has been shown that the activation of eIF2 $\alpha$  increases at 72 hpi. (Abraham *et al.* 2013). To conclude, autophagy and ER stress occurring during the CHIKV infection of U-87 MG cells could delay apoptosis and subsequently induce efficient viral replication.

Joubert *et al.* also showed that during CHIKV infection of human and mouse cells and *in vivo* mouse, autophagy was triggered by ER and oxidative stress, delaying the induction of apoptosis. ER stress was regulated by the phosphorylation of Inositol-requiring transmembrane endoribonuclease 1  $\alpha$  (IRE1 $\alpha$ ). IRE1 $\alpha$  is present in the ER membrane and transduces the signal of the unfolded protein response (UPR). The UPR response is involved in CHIKV infection and can be disabled by nsP2 mediated-shut off (Fros *et al.* 2015; Khongwichit *et al.* 2016). IRE1 $\alpha$  was activated by phosphorylation (p-IRE1 $\alpha$ ), and during CHIKV infection p-IRE1 $\alpha$  was detected during the early phase of infection with an increase between 4 to 24hpi. The kinetics of IRE1 $\alpha$  phosphorylation correlated with the induction of LC3-II. Finally, the production of ROS during CHIKV seemed to participate in CHIKV-induced autophagy and both ER and ROS could be essential for the induction of autophagy. Finally, in CHIKV infected-human HFF *Atg5* $^{-/-}$  the percentage of cleaved-caspase-3 increased, whereas in MEFs *bax* $^{-/-}$ , *bak* $^{-/-}$  the percentage of cleaved caspase-3 decreased drastically 24 hours after infection. These results confirm first, that extrinsic and intrinsic apoptosis are activated and, second, demonstrate the antiapoptotic function of autophagy during CHIKV infection (P. E. Joubert *et al.* 2012), (Figure 16).



**Figure 16: Schematic representation of apoptosis and autophagy crosstalk during CHIKV infection in mammals (Adapted from Joubert *et al.* 2012).**

Another interesting analysis showed a pro-viral effect of apoptosis influencing CHIKV infection of through the detection of viral particles in apoptotic bodies. Indeed, CHIKV infection of HeLa cells and primary fibroblasts led to the detection of viral particles in apoptotic blebs during apoptosis and demonstrated the ability of infected apoptotic bodies to infect neighboring non-infected cells through the phagocytosis of apoptotic bodies containing CHIKV particles. More interestingly, the apoptotic blebs containing CHIKV were capable of infecting and replicating in macrophages, previously shown to be refractory to infection, without inducing a pro-inflammatory response (Krejbich-Trotot, Denizot, *et al.* 2011).

Finally, another strategy has been developed by several different enveloped viruses employing apoptosis features. At the late stage of apoptosis, the plasma membrane of apoptotic cells exposes a marker recognized by macrophages for the engulfment of apoptotic blebs, named phosphatidylserine (PS). The PS is recognized by PS receptors belonging to the TIM1, TIM4 and

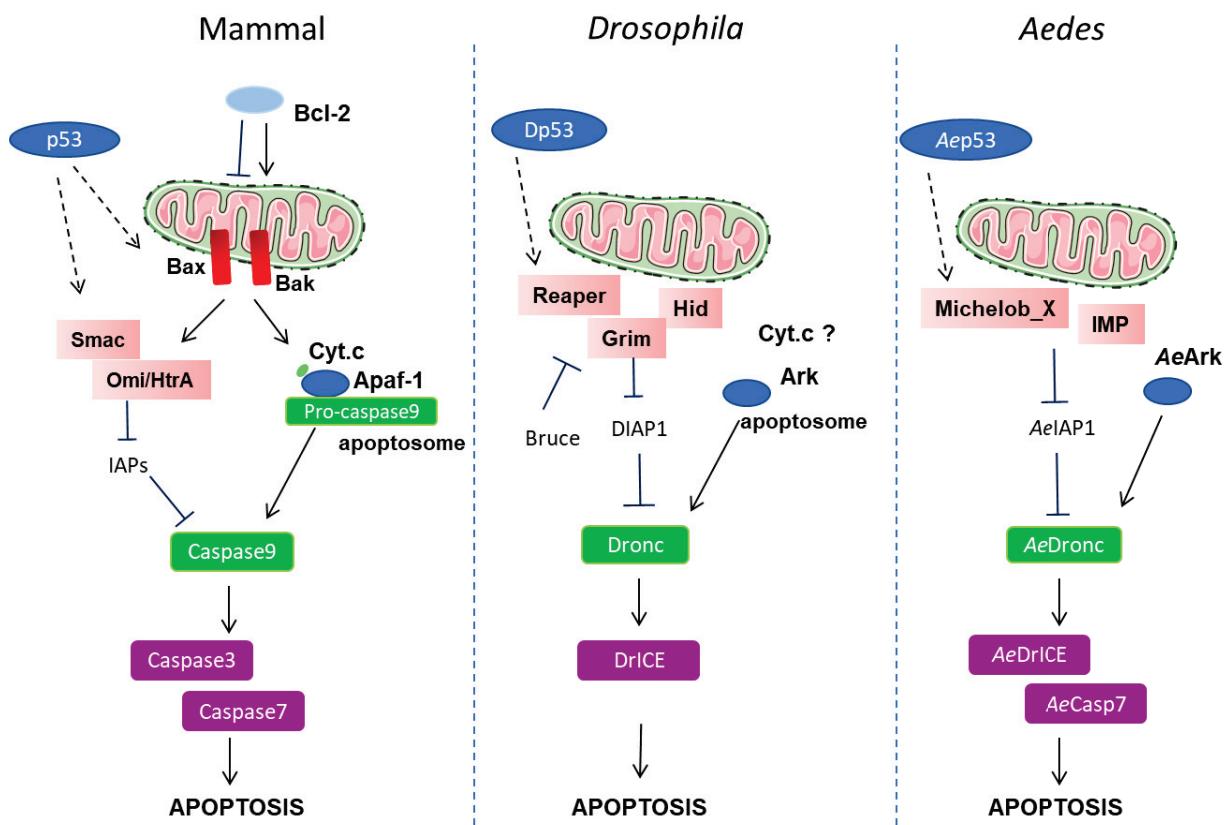
TAM (Axl, Tyro3 and Mer) families. In viable cells, the PS are retained on the cytoplasmic side by flippases, and during apoptosis the distribution of phospholipids is disrupted, resulting in the externalization of PS guided by scramblases (Hankins *et al.* 2015). The budding of new viral particles at the plasma membrane can lead to the passive distribution of PS, in the absence of flippases, between the inner and outer part of the membrane. Consequently, the new virions present PS at the outer leaflet of their membranes, leading to the binding to nonspecific receptors and uptake of the virus, for example, by macropinocytosis, allowing the infection to hijack the innate immune system. This mechanism has been named “apoptotic mimicry” and was discovered for different virus genera such as *alphaviruses* (EEEV, RRV, CHIKV and SINV), *flaviviruses* (DENV, WNV, Yellow fever virus YFV), *polyomavirus* SV40, *vesiculovirus* VSV, *ebolavirus* EBOV and *Marburgvirus* MARV (Amara and Mercer 2015).

### **3.2.2 In insects**

#### **3.2.2.1 The apoptotic pathway in *Drosophila* and *Aedes***

Apoptosis in insects has been well characterized and described thanks to *in vivo* genetic manipulation of the fly *Drosophila melanogaster*. As in vertebrates, cell death in invertebrates plays important roles during the development of the embryo and in the adult, in the homeostasis of tissues and during pathogen infections (Mollereau 2009). Apoptosis in *Drosophila* is also controlled by initiator and effector caspases that are expressed ubiquitously and synthesized as inactive procaspases negatively regulated by members of the *Drosophila* IAP (DIAP) family (Hay and Guo 2006). Based on sequence homology with *Drosophila* genes involved in apoptosis, numerous genes presumed to regulate apoptosis have been identified in other insects, like the mosquito *Aedes* (Bryant *et al.* 2008; Liu and Clem 2011). In *Drosophila*, the expression of the DIAP antagonist reaper, hid and grim (RHG) induces the inhibition of DIAP1 and subsequently caspase-9 orthologue Dronc activation (Figure 17). It has been shown that the inhibition of DIAP1 is sufficient to trigger apoptosis in fly cells compared to mammal cells (Vasudevan and Ryoo 2015). In *Aedes* homologues of IAP-antagonists have been characterized named Michelob\_x and IMP which induce the activation of AeDronc (Wang and Clem 2011). The activation of initiator caspases leads to the activation of effector DrICE in *Drosophila* and

*AeDrICE* or *Aecasp7* in the mosquito (Feng *et al.* 2016) (Figure 17). Controversy exists regarding the role of the *Drosophila* mitochondria in apoptosis. On the one hand, Ark apoptosome formation is required for the induction of apoptosis, without cytochrome c (Zimmermann *et al.* 2002). On the other hand, it has been shown that caspase activation occurs at the mitochondrial level and includes RHG proteins and proteins of the Bcl-2 family (Clavier *et al.* 2016).



**Figure 17: Comparison between the apoptotic pathway in *Drosophila*, *Aedes* and mammals.** In insects the adaptor Ark (in *Drosophila*) and in AeArk (in *Aedes*), which are homologues of Apaf-1 in mammals, promote the activation of initiators caspase Dronc and AaDronc (homologue of caspase-9). The inhibitor DIAP1 in *Drosophila* and AelAP1 can negatively regulate the activity of the initiator caspase and subsequently the effector caspases DrICE and AeDrICE or Aecasp7. Several antagonists of IAP in *Drosophila* have been described, Reaper, Hid and Grim and two in *Aedes*, called Michelob\_X and IMP, and can be p53 target genes.

Few of the many different mosquito species vectors. However, for a given vector species, all females can potentially be chronically infected and transmit the virus during a new bloodmeal. There is a very high degree of vector-virus specificity for transmission. The role and process of the immune system in the modulation of infection through different signaling pathways as a

function of the vector-virus has been described previously. Apoptosis is another known efficient antiviral response in vertebrates and invertebrates. However, it seems that apoptosis is not always antiviral and can be used by some viruses using caspase cleavage for their own viral replication (Richard and Tulasne 2012) or for dissemination using apoptotic bodies.

### 3.2.2.2 The effect of apoptosis on arbovirus outcome

In this part we present the significance of apoptosis during the arbovirus infection of permissive and resistant mosquitoes and the opposite conclusion about the role of apoptosis in determining vector competence.

Regarding the lack of tools for studying apoptosis in the mosquito, two different approaches using SINV/*Ae.aegypti* have been developed through the sequencing and annotation of the *Aedes aegypti* genome and the well conserved protein function between *Drosophila* and *Aedes* (Bryant *et al.* 2008).

#### - Effect of apoptosis inhibition or induction on virus outcome

The first approach used RNAi to inhibit or induce apoptosis during SINV infection and the second one by the engineering expression of pro-apoptotic genes in the SINV genome.

In the former approach, Wang and colleagues showed that silencing *Aeiap1* by the injection of dsRNA in *Ae. aegypti* increased the mortality of mosquitoes and increased the apoptotic level detected by caspase activity. The activity of caspase was higher in the midgut of *Aeiap1* dsRNA than in the rest of the mosquito (carcass). The mortality rate can be rescued by silencing *Aedronc*. Then, they analyzed the impact of inducing or inhibiting apoptosis on the SINV-GFP escape barrier. They showed a higher GFP intensity in the midgut of *Aeiap1* dsRNA mosquitoes than in control and a lower GFP intensity in the midgut of *Aedronc* dsRNA mosquitoes, suggesting that the activation of caspase increased viral replication in the midgut while the inhibition of caspase activation decreased SINV replication. Moreover, *Aedronc* infected-mosquitos presented a lower rate of SINV dissemination in their salivary glands, suggesting that the inhibition of apoptosis by silencing caspase *Dronc* decreased dissemination from the midgut

(Wang et al. 2012). It seems that a caspase activity is required for the dissemination of SINV from the midgut to the secondary organs and could participate in the remodeling of the basal lamina. It was previously demonstrated that caspase activity in a baculovirus-infected lepidopteran host induced the remodeling of the basal lamina and facilitated dissemination across the midgut barrier (Means and Passarelli 2010). These first studies seem to indicate that apoptosis does not present an antiviral effect on SINV. However, the injection of dsRNA in whole mosquito affects the apoptosis pathway in a nonspecific manner (infected or not by SINV), and it could be a bias in a context of arbovirus infection.

Another study, during the DENV infection of permissive and refractory (Midgut Infection Barrier MIB profile, *cf.* Figure 7) *Aedes aegypti*, showed an increase of initiator *Aedronc* and *Aedredd* (orthologue caspase-8) and effector *Aecaspase16* expression at 24 and 36 hours post-infection in the midguts of DENV-MIB refractory mosquitoes compared to DENV-infected permissive mosquitoes. A drastic decrease of caspase expression was detected after 48 hours in DENV-MIB. The authors then analyzed the effects of RNAi knockdown of *Aedronc* and *Aecaspase16* on DENV development in DENV-MIB refractory mosquitoes. The knockdown of initiator *Aedronc* but not *Aecaspase16* increased the development of DENV in infected MIB-mosquitoes at day 13 (Ocampo et al. 2013). Taken together, it seems that Dronc activity is important in SINV and DENV infection and could be involved in midgut infection and/or the midgut escape barrier.

It seems be more relevant to analyze the role of apoptosis only in infected cells, and moreover it was previously reported that only a small number of epithelial cells were infected by arbovirus, suggesting that the manipulation of whole mosquitoes in a non- tissue-specific manner could induce a misleading interpretation.

In the second approach, the engineering of pro-apoptotic genes directly in the SINV genome is a solution to analyze the role of apoptosis only in infected cells (O'Neill et al. 2015). In this study, they cloned the pro-apoptotic protein Reaper (DIAP antagonist) from *Drosophila* (SINV/reaper) in the SINV genome to examine the ability of SINV to infect *Aedes aegypti* *in vivo* after the induction of apoptosis. The induction of apoptosis in the midgut of infected mosquitoes has been detected by the TUNEL assay and effector caspase-7 orthologue activity. At 3 days post-bloodmeal infection, the area of infection in the SINV/reaper mosquito was lower than in infected control and at 5 and 7 dpi, the area of infection in the midgut was not

different between the SINV/reaper and SINV control mosquitoes. The same results were obtained by achieving viral replication and dissemination in the carcass of infected mosquitoes. These results suggested that the expression of reaper in infected cells delays viral replication at the initial stage of infection but does not avoid the dissemination from the midgut. The analysis of the amount of virus in the saliva of infected mosquitoes also showed a delay in SINV replication at 10 dpi but no differences at 14dpi. Finally, the SINV/reaper infected mosquitoes presented a lower survival rate than SINV infected control mosquitoes. Moreover, nucleotide sequencing of SINV/reaper infected mosquitoes showed rapid and strong selection against the maintenance of Reaper expression.

In addition to these two approaches, another study was conducted, based on the expression of the *Culicoides* Inhibitor Apoptosis Protein (*CsIAP*) from *C. sonorensis* cells in mammalian cells before Bluetongue BTV virus infection. The stable expression of *CsIAP* in the BSR mammal cell line suppressed apoptosis induced by BTV infection and the same result was obtained after infection with African horse sickness virus AHSV (another arbovirus of the genus *Orbivirius*). They indicated that *CsIAP* was an inhibitor of mammalian caspase-9 and then suggested that the suppression of apoptosis in insect cells could involve a caspase-9 homologue (Vermaak, Maree, and Theron 2017). Previously, another study showed that the expression of *Aedes albopictus* IAP1 in BTV-infected BHK cells delayed BTV-induced apoptosis for 24 hours and decreased virus production (Li *et al.* 2007). These results suggested that arbovirus-induced mammal cell death can be controlled and inhibited, participating in the persistence of the virus in mammal cells. Moreover, we wonder whether the virus could block IAPs in the insect vector and in the mammal host. Interestingly, African Swine Fever virus (ASFV) was revealed to encode a homologue of IAP that promotes cell survival and is able to activate the transcription of NF- $\kappa$ B in human Jurkat cells, allowing the modulation of immune response; moreover, NF- $\kappa$ B can inhibit apoptosis by the transcription of antiapoptotic genes such as IAP or Bcl-2 (Rodríguez *et al.* 2002). These results suggested an important role of IAPs in the host and vector in controlling cell fate and it could be interesting to overexpress mammalian IAPs in cells infected by arbovirus, inducing the lytic cycle, to see if mammalian IAP is sufficient to block apoptosis through initiator caspases.

Taken together, these results suggest that the induction of apoptosis during infection plays an anti-viral defense role and that arbovirus could have evolved mechanisms to avoid the induction of apoptosis in natural vectors.

- *Rapid and long-term effect of cell death in determining vector competence*

In a permissive (susceptible) infected mosquito, the cellular response in the initial stage of infection in the midgut seems important and even decisive for vector competence. Vaidyanathan and Scott examined the midgut epithelial cells of laboratory refractory *Culex pipiens pipiens* infected by WNV at 3 days post-feeding (Vaidyanathan and Scott 2006). Transmission electron microscopy (TEM) revealed cellular changes consistent with apoptosis such as chromatin condensation, the detachment of cells and the engulfment of apoptotic bodies by neighboring cells. Moreover, the cytoplasm and vesicles in the apoptotic cells were positive for virion detection. To analyze the dissemination of WNV in the mosquito, the viral titer of WNV fed *Culex* was detected at 7- and 10-days post feeding, and no mosquito was detected positive (Vaidyanathan and Scott 2006). Unfortunately, the midgut epithelial cells of susceptible strains were not examined. These results suggest that apoptosis is associated with WNV infection and the midgut infection barrier but did not allow concluding that apoptosis contributes to resistance.

The infection of susceptible and less susceptible *Culex tarsalis* strains with Western Equine Encephalomyelitis virus (WEEV) showed that the infection of susceptible strains causes pathology in the midgut epithelium between 2- and 4-days post feeding detected by sloughed cells in the lumen. Moreover, *in situ*, necrotic cell death was detected in the more susceptible strain and not in the less susceptible and control strains (Weaver, Lorenz, and Scott 1992). Another study reported cytopathologic lesions in epithelial cells of the midgut of mosquito *Culiseta* orally infected with Eastern Equine Encephalomyelitis virus (EEEV) at 3 days post feeding. The degeneration of midgut epithelial cells was accompanied by the disruption of basal lamina. They suggested that EEEV virus-associated pathologic changes promote dissemination to secondary organs (Weaver *et al.* 1988).

More recently, a midgut transcriptome analysis of *Aedes aegypti* fed with CHIKV has shown increased activity of matrix metalloproteinase (MMP) associated with a decrease of the basal lamina component collagen IV between 20-48 hours post infection (Dong, Behura, and Franz

2017). The remodeling of the basal lamina could participate in the midgut escape barrier. However, the MMP could have a proapoptotic effect (Tamura *et al.* 2004) and activate the effector caspase (Means and Passarelli 2010), thus it is tempting to combine the apoptotic effect and/or caspase activity on MMP in midgut barrier escape.

The detection of arbovirus in salivary glands in laboratory conditions occurred between 10- and 14-days post feeding. Structural pathology in the salivary glands of *Ae albopictus* intrathoracically inoculated with SINV at 10 days post-infection was reported (Bowers, Coleman, and Brown 2003) and apoptotic TUNEL positive cells in the lateral lobes of the salivary gland where SINV were detected but not in the median lobe (Kelly, Moon, and Bowers 2012). It has been shown that SINV can infect the lateral lobe but does not seem to infect the median lobe of *Ae. aegypti* and *Ae. albopictus* (Bowers *et al.* 1995; Gaidamovich *et al.* 1973). The dissemination of WNV to the salivary glands of *Culex pipiens quinquefasciatus* was revealed at 14 days post infection and the WNV-induced pathology detected at 14, 21 and up to 25 days post infection (Girard *et al.* 2006). In another study, they showed that the long-term infection of salivary glands was still associated with apoptosis and decreased virus release into saliva. They hypothesized that salivary gland pathology induced by WNV could affect the feeding behavior of mosquitoes and subsequently transmission (Girard *et al.* 2007).

To conclude, it seems that the cell death could be involved during the infection of the insect vector and could have a pro- and/or an anti- viral effect, maybe depending on the timing and the organ infected. As it is known that arboviruses cause persistent infection with no cytopathic effects in the insect vector, the pathology of the mosquito for instance, may not have been sufficiently investigated. Studies presenting the permissiveness and resistance of vectors have shown the increasing complexity of virus-insect interactions.

#### - *Cellular factors involved in cell death regulation*

Quite recently, using an *in vivo* *Drosophila* model, a team identified that the injection of *Drosophila* with baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and Flock House virus (FHV) rapidly induced (1 hour post injection) the upregulation of *hid* and *reaper* in the gut and fat body. The infection of the *Drosophila* DL-1 cell line showed the same results at 24 and 36 hpi. Because *reaper* and *hid* are p53-target genes, they injected

viruses in *Drosophila* p53-/- mutants and first observed a decrease of the target genes *reaper* and *hid* and indirect apoptotic features with the activation of caspase Dronc. The inhibition of apoptosis has been associated with an increase of viral infection and the necrotic evolution of infected cells at 4-7 days post injection. They suggested that a rapid induction of apoptosis p53-dependent as an innate immune response could be associated with the susceptibility and resistance of arbovirus infections in their vector. To investigate this hypothesis, they infected susceptible and resistant laboratory strains of *Aedes aegypti* with DENV and identified an increase of *Michelob\_mx* mRNA at 3 and 18 hours post blood meal in resistant mosquito but not in susceptible mosquitoes (Liu *et al.* 2013). However, the signal responsible for p53 activation during arbovirus infection was not elucidated.

Finally, alphaviruses cause significant cytopathic effects in vertebrate cells. The hypothesis that host transcriptional shut-off is linked to the cytopathicity and/or cell death of alphavirus infections is still misunderstood. Interestingly, in the mosquito, alphavirus infection is not associated with RNA polymerase II subunit RPB1 homologue degradation. It has been shown that SINV infection does not induce the degradation of the RPB1 homologue in mosquito cells, suggesting that viral infection does not induce transcriptional shut-off as in vertebrate cells (I. Akhrymuk *et al.* 2012).

- *Oxidative stress response*

During arbovirus infection, oxidative stress was detected in both mammalian and insect cells. In insects, oxidative stress is defined as the loss of homeostasis between the accumulation of reactive oxygen species (ROS) and the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) or glutathione transferase and reductase (Felton and Summers 1995). During blood feeding, the midgut is in contact with sugar, iron, heme and other components of vertebrate blood and mosquitoes have evolved a protective adaptation against damage caused by heme and iron uptake. Indeed, heme can induce lipid peroxidation, protein degradation and ultimately cell death. Once in the epithelial cells, these components are detoxified, and a strong antioxidant and protective response is engaged (Whiten, Eggleston, and Adelman 2018). Concomitantly, pathogens present in the blood could take advantage of this antioxidant response, blocking cell death, to infect and replicate into midgut epithelial cells. To test the hypothesis that arbovirus infection and subsequently vector competence could be

influenced by the antioxidant response of blood feeding, Henrique and colleagues performed catalase knockdown in *Aedes aegypti* and infected them with DENV and ZIKV. The RNAi-mediated knockdown of catalase challenged with H<sub>2</sub>O<sub>2</sub> resulted in the reduced lifespan of mosquitoes and oviposition. Then, the incapacity of ROS degradation after H<sub>2</sub>O<sub>2</sub> treatment reduced the ability of DENV to infect the midgut of *Ae. aegypti* but curiously not that of Zika virus (Henrique *et al.* 2017). The same result was obtained in *Anopheles gambiae* infected with the parasite *Plasmodium falciparum*: by reducing mitochondrial ROS generation in the midgut, they observed an increase in mosquito susceptibility to the malaria parasite (Gonçalves *et al.* 2012). A proteomic approach was used to characterize several protein modifications in the midgut of *Aedes aegypti* infected by DENV and CHIKV seven days after oral infection (Tchankouo-Nguetcheu *et al.* 2010). The CHIKV infection induced an increase in the protein expression involved in detoxification, and both viruses increased proteins involved in antioxidant response but not the same. Taken together, these results suggest that the oxidative response first induces a protective response in favor of virus infection and, second, the responses in the midgut of the mosquito is not similar in virus/mosquito combinations.

Interestingly, oxidative stress in mammals can lead to the post-translational modification of p53, leading to the regulation of genes involved either in survival or in cell death (Liu, Chen, and St. Clair 2008).

- Apoptosis-Autophagy crosstalk during arbovirus infection of the mosquito

The well conserved autophagic flux between vertebrates and invertebrates allows analyzing insect autophagy-arbovirus interactions for the first time in a useful *Drosophila* organism. The first studies identified the ortholog adapter protein p62 in the insect called *ref(2)p* and showed that the *ref(2)p* null allele mutant renders sigma virus (which is not an arbovirus, family: *Rhabdoviridae*) infection in *Drosophila* permissive, suggesting an anti-viral response (Contamine, Petitjean, and Ashburner 1989). While *Drosophila* is neither hematophagous nor a natural vector of arboviruses, it provides very useful information thanks to easier genetical manipulation compared to *Aedes aegypti* and *Aedes albopictus*.

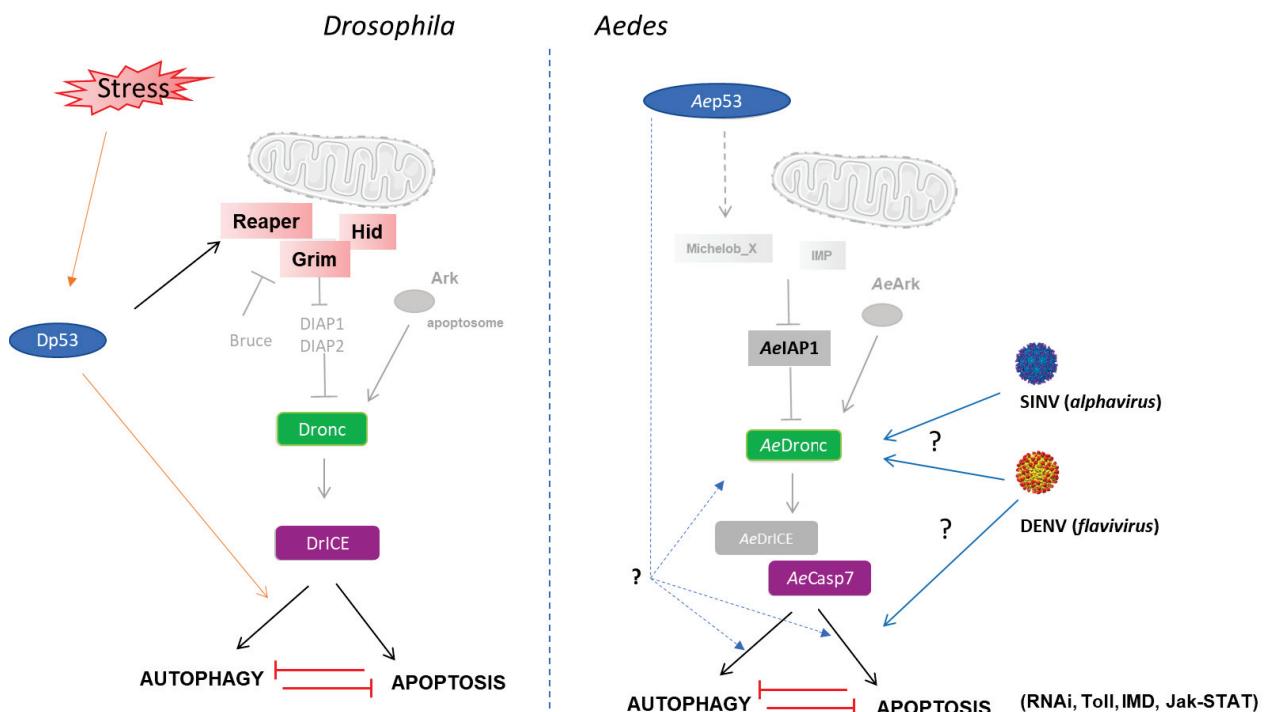
The *in vivo* genetic model *Drosophila* has been used as an insect model for SINV analysis. It has been shown that the over-expression or inhibition of the Akt-mTOR pathway (involved in the negative regulation of autophagy) in flies, increases or decreases SINV replication, respectively. In parallel, SINV infection of *Drosophila* cells led to the phosphorylation of Akt and its activation.

Moreover, using an *Aedes albopictus* mosquito C636 cell line, the chemical inhibition of Akt and TOR induced a decrease in viral replication and viral particle production. It was shown that the pathway was in fact involved in the control of the invertebrate cellular cap-dependent translation pathway as a survival response and further investigations were needed to characterize the regulation of autophagy (Patel and Hardy 2012).

Using a natural vector, the infection of *Aedes aegypti* with DENV resulted in the induction of autophagy and apoptosis while the knockdown of *Aedronc* decreased DENV infection (Eng, van Zuylen, and Severson 2016). The role of caspase *Aedronc* in viral infection has been described previously with DENV and SINV by Ocampo *et al* (2013) and O'Neill *et al* (2015), respectively. Eng *et al* also showed that in the midgut of resistant mosquitoes, DENV infection led to the upregulation of *Aedronc*, *AeLAP1* and *Mx* at 48 hours post blood meal compared to the midgut of susceptible mosquitoes. In parallel, in whole resistant mosquitoes infected by DENV, autophagy genes (*Atg1*, *Atg5*, *Atg8*, *Atg12*) were upregulated from 24 to 60-72 hours post blood meal and autophagosome biogenesis was confirmed. More interestingly, several other apoptosis-related genes were transcriptionally activated during autophagy, like *Aedes p53* (*Aep53*) and IAP orthologs (*IAP5*, *IAP9* and *AeBruce*). They proposed a mechanism in which effector *Aecasp7*, activated by *AeDronc*, could promote either apoptosis or autophagy. The latter is an important process in vector infection (Figure 18); however it is unknown how caspase7 activates autophagy (Eng *et al.* 2016).

Recently, impaired autophagic flux accompanied by higher caspase activation and higher fly mortality was shown in *Drosophila* lacking *p53* (*p53*-/-) and treated with paraquat (ROS inducer). Indeed, they observed that *p53*-/- flies were more sensitive to paraquat than control *w1118* flies. More interestingly, they also showed that the *p53* isoforms called *p53A* ( $\Delta$ *Np53*) and *p53B* (*p53*) were differentially regulated in the context of oxidative stress with an increase in *p53B* transcripts but not *p53A* ones. Thus, as the sensitivity of *p53*-/- flies to paraquat was similar to autophagy-defective mutant flies and as it has been demonstrated that *p53* is a regulator of autophagy, they hypothesized that *p53*-/- could present a defective autophagic flux. Targeting *ref(2)P/p62*, which is normally degraded during autophagy and accumulated in *atg8*-/- mutants, they observed a higher level of *ref(2)P* in *p53* null flies. Since no *Atg8* expression was identified in *w1118* flies, they suggested that the sensitivity of *p53*-/- flies treated with paraquat was associated with impaired autophagy. In addition, they proved that

oxidative stress induction in the *p53*-/- mutant led to an increase in apoptosis, reflected by Drice and Dcp-1 caspase activity (Figure 18). Finally, they demonstrated that the p53A isoform allowed the activation of caspases which in turn inhibited autophagy. On the contrary, by overexpressing p53B they did not observe caspase activation but the activation of functional autophagy instead. This study demonstrated the importance of p53 A and B isoforms in response to oxidative stress and their opposite responses (Robin *et al.* 2019).



**Figure 18: Illustration of apoptosis – autophagy balance and the role of p53 during stress induction in *Drosophila* and arbovirus infection in *Aedes*.**

Finally, to my knowledge the existence of phosphatidylserine (PS) orthologs and equivalent mechanisms of “apoptotic mimicry” in mosquitoes and other insects has not been studied or published. Nevertheless, in the *Drosophila* fly, PS ortholog receptors at the cell surface of hemocytes (macrophages), called Draper and Integrin  $\beta$ v (Tung *et al.* 2013) have been identified. The abdominal injection in *Drosophila* with *Drosophila* C virus (DCV) and the infection of S2 cells revealed the induction of apoptosis and the presence of DCV in the hemocytes of flies. By inhibiting PS recognition and blocking the receptors, they showed that infected flies died earlier and suggested that PS-mediated phagocytosis plays an important antiviral role against DCV infection (Nainu *et al.* 2015).

To conclude, during arbovirus infection in insects, several signaling pathways such as apoptosis, oxidative stress, autophagy and other interconnected pathways seem to be involved in the cellular outcome and subsequently in permissiveness and resistance to vectors.

## 4 Role of p53 and p53 isoforms during arbovirus infection in mammals and insects

### 4.1 Human p53

#### 4.1.1 Discovery of p53

Thanks to a virological approach p53 was first described as a protein that binds to the large A antigen of the Simian virus SV40 (DeLeo *et al.* 1979; Lane and Crawford 1979; Linzer, Maltzman, and Levine 1979). Then, p53 was studied extensively in the field of cancer research, firstly as an oncogene in 1980 (Jenkins, Rudge, and Currie 1984; Land, Parada, and Weinberg 1983) and finally as a tumor suppressor in 1990 (Baker *et al.* 1989; Chandar *et al.* 1992; Masuda *et al.* 1987). The clue to p53's function as a tumor suppressor came from the finding that p53 deficient mice developed spontaneous tumors (Donehower *et al.* 1992). Another important discovery revealed mutations in p53 alleles in a family presenting the Li-Fraumeni syndrome. These patients presented an increased risk of developing tissue sarcoma, and breast, bone and brain tumors (Srivastava *et al.* 1990). At the time it was noticed that nearly half of all human tumors were linked to mutations in the p53 gene.

p53 was given the title that clearly defines its function: "the guardian of the genome". Indeed, tumor suppressor p53 is responsible for ensuring the maintenance of cellular homeostasis in response to several stimuli and defining cell fate. Since the discovery of p53 in 1979, more than one hundred thousand articles on it have been published, making it the most studied protein. One of the first activities of p53 investigated was its ability to bind DNA in a specific sequence, mediating its transcriptional activation function; indeed, hundreds of genes are targeted by p53. The activation of p53 can be engaged by several signals such as DNA damage, hypoxia, oncogene signaling, pathogen detection and oxidative stress, and the cellular downstream

responses controlled by p53 are involved in cell cycle arrest, apoptosis, autophagy, immune response, metabolism, DNA repair and senescence.

#### **4.1.2 The p73/p63 and p53 family**

Almost twenty years after the discovery of p53, two additional members, called p63 and p73, have been identified, suggesting that p63 and p73 were ancestors of p53 and that p53 has evolved by duplication and mutation (Blandino and Dobbelstein 2004). The disruption of the *p73* gene in mice has been associated with profound defects in homeostasis and neuronal development but no susceptibility to spontaneous tumorigenesis was observed (Yang *et al.* 2000), whereas *p63*<sup>-/-</sup> mice presented major defects in their limb and epithelial development (Yang *et al.* 1999), suggesting that both ancestor functions were principally involved in development and morphogenesis. Regarding the structure of the mammal gene and protein domain, p53 and p73/p63 share a similar N-terminal domain and only p73/p63 contain a sterile alpha motif SAM in the C-terminal region. The SAM domain is necessary in protein-protein interactions (Thanos and Bowie 1999).

#### **4.1.3 Human p53 and p53 isoforms**

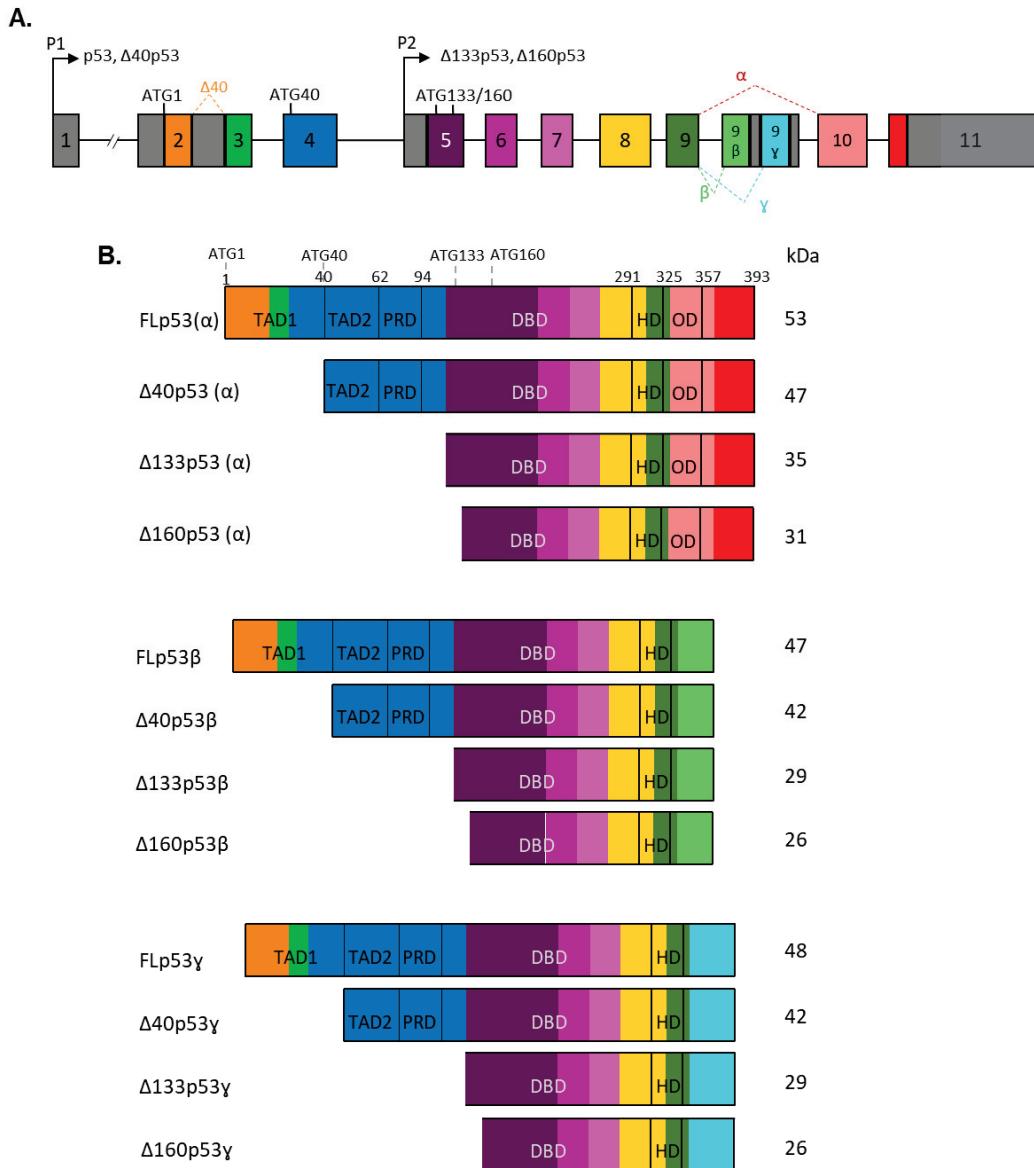
The p53 gene is located on the short arm of chromosome 17 and presents 13 exons. To date, it has been reported that the human p53 gene can express twelve p53 mRNAs in a tissue and cell-dependent manner (Figure 19). The twelve different mRNAs result from alternative promoter (P1 and P2) usage and alternative splicing (intron-2 and intron-9). The human p53 gene contains two promoters: one upstream of exon-1 and an internal promoter in intron-4, resulting in four C-terminal mRNA variants (full-length p53, Δ40p53, Δ133p53 and Δ160p53). The transcript from the P1 promoter can encode the full-length p53 (FLp53 or p53α) and Δ40p53 (or Δ40p53α) due to the internal initiation of translation from codon 40 (ATG40) or the alternative splicing of intron-2. These transcripts can also exist with two other different 5' regions from the alternative splicing of exon-9 by retaining either exon-9β or exon-9γ and

encoding p53 $\beta$  or  $\Delta$ 40p53 $\beta$  proteins, and p53 $\gamma$  or  $\Delta$ 40p53 $\gamma$  proteins, respectively (Khoury and Bourdon 2010).

The transcript from the P2 promoter, located within intron-4, encodes  $\Delta$ 133p53 (or  $\Delta$ 133p53 $\alpha$ ) which is a N-terminally truncated protein, initiated at start codon 133, and  $\Delta$ 160p53 (or  $\Delta$ 160p53 $\alpha$ ) translated due to ATG160 in the  $\Delta$ 133p53 transcript (Marcel *et al.* 2010). These transcripts can also exist with two other different N-terminal regions by alternative splicing and encode  $\Delta$ 133p53 $\beta$ ,  $\Delta$ 133p53 $\gamma$ ,  $\Delta$ 160p53 $\beta$  and  $\Delta$ 160p53 $\gamma$  (Figure 19, A).

#### 4.1.4 Structure of p53 and p53 isoforms

The FLp53 (p53 $\alpha$  or p53) is active as a tetramer, dimers of two homodimers, each containing the 393 amino acid long protein composed of seven functional domains (Figure 19, B). The N-terminus is composed of two transactivation domains (TAD1 -residues 1 to 40 and TAD2 - residues 40 to 62) which are essential for the activation of the subset of p53-target genes (Venot *et al.* 1999). FLp53 is composed of a proline-rich domain (PRD), a DNA-binding domain (DBD) and a hinge domain (HD). The C-terminus is composed of an oligomerization domain (OD) which allows p53 to form a tetramer organized as a dimer of dimers and a regulation domain ( $\alpha$ ) where many post-translational modifications occurs (acetylation, phosphorylation, ubiquitination, SUMOylation, methylation, etc.) which are involved in the stability and activity of p53. The export and import of p53 between the nucleus and cytoplasm occur due to one NES and three NLS, distributed in oligomerization and regulation regions.



**Figure 19: Human p53 gene organization (A) and the structure of the p53 protein and p53 protein isoforms (B).**

**A)** The human p53 gene is composed of eleven exons (marked as colored boxes from 1 to 11) and theoretically expresses twelve isoforms from alternative promoters -  $\uparrow$  - P1 and P2, splicing sites  $\alpha$ ,  $\beta$  and  $\gamma$  or translational initiation codon ATG. The promoter P1 located upstream from exon-1 allows transcribing the full-length p53 from ATG1 and the  $\Delta 40p53$  isoform from ATG40. Internal promoter P2 is in exon-5 and produces  $\Delta 133p53$  and  $\Delta 160p53$  from ATG133 and ATG160, respectively. The  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms are produced by the different alternative splicings of intron-9:  $\alpha$  results from the entire splicing of intron-9, whereas  $\beta$  and  $\gamma$  are generated by the retention of small parts. Legend: grey box regions =non-coding sequence.

**B)** The human p53 protein isoforms. The full-length p53 (from ATG1) contains two complete transactivation domains (TAD1 and 2), rich proline domain (PRD), DNA-binding domain (DBD), hinge domain containing NLS (HD), oligomerization (OD) and at the C-terminal regulatory region (in red). The  $\Delta 40p53$  isoform lacks the 40 first amino-acids included in the 1<sup>st</sup> TAD, the  $\Delta 133p53$  and  $\Delta 160p53$  isoforms lack both TAD and a part of DBD. The C-terminal isoforms from alternative splicing are represented in red ( $\alpha$ ), in green fuchsia ( $\beta$ ) and blue fuchsia ( $\gamma$ ). The molecular weight of each isoform is indicated on the right (kDa) and the name of the isoforms on the left. (Adapted from Khouri and Bourdon 2010).

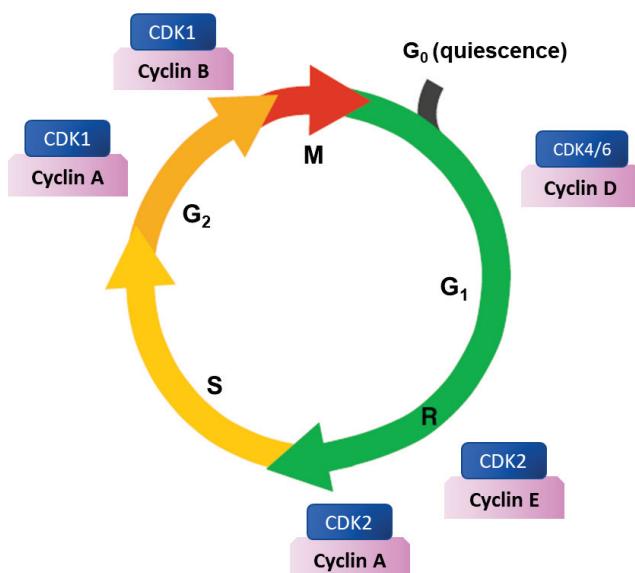
#### 4.1.5 Stabilization of p53

In undamaged cells, the low level of p53 is strictly controlled and engages the regulation of transcript expression but mostly protein stabilization. Indeed, the amount of p53 protein is determined more by its rate of post-translational degradation than by the translation of mRNA, and this degradation is ensured by the E3 ubiquitin-protein ligase MDM2 for mouse double minute 2 (also called HDM2 for human double minute 2). Without stressful conditions, MDM2 binds to p53 in the nucleus and ubiquitinates it, promoting its exportation to the cytoplasm where it is polyubiquitinated and degraded by the 20S and 26S proteasomes. Under diverse stress conditions, modifications of p53 will induce its stabilization, accumulation, and activation. It has been shown that the major mechanism controlling p53 stabilization is the impact on MDM2-p53 interaction. The phosphorylation of p53 in the N-terminal and C-terminal regions leads to a conformational change of the molecule which influences the interaction between MDM2-p53. These phosphorylations are guided by protein kinases such as ATM (ataxia-telangiectasia), JNK (Jun NH<sub>2</sub>-terminal kinase), p38, ATR (A-T and Rad3-related) and others. One of the phosphorylations described most is on Serine 15 (Ser15) located in the transactivation domain: for example, after DNA damage, the mediator ATM can phosphorylate Ser15 on p53 as well as MDM2 (Banin *et al.* 1998). After Ultra-Violet or oxidative H<sub>2</sub>O<sub>2</sub> treatment, JNK is responsible for the phosphorylation of p53 at Threonine 81, Thr81 (Buschmann *et al.* 2001). The phosphorylation of specific sites allows the induction of other modifications in the process of p53 activation like the acetylation of serine, for example by acetylase p300 belonging to histone acetylase family members CBP/p300. These mediators enhance the DNA-binding activity of p53 and subsequently its transcriptional activity (Avantaggiati *et al.* 1997). These few post-translational modifications give an overview of the complex web governing the stabilization of p53 (Figure 21 “Regulation” and “Stabilization” frames). In response to p53 activation, different pathways can be activated or inhibited due to the transcription of p53-target genes. Pathogens have evolved various means to control cell fate by p53 stabilization at the post-translational level in vertebrates, which will be discussed in the following sections.

#### 4.1.6 A transcription factor

- Cell-cycle

The cell cycle is a fundamental cyclic sequence in the life of a cell and is divided into four phases (Figure 20): 1) daughter cells produced after cell division of the mother cell undergo a sequence of growth ( $G_1$ ) where proteins and membranes are synthesized; 2) the replication of DNA (S); 3) a second phase of growth ( $G_2$ ); and 4) the phase of mitosis where DNA is divided equally to obtain two new daughter cells. A quiescent, nondividing phase can be adopted by cells ( $G_0$ ). The cell cycle process is guided by an evolutionary conserved family of protein kinases called cyclin-dependent kinases CDKs. The on/off switching of different CDKs and checkpoints allows promoting or putting on standby the different stages of the cell cycle. The activities of CDKs that allow the accumulation of corresponding cyclins are regulated by phosphorylation, protein-protein interactions, and ubiquitin-mediated degradation. In addition to cyclin-CDK, sensors regulate the passage from  $G_1$  to S or S to  $G_2$ , etc. in the different steps of the cell cycle. For instance, during the  $G_1$  phase, a transcription factor E2F is sequestered by a protein of the retinoblastoma (pRb) participating in the efficient progress of the  $G_1$  phase and while this stage is finishing, cyclin-CDK complexes (cyclinD-CDK4/6) induce the hyperphosphorylation of pRb. The phosphorylation of pRb induces the release of E2F. E2F-dependent transcription participates in the entry of the S phase through the restriction point, called R and induces the transcription of cyclin A and B which activate CDK2 in a positive feed-back loop by phosphorylation of pRb (Ezhevsky *et al.* 1997; Poon 2016).



**Figure 20: The four phases in the cell cycle and CDK-cyclin complexes.** The cell cycle is divided into four phases: 1)  $G_1$  where cell growth occurs; 2) the DNA replication phase called S; 3) a second growth stage,  $G_2$ ; and 4) the mitosis stage. A nondividing cell can rest in  $G_0$  quiescent phase. The cyclin/cyclin dependent kinase (cyclin/CDK) strictly controls the efficient process of a stage and the passage to the new one (Adapted from Poon 2016).

Another checkpoint during G<sub>1</sub> allows verifying if any DNA damage is detected and, if this is the case, DNA repair is necessary. The mechanism assessing this checkpoint is a p53-dependent mechanism: in the absence of DNA damage p53 is degraded through proteasome and DNA damage detection by sensors inducing the activation of ATM and ATR, presented previously, and p53 is activated. One of the transcriptional targets is p21, also called CDK interacting protein 1 or cyclin-dependent kinase inhibitor protein 1 (p21<sup>CIP1</sup>), which binds and inhibits the complex cyclin A/E-CDK2, diminishing pRb phosphorylation and stopping the cell in the G<sub>1</sub> phase (Deng *et al.* 1995; Dulić *et al.* 1994) (Figure 21 “Cell cycle”). Another target gene, *gadd45*, is activated after different stress and DNA damage. Gadd45 participates in the checkpoint in the G<sub>1</sub> phase cell cycle arrest and apoptosis, and can contribute to p53 stabilization via p38 (Salvador, Brown-Clay, and Fornace 2013). Several DNA viruses, which integrate their genome inside the cell genome, oncogenic viruses and even RNA viruses, take advantage of cell cycle control via p53 and other transcription factors, or p53 stabilization, for their own replication. They will be presented in the following section.

- Apoptosis

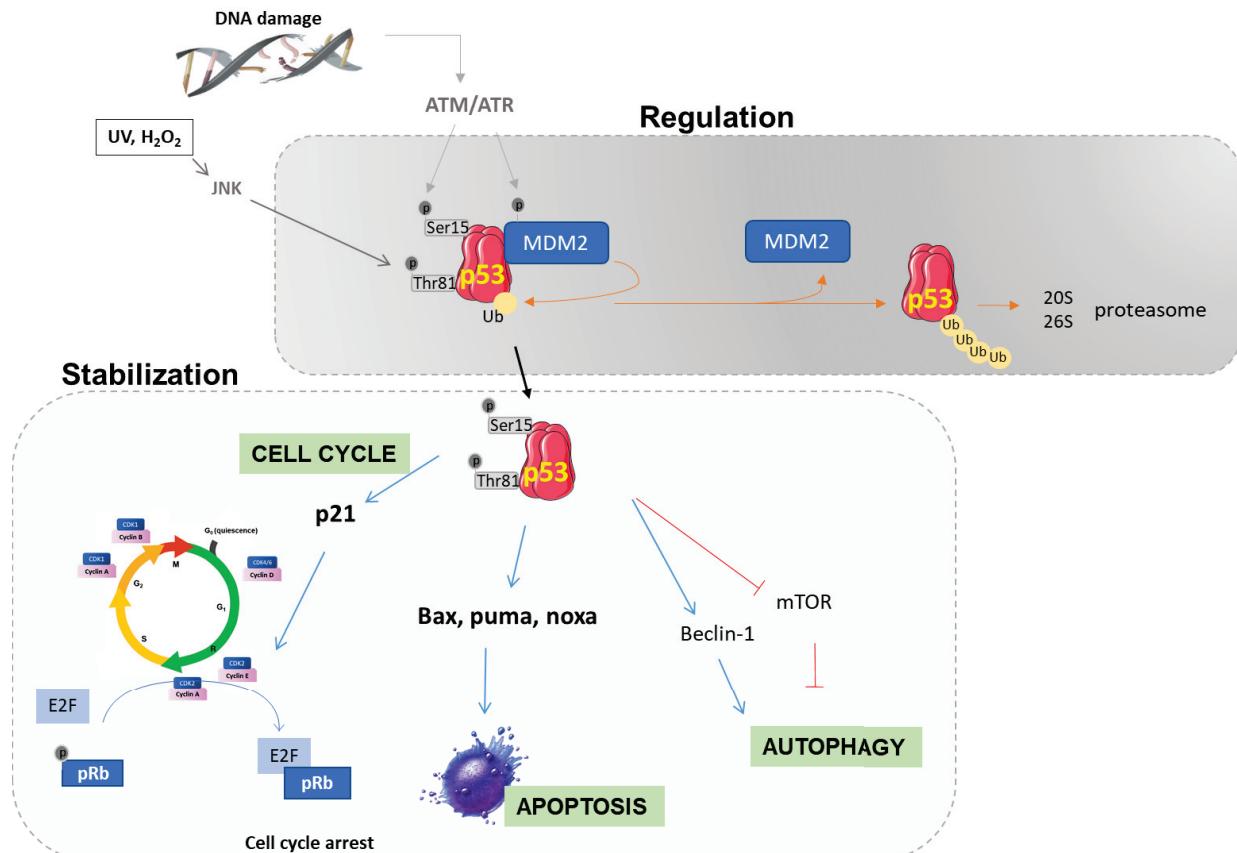
The apoptosis signaling pathway in mammals has been described previously and p53-mediated apoptosis has been introduced listing p53-target genes such as *bax*, *noxa*, *puma* and *apaf-1* (Figure 21). Moreover, the stabilization of p53 can be controlled by phosphorylation and specific p53 response is mediated by the particular site of phosphorylation.

- Autophagy

The regulation of autophagy can also be dependent on p53 and p53 plays a key role for the activation or inhibition of autophagy (Figure 21). Indeed, under glucose starvation stress, autophagy can be initiated indirectly by p53 through the inhibition of mTOR by the direct transcription of Adenosine monophosphate-activated protein kinase (AMPK) or by blocking Akt signaling that ultimately inhibits mTOR (Feng *et al.* 2007).

The inhibition of autophagy by p53 can be mediated by direct binding to the RB1-inducible coiled-coiled protein 1 (RB1CC1) involved in the stabilization and phosphorylation of ULK1 (Morselli *et al.* 2011). Another function of p53 in autophagy is the accumulation of dsRNA in the cell, from viral infection or DNA damage and abnormal transcription or stress, which can be controlled by protein kinase RNA-activated (PKR).

The PKR phosphorylates the eukaryotic translation initiator factor 2 $\alpha$  (eIF2 $\alpha$ ) to shutdown protein translation of the cell and it has been hypothesized that cytoplasmic p53 could degrade dsRNA and indirectly activate PKR (Galluzzi, Kepp, and Kroemer 2010).



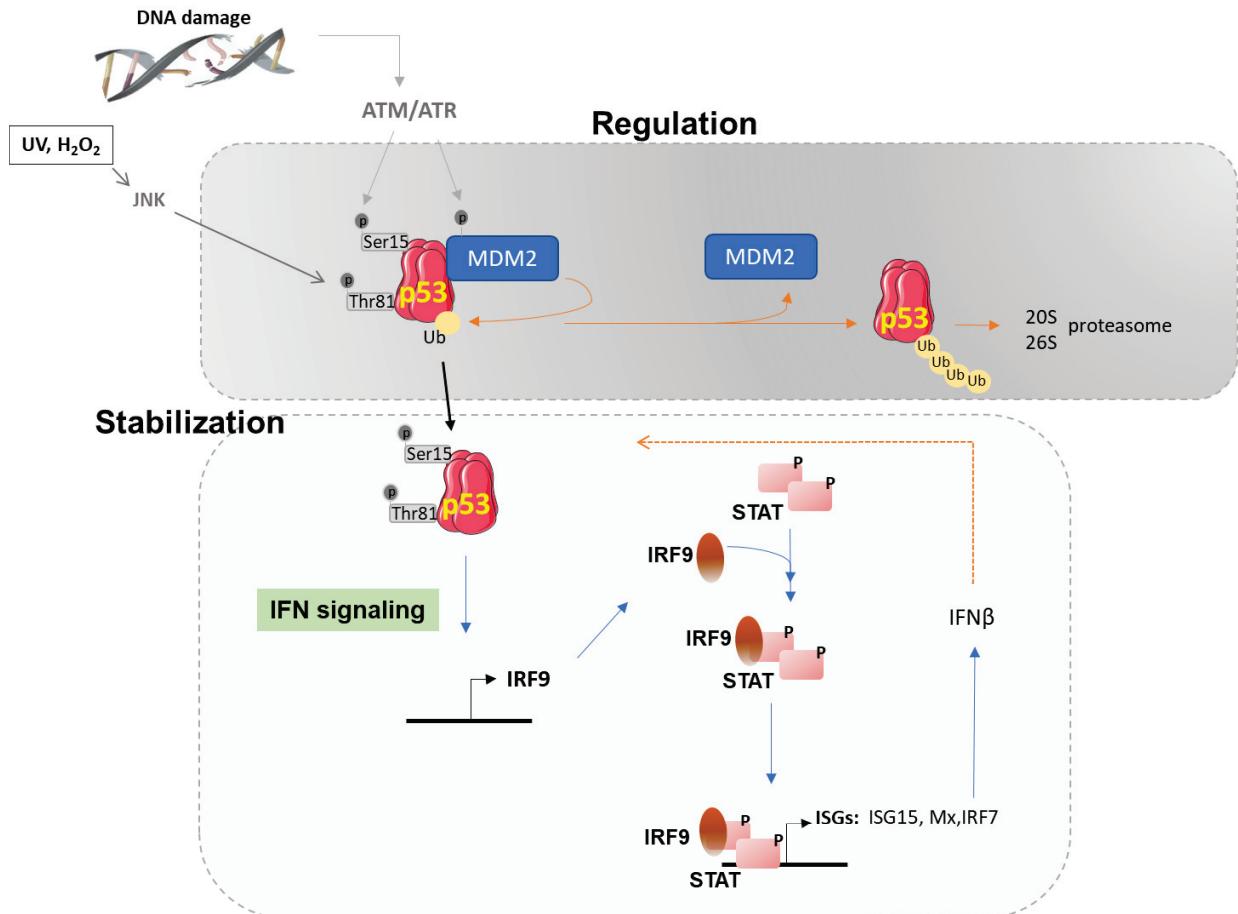
**Figure 21: The complexity of the p53 regulation-activation stage and regulation of targeted pathways. The control of p53 stabilization is influenced by the interaction of MDM2-p53. Under nonstress cellular conditions, MDM2 binds p53 and monoubiquitinates before export to the cytoplasm for polyubiquitination and proteasome degradation. Under stress conditions, like DNA damage or oxidative stress, sensors and mediators (ATM/ATR and JNK) will phosphorylate p53, preventing its degradation and so induce its accumulation. A sufficiently high level of p53 protein participates in the control of adequate cellular response: 1) the control of cell cycle arrest through p21 pathway; 2) the transcription of pro-apoptotic proteins for apoptosis; 3) indirect action on mTOR and Beclin-1 for autophagy induction.**

#### - Type-I interferon signaling and p53 interaction during viral infection

Several reports in the field of cancerology have hesitated to link p53 and IFNs based on the observation that p53 and interferon share antioncogenic activities. In 2000, Mecchia and colleagues observed in melanoma cell lines an increase of the IFN-dependent accumulation of p53 (Mecchia *et al.* 2000), and in 2003 Takaoka *et al.* demonstrated a link between p53 and IFN $\alpha/\beta$  through the direct transcription of p53 mRNA by ISGF3 activated by IFN and its key role

in antiviral response at an early stage of MEF infection with VSV (Takaoka *et al.* 2003). It was shown that VSV and herpes simplex virus (HSV) infections induced the Ser15 phosphorylation of p53 *via* ATM kinase and certain p53 target genes like *mdm2* and *puma* were upregulated, whereas *p21* and *noxa* were not, indicating that p53 was efficiently activated and the response engaged was different between viral infection and DNA damage. Interestingly, p53 mRNA increases in infected WT MEFs but not in IFNAR-/- MEFs, whereas the Ser15 phosphorylation of p53 was still detectable in IFNAR-/- MEFs, indicating that the transcription of p53 is dependent on IFN but not its activation. MEFs p53-/- were infected more by VSV than WT MEFs and VSV-induced apoptosis was reduced in p53-/-, suggesting a pro-apoptotic role of p53 in MEFs. In parallel, *in vivo* mice p53-/- infected with VSV all died while 80% of the WT infected survived. They suggested an increase of p53 mRNA dependent on IFN leading to an increase of apoptosis dependent on p53. Previously, several investigators linked IFN Type-I and apoptosis (Clemens 2003).

A few years later, Muñoz-Fontela *et al.* demonstrated another p53 function in the activation of the IFN signaling pathway during the VSV infection of *in vitro* mouse MEF and human HCT116 and mice (Muñoz-Fontela *et al.* 2008). Firstly, to analyze the proapoptotic function of p53 to impair viral infection they treated MEFs WT and p53-/- or not with broad-spectrum caspase inhibitors and infected with VSV-GFP. As reported previously, p53-/- infected animals produced a higher viral titer than WT. However, the WT treated with caspase inhibitors still reduced viral replication while the cell-death level was like that obtained in p53-/. Additional experiments confirmed the higher VSV-GFP replication in the absence of p53 expression in EJp53 cell lines. EJp53 presents a (TET)-regulatory p53 system accompanied with cell cycle arrest without the induction of apoptosis: the inhibition of VSV-GFP replication seems to be independent of the apoptotic-p53 function. They then showed the upregulation of ISGs Mx1, IRF7, RIG-I and IRF9 (containing ISGF3 complex, *cf.* Figure 10). Using a Chromatin immunoprecipitation (ChIP) assay, they demonstrated a specific p53 binding region in the IRF9 promoter and determined that the role of p53 in enhancing the IFN signaling pathway was clearly due to its transcriptional activity and not its post-translational activity (Figure 22).



**Figure 22: Type I interferon signaling pathway mediated by p53 activity.** The Type-I interferon production and signaling pathway can be influenced by p53 activity during viral infection through a positive feedback loop of the transcriptional induction of Interferon regulatory factor 9 (IRF9).

Interestingly, IFN can upregulate IFIX $\alpha$ 1 which downregulates post-translationally MDM2, allowing the accumulation of p53 (Ding *et al.* 2006). Finally, it has been reported that after IRF7 expression through ISGF3, IRF7 can activate IFN $\alpha$  transcription, and that IFN $\alpha$  expression is delayed by IFN $\beta$  expression (Marié *et al.* 1998).

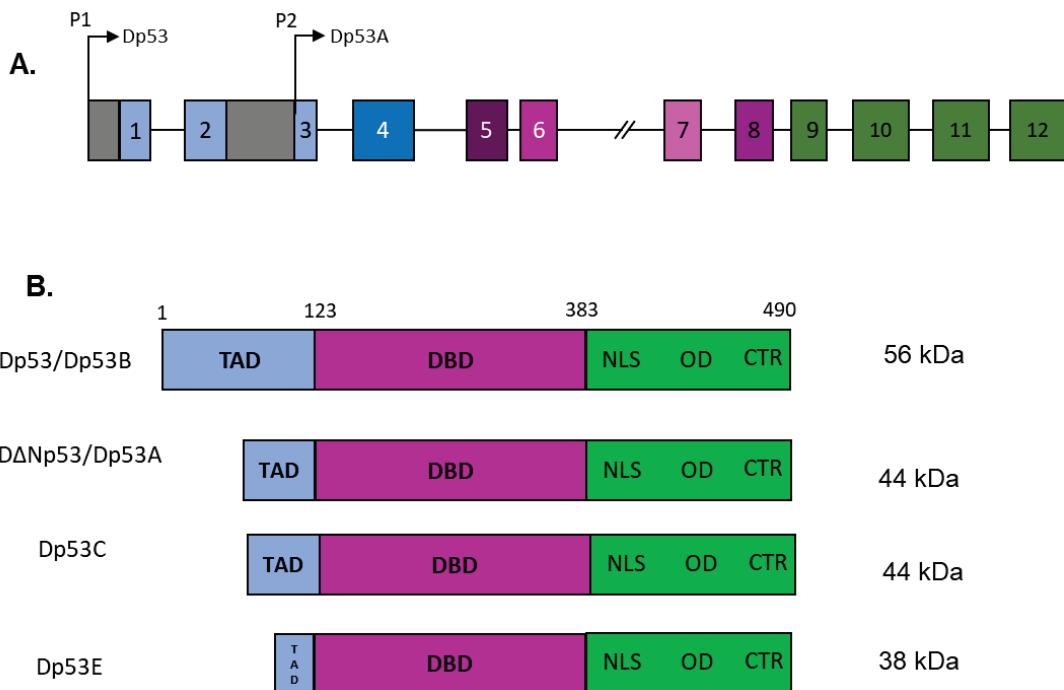
Taken together, these results suggest that under viral infection, p53 regulates the IFN $\beta$  signaling pathway by the direct transcription activation of IRF9 which associates with STAT and translocates to the nucleus for the expression of ISGs including IRF7. IFN expression indirectly allows the stabilization of p53 by the expression of Mdm2 antagonists and the positive feedback loop of IFN production through IRF7 allows participation in p53 accumulation (Figure 22).

To conclude, these discoveries indicate that p53 plays a key role in innate immune response by rapid apoptosis and autophagy crosstalk or by reinforcing the type I IFN signaling pathway. Moreover, this system guides p53 stabilization in infected cells and in neighboring uninfected cells, suppressing viral dissemination more rapidly. The guardian of the genome is a suitable name and we can clearly understand the evolutionary reasons that drove viruses to induce p53 agonists and antagonist factors.

## 4.2 *Drosophila* and mosquito p53

### 4.2.1 Dmp53/Dp53 and p53 isoforms in *Drosophila*

The *Dmp53* gene resembles the mammalian *p53* gene in its structure and functionality, however *Drosophila* presents a single p53 family gene that can potentially encode four p53 isoforms (Zhang *et al.* 2015). Dmp53/Dp53 or Dp53B is an orthologue of mammal FLp53, Dp53A, also known as DΔNp53 and Dp53C, resembles human Δ133p53 and Δ40p53 like mRNA is transcribed from an internal promoter and contains an N-terminal truncated TAD. Finally, the Dp53E isoform bears a smaller TAD (Figure 23). The Dp53B protein includes one full transactivation domain (TAD) where Dp53A and Dp53C contain a truncated TAD for almost the 60 first amino acids, a DNA-binding domain (DBD), nuclear localization signal (NLS), oligomerization domain (OD) and C-terminal regulatory domain (CTR) (Marcel *et al.* 2011). Studies on *Drosophila* p53 provided new insights on the role of p53 in cellular fate and on p53 and p53 isoform interactions and the consequences on p53-mediated cellular responses. In the absence of p63 and p73, it has been understood that Dp53 and p53 isoforms ensure the control of cell death, autophagy, cell differentiation, embryogenesis and lifespan in a sex-dependent manner (Ingaramo, Sánchez, and Dekany 2018).



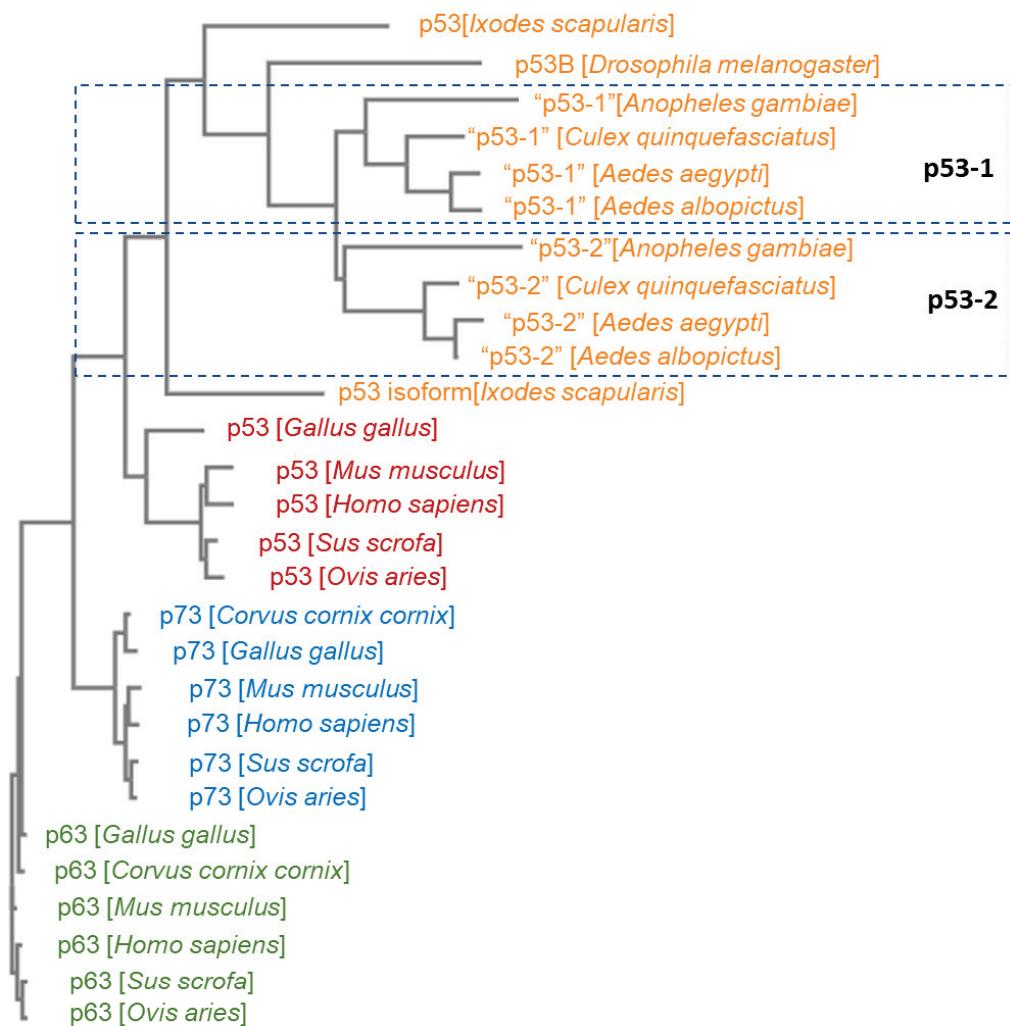
**Figure 23: The p53 gene of *Drosophila* (A) and p53 isoforms (B).**

Historically, Dp53A was the first p53 isoform discovered but it was mistaken for Dp53, indicating why this isoform is that most studied (Ollmann *et al.* 2000). Studies on irradiated imaginal discs referring to the post-embryonic development of fly, mutated for Dp53, have shown reduced apoptosis and normal cell cycle arrest, suggesting a specific apoptotic engagement of Dp53. Then, the Dp53A isoform was characterized as the main isoform mediating apoptosis through *reaper* transcription following DNA damage (Brodsky *et al.* 2000).

#### 4.2.2 Recent discovery of two p53 isoforms in *Aedes*

The sequencing and incomplete annotation of the mosquitoes *Aedes albopictus*, *Aedes aegypti* and *Anopheles gambiae* have led to the identification of two p53 sequences in the mosquito (Figure 24). Indeed, Chen *et al.* created a phylogenetic tree based on p53 amino acid sequences derived from 22 mosquito species, several other insects (order Diptera) and a control outgroup from a snail sequence. Then, by predicting amino acid sequences from p53 homologues derived from mosquito species and species of flies they identified two paralogues of p53, named “p53-1” and “p53-2” in the four mosquitoes species studied (*Aedes aegypti*, *Aedes albopictus*, *Culex*

*quinquefasciatus* and *Anopheles gambiae*) with a single homologue in fly species (*Drosophila melanogaster*, *ceratitis capitata* and *Bactrocera dorsalis*). They confirmed this result using two different phylogenetic methods (neighbor joining and stronger method, namely the maximum-likelihood method) (Chen *et al.* 2017). They suggested a duplication origin of p53 isoforms of mosquitoes and further studies are needed to better characterize p53-1 and p53-2. Based on DBD amino acid predicted sequences it was estimated that the p53-1 of *Aedes aegypti* shared 67% similarity with *Ae. albopictus* and 50% with *Cx. quinquefasciatus* and for p53-2, 82% and 63%, respectively (Figure 24). *Anopheles gambiae* showed less than 28% similarity for both paralogues with three other mosquito species.



**Figure 24: Phylogenetic tree of selected p53 homologues and orthologue sequences, created on the EMBL-Simple phylogeny website.**

## 4.3 Control of p53 pathways during viral infection

### 4.3.1 Function of p53 during “non-arboviral” infections

- SV40 and p53

The discovery of the large T-Antigen protein of SV40 (*Polyomavirus*, small DNA genome 5.2 kb) has started the investigation of interactions between oncogenic virus proteins and p53. In humans, the question of whether Simian Virus 40 (SV40) can cause human tumors is highly controversial (Poulin and DeCaprio 2006). The infection of host cells with SV40 leads to cell transformation by the control of the cell cycle by the passage from G<sub>1</sub> to the S phase (O'Reilly 1986). It has been shown that the T-antigen can bind to pRb, releasing E2F and activating its transcriptional activity that mediates the passage to the S phase (Figure 25). In parallel, the T-antigen can also block p53 then sense the aberrant passage to the S phase and thus inhibit apoptosis (Ludlow 1993). In mouse fibroblast 3T3 cells it has been shown that the T-antigen can also bind to TAD1 of p53 and control acetylation by the recruitment of p300/CBP, encouraging p53 DNA-binding activity and maybe target genes for cell transformation (Hermannstädtter *et al.* 2009). However, p53 still controls SV40 at an early stage of infection before T-antigen detection. Indeed, it has been demonstrated that p53 was activated in non-T-antigen positive cells and could bind to the T-antigen promoter, stopping its transcription by preventing factor Sp1 (specificity protein 1) binding (Drayman *et al.* 2016).

- p53 and HPVs

Another DNA virus, Human papilloma virus (HPV), leads to persistent infection associated with cervical cancer. HPVs viruses are small double-strand DNA viruses which present a tropism for squamous epithelial cells. HPVs are classified into five genera, named alpha  $\alpha$ , beta  $\beta$ , gamma  $\gamma$ , mu  $\mu$  and nu  $\nu$  which are also subdivided into species referenced by numbers. The majority of HPV infections are benign and resolved without progression to cancer. The different HPV types are usually referred as “low risk” (wart-causing) or “high risk” (cancer-causing) and the diversity of pathology is reflected by phylogenetic relationships between HPV types. The high risk HPVs can cause several types of cancer such as cancer of the oropharynx, vagina, penis and anus, and it has been revealed that oncoproteins E6 and E7 are essential for the oncogenic cell

transformation phenotype (Ghittoni *et al.* 2010; Moody and Laimins 2010). Indeed, the E6 oncoprotein mediates the degradation of p53 involving the ubiquitin-dependent proteasome (Scheffner *et al.* 1990; Scheffner *et al.* 1993). In parallel, it has been demonstrated that the E7 oncoprotein can bind to pRb, promoting its degradation and subsequently perturbing cell proliferation (Dyson *et al.* 1989). Moreover, studies of the efficiency of E7 in targeting pRb showed that the oncogenic potential of some HPVs was dependent on this efficiency of E7 to bind to pRb (Caldeira, Dong and Tommasino 2005). More recently, it has been proposed that E7 can also indirectly interfere with p53 function independently of E6 through the deregulation of cell cycle genes under the control of the p53-p21-DREAM pathway (Fischer *et al.* 2017). The DREAM (Dimerization partner DR, RB-like, E2F and multi-vulval class B MuvB) complex is essential for the good progress of the cell cycle. Indeed, the DREAM complex mediates the repression of cell cycle genes during the G0 phase (quiescence) and organizes periodic gene expression with peaks during the G1/S and G2/M phases of the cell cycle (Figure 25). This strict coordination is provided by additional factors: the RB-like pocket proteins p130 and p107 in the DREAM complex, which repress cell cycle gene expression during the G0 phase, whereas MYB-like 2 MYBL2 and forkhead box M1 FOXM1 coordinate gene expression during replication and G2/M phases (Sadasivam and DeCaprio 2013). The HPV E7 oncoproteins can disrupt the p130-DREAM complex, promoting the S phase, either by interaction and disruption of p130, or by interaction with p21, the inhibitor of Cyclin Dependent Kinase 2 activation (Nor Rashid *et al.* 2016).

#### - p53 and HIV

The *lentivirus* HIV-1 responsible for the development of AIDS (Acquired ImmunoDeficiency Syndrome) also evolved to block p53 pathways in immune cells through viral factors. Greenway and colleagues reported that the viral protein Nef can interact with p53 and decrease its transcription activity in MOLT-4 cells that subsequently inhibit apoptosis and could allow longer cell viability beneficial for viral replication (Greenway *et al.* 2002) (Figure 25). In parallel, another viral factor, Vif, can control the stabilization of p53 acting on p53/MDM2 interaction in order to mediate G<sub>2</sub> cell cycle arrest, suggesting a contributable effect on virus production (Goh *et al.* 1998; Izumi *et al.* 2010). The dual effect on p53 seems to be perfect control of cell fate during viral infection.

The control of p53 and p53 pathways seems to be an important mechanism shared by several different viruses, making research necessary to elucidate the issue of therapeutic strategies targeting p53 in a tissue dependent manner.

#### **4.3.2 Different roles of p53 during arboviral infections: DENV, WNV and ZIKV**

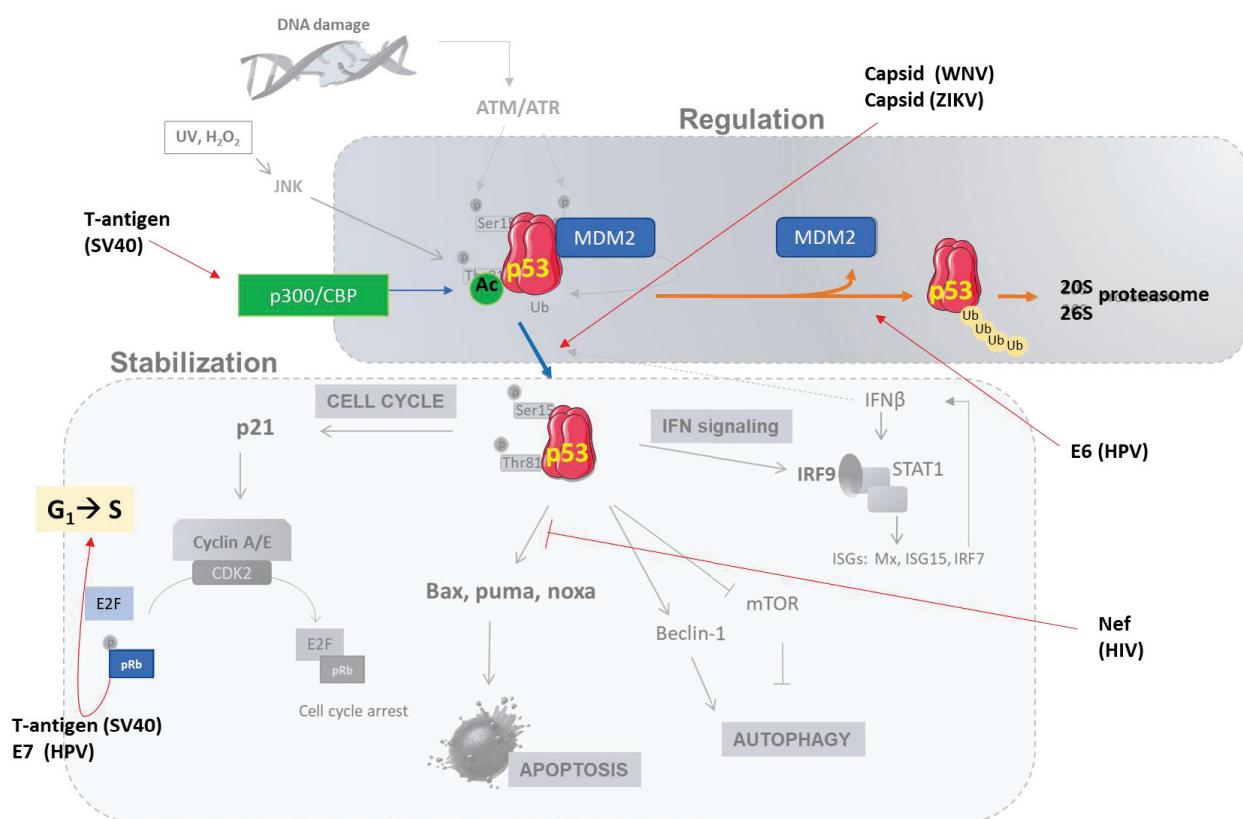
In the old-world alphavirus infections, only one study has shown that SFV induced apoptosis in BHK cells and also in lung carcinoma cells deleted for the *p53* gene, suggesting a p53-independent apoptosis (Glasgow *et al.* 1998). To my knowledge, no studies on the involvement of p53 during CHIKV in mammals and insects have been published.

In parallel, more investigations into the role of p53 during *flavivirus* infections have revealed p53-dependent cell death. The infection of the human liver Huh-7 cell line with DENV led to apoptosis detected by TUNEL and PS externalization and a decrease of mitochondrial transmembrane potential. In a p53-deficient cell line (H1299), virus-induced cell death was non-detectable (Nasirudeen, Wang, and Liu 2008). Curiously, previous studies have shown the induction of apoptosis in liver cell lines HepG2 (WT p53) and in Hep3B (p53<sup>-/-</sup>) and at 7 dpi. All the infected p53<sup>-/-</sup> cells died, whereas p53 WT was persistently infected for 3 months with DENV. Moreover, viral particles produced from Hep3B were significantly more efficient in new cell attachment compared to HepG2 (Thongtan, Panyim, and Smith 2004). They proposed that DENV-induced apoptosis was p53-independent, however, p53 could play a role in lowering viral particle production, thereby avoiding apoptosis.

Interestingly, a recent study, using the HepG2 cell line, has shown that RNAi knockdown of p53 was linked to increasing virus production and decreasing Type-I interferon (IFN $\beta$ ) secretion in supernatant at 24 hours post infection. The percentage of apoptotic cells was the same in both infected cells (Hu *et al.* 2017). Taken together, these results seem to indicate that in both studies, Dengue virus infection was controlled by p53, through the IFN pathway and not the apoptotic pathway.

In the mosquito, it has been shown that infection of the *Ae.albopictus* C636 cell line with DENV induced the upregulation of isoform p53-2 and not p53-1 at 24, 36 and 48hpi (Chen *et al.* 2017). In order to investigate the function of the p53-2 isoform during DENV infection, they

hypothesized different functions of p53-1 and p53-2 in oxidative stress response and cell death, as has been previously shown for oxidative stress response in DENV-infected C636 associated with antioxidant defense and antiapoptotic effects (Chen *et al.* 2012). The knockdown (KD) of p53-2 but not p53-1 increased cell death and oxidative stress in a DENV-infected C636 cell line over time. Moreover, among six antioxidant genes, only one has been shown to significantly decrease in KD p53-2 infected cells, i.e. the catalase CAT. The activation of CAT confirmed the transcriptional activity of p53 during DENV infection (Chen *et al.* 2018).



**Figure 25: The viral control of mammal cell fate by targeting p53 and p53 pathways.**

The outbreak of Zika virus in 2014 in Brazil is associated with microcephaly in newborn infants. Indeed, infections during pregnancy were linked to spontaneous abortion, stillbirth, fetal malformations, or microcephaly. ZIKV was first isolated in Uganda in 1947 and it has been detected in Africa and Southeast Asia, causing self-limiting febrile disease in humans. It has been demonstrated that there are two distinct lineages: African and Asian. Following the outbreak in Brazil there have been extensive efforts to develop a new animal model as ZIKV does not cause infection and disease in wild type adult mice. Even if

the mice present significant differences in their placenta anatomy and gestation period compared to human, they are very useful for reproducing ZIKV pathogenesis regarding its neurotropic nature of infection. It has been shown that ZIKV infection in animal models (mice, macaques) is associated with the activation of immune responses, inflammation, and neural damage in the Central Nervous System (CNS). Moreover, the comparison of African and Asian lineages in *Stat2*<sup>-/-</sup> mice has revealed a higher expression level of type I and type II IFNs and cytokines in African lineages compared to Asian ones (Tripathi *et al.* 2017).

Several other investigations have rapidly identified a considerable induction of apoptosis and cell cycle arrest response in humans proliferating neural progenitors. In 2016, Ghouzzi *et al.* showed the accumulation of p53 perhaps by Ser15 phosphorylation, associated with the induction of cleaved caspase-3 apoptosis in human neural progenitor cells (hNPCs) (Ghouzzi *et al.* 2016). Later, two additional studies reported a central role of ZIKV capsid in apoptosis induction by p53 stabilization acting on the p53/MDM2 axis, and suggesting a role of capsid-mediated pathogenic process in neurodevelopment disruption and subsequently microcephaly (Slomnicki *et al.* 2017; Teng *et al.* 2017). Finally, Frumence and colleagues showed that human lung epithelial A549 cells were susceptible to the South Pacific epidemic strain of Zika virus and demonstrated that their infection led to IFN $\beta$  production, *IRF3* and *IRF7* expression and then the expression of ISGs genes such as *IFIT1/ISG56* and *IFIT2/ISG54*. Moreover, they demonstrated that A549 infected cells undergo apoptosis through caspase-9, caspase-3 activation, and the cleavage of PARP at 48 hours post infection. The induction of apoptosis occurred at the maximum of virus production and was also associated with the accumulation of mitochondrial ROS. Interestingly, they challenged A549 cells with IFN $\beta$  prior to and post Zika virus infection and revealed a drastic diminution in the production of ZIKV particles at 48 hpi and an increase in cell viability at 48 and 72 hpi. Moreover, the activity of caspase-3/7 decreased drastically, by the same magnitude as cells incubated with *pan*-caspase inhibitor ZVAD-fmk (Frumence *et al.* 2016). They showed that the infection of A549 induced the release of IFN $\beta$ , which presents an antiviral effect. However, IFN $\beta$  also presented a protective role by preventing apoptosis induction. Thus, they hypothesized that the early release of IFN $\beta$  could lead to the sufficient survival of ZIKV-infected epithelial cells that promote viral

replication by delaying apoptosis. The accumulation of p53 was investigated but it would be most interesting to study the effect of A549 p53  $+/+$  and A549 p53  $-/-$  on ZIKV infection.

The pathogenicity of West Nile virus has been also related to p53 apoptosis in infected U2OS and SH-SY5Y cell lines where the viral capsid can sequester HDM2/MDM2 to interfere with p53/HDM2 interaction (Figure 25). The complex of non-structural viral protein NS2B-NS3 induces caspase 8 activation and the viral capsid, caspase 9 and 3 activation (Yang *et al.* 2008). The induction of apoptosis seems to promote viral infection and could explain the pathogenic process.

To conclude, the activity of p53 during several different viral infections seems to be linked either to pro- or anti-viral effects, but in any case, it seems to play a central role.

#### **4.3.3 Modulation of p53 transcriptional activity by p53 isoforms during viral infection**

Human p53 isoforms can regulate p53 transcriptional activity and subsequently regulate the p53 signaling pathway, and even this axis has been shown to be regulated or exploited during viral infection.

Once again, the regulation of p53 transcription activity by p53 isoforms has been investigated in the field of cancer, because it has been observed that p53 isoforms appear to be dysregulated in different types of cancer (Bourdon *et al.* 2005). It has been hypothesized that p53 isoforms could exert an effect either on their own, thanks to their intrinsic properties and/or by modulating full length p53 activity. Firstly, it has been shown that in transfected cells,  $\Delta 40p53$  can associate p53 as heterodimers and promote the translocation of p53 from the cytoplasm to the nucleus *via* the reduction of MDM2-mediated polyubiquitination and inhibit p53-mediated growth suppression (Ghosh, Stewart, and Matlashewski 2004). Interestingly, in cell lines H1299 (p53  $-/-$ ) expressing  $\Delta 40p53$  or not and cultured in starvation condition, it has been reported that  $\Delta 40p53$  alone can decrease the conversion of LC3-I to LC3-II, which is a parameter of autophagosome formation (Zang *et al.* 2017). Previously, it was indicated that p53 can inhibit autophagy through the phosphorylation of PKR/eIF2 $\alpha$  by dsRNA degradation and in this study it was demonstrated that  $\Delta 40p53$  and p53 present an exonuclease activity

participating in dsRNA degradation and the inhibition of starvation-induced autophagy by blocking PKR phosphorylation. They also showed that after DNA damage induction,  $\Delta 40p53$  promoted p53 nuclear translocation and inhibited p53 activity-induced cell death; however, the dominant negative effect of nuclear  $\Delta 40p53$  on p53 is contradictory and further studies are needed. Nonetheless, taken together, these results suggested that during viral infection producing dsRNA,  $\Delta 40p53$  and p53 could promote an antiviral response by inhibiting autophagy and cell death activation. Moreover, it has been reported that ISG15 could be directly targeted by p53 in response to dsRNA but not in a p53-dependent manner during an RNA virus NDV (Newcastle Disease virus, family *Paramyxoviridae*) infection (Hummer, Li, and Hassel 2001).

Studies on two other isoforms, p53 $\beta$  and  $\Delta 133p53$ , have shown that in human fibroblasts p53 $\beta$  could increase p53 transcriptional activity in a promoter-dependent manner to promote cellular senescence, whereas  $\Delta 133p53$  diminished this p53-mediated replicative senescence (Fujita *et al.* 2009). Indeed, in a Luciferase reporter system, it was demonstrated that p53 $\beta$  could associate p53 and upregulated *bax* but not *p21*, suggesting that this isoform favors the expression of apoptotic genes. As the  $\Delta 133p53$  isoform lacks TADs, it cannot induce transcription and play a dominant-negative role by association with p53. For instance, overexpression of  $\Delta 133p53$  with p53 was associated with the inhibition of apoptosis. Moreover, the association of heterodimers  $\Delta 133p53/p53$  promoted cell survival at a low level of oxidative stress (Gong, Pan, Yuan, *et al.* 2016) in HCT116 cell lines and DNA double-strand break (DSB) repair in human induced pluripotent stem (iPS) cells (Gong, Pan, Chen, *et al.* 2016). Finally,  $\Delta 133p53$  seems to be involved in the development of inflammation and autoimmune disease (Campbell *et al.* 2012).

Concerning viral infection, the first evidence of p53 isoform ( $\Delta 133p53$  and p53 $\beta$ ) regulation was highlighted during Influenza A virus infection of human epithelial A549 cells (Terrier *et al.* 2012). Firstly, they observed that total knockdown of p53 in A549 cells was associated with an increase of viral replication and production, and then siRNA silencing of  $\Delta 133p53$  induced a decrease of viral production. In parallel, the siRNA silencing of the p53 $\beta$  transcript led to an increase in viral production. In the absence of p53, no viral decrease has been observed in the presence of isoforms, whereas the co-expression of p53 and  $\Delta 133p53$  led to a considerable increase of viral production and it was inhibited in the presence of p53 $\beta$ . Taken together, these results suggest an inter-connected response of p53 and p53 isoforms during viral infection

where  $\Delta 133p53$  presented a pro- and  $p53\beta$  an anti-viral effect. The different responses involved in p53 and p53 isoforms like apoptosis, cell cycle and immune response could be controlled by viruses.

Recently, Wang and colleagues have shown that KO of p53 in A549 using CRISPR/Cas9 technology was associated with a lesser percentage of infected cells and viral RNA copies compared to WTp53. They demonstrated that attenuated viral dissemination was linked to neither an initial entry process nor a decrease of caspase-3 activity in infected p53 KO cells, but to a direct inhibiting effect of p53 on Interferon-induced transmembrane proteins (IFITMs) controlled by IAV (Wang *et al.* 2018). The mechanisms by which IFITMs inhibit virus infection is not clearly understood, however it has been demonstrated that IFITMs may block virus entry and virus-endosome fusion. In the context of IAV infection, it has been shown that IFITMs restrict IAV exit by blocking fusion pore formation from late endosomes (Brass *et al.* 2009). Moreover, Wang and colleagues have reported that  $\Delta 40p53$  isoform expression in infected A549 cells is associated with a higher percentage of infected cells and fewer transcript levels of IFITMs, revealing a new role of  $\Delta 40p53$  during IAV infection. To explain the effect of p53 during IAV infection, they suggested that p21 may impact IAV infection *via* protein kinase PAK1 previously reported to enhance the viral titer of IAV (Pascua *et al.* 2011). Interestingly, the decrease in IAV infection contradicted the results obtained previously by Terrier's study where the decrease of p53 was associated with an increase in IAV production (Terrier *et al.* 2012). These two studies used A549 cell lines but not the same system to knockout p53.

To conclude, studies on p53 and p53 isoforms in the context of viral infection have revealed new mechanisms by which the cells and virus exploit the effect of the protein on cell fate. Therefore, it seems obvious that the relative expression of p53 isoforms in a cell, tissue and time-dependent manner is one of the most important factors involved in p53 signaling pathways and highlights the attention that should be given during p53 research.



## OBJECTIVES

The chikungunya virus is a re-emerging virus representing a public health issue worldwide. The recent outbreaks have caused disabling diseases in humans with joint and muscle pains. Currently, there are no vaccines or adapted treatments for the disease. Thus, the investigation of alphavirus biology in the mammal host and insect vector is crucial. Indeed, the chikungunya virus is an arbovirus transmitted to vertebrate hosts by the *Aedes* mosquito vector. At the cellular level, the chikungunya virus exhibits lytic infection in mammalian cells, whereas it causes persistent infection in mosquito cells. The involvement of apoptotic cell death as an anti-viral activity has been investigated in mammalian and mosquito cells. However, the cellular mechanisms mediating the induction of apoptosis and its function following viral infection remain enigmatic. As presented in the introduction, the central role of p53 for the control of apoptosis, cell cycle and immune response is increasingly studied in the context of virus infection. The main objective of my project was to study the potential function of p53 during chikungunya infection in mammals and insects.

The first objective was focused on the implication of full length p53 during chikungunya virus infection in a human muscle cell line and in insects using the *in vivo* model *Drosophila melanogaster* and *Aedes* mosquito cell lines. In the human muscle cell line and in *Drosophila melanogaster*, we have investigated the effect of p53 depletion on viral replication and cellular response, focusing on cell death and interferon Type-I signalling pathways. For the mosquito cells we have examined the impact of two CHIKV particles, one produced in mammals and other one in mosquito cells, on p53-target genes.

In the second area of investigation, we have developed molecular and cellular tools to study the potential function of p53 isoforms  $\Delta 40p53\alpha$  and  $\Delta 133p53\alpha$  during chikungunya virus infection in a human muscle cell line. To do this, we have generated CRISPR/Cas9 cell lines overexpressing endogenous  $\Delta 40p53$  in p53 wildtype and p53-/- cell lines. In parallel, we have generated inducible cell lines overexpressing  $\Delta 40p53\alpha$  and  $\Delta 133p53\alpha$ .



# EXPERIMENTAL REPORT

## CHAPTER 1) Opposite effect of p53 on CHIKV infection in a human cell line and *in vivo* in *Drosophila melanogaster*

### 1 Objectives

In this first part, the objective was to investigate the potential role of p53 protein during chikungunya virus infection in a human muscle cell line and in insects using the *in vivo* model *Drosophila melanogaster* and *in vitro* mosquito cell lines. In order to study the potential function of p53 we first analyzed the p53-target genes involved in cell cycle arrest and apoptosis. In parallel, interested by the recent discovery concerning the influence of p53 on antiviral innate immunity we investigated the Type-I interferon production and signaling pathway. Using another approach, CRISPR/Cas9 technology, we investigated the function of p53 in a human primary immortalized muscle cell line and in *Drosophila melanogaster*.

### 2 Material and Methods

#### 2.1 Cell lines and viruses

HEK 293T, BHK-21, VeroE6 and U2OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco<sup>TM</sup> Thermo Scientific) supplemented with 10% Foetal Bovine Serum (FBS, HyClone).

LHCN-M2 (human skeletal myoblasts) immortalized cells were cultured in medium 4:1 Dulbecco's Modified Eagle Medium (DMEM, Gibco<sup>TM</sup> Thermo Scientific)/ Medium 199 (Gibco<sup>TM</sup> Thermo Scientific) supplemented with 15% FBS, 0.02% HEPES, 0.03µg/mL Zinc Sulfate, 1.4µg/mL Vitamin B12, 0.055µg/mL Dexamethasone, 2.5ng/mL recombinant human Hepatocyte Growth Factor and 10ng/mL recombinant human FGF-basic. LHCN-M2 cells were a kind gift of Drs Chun-Hong Zhu and Woodring E. Wright (Zhu et al. 2007). HEK 293T, BHK-21, VeroE6, U2OS and LHCN-M2 cell lines were maintained at 37°C, 5% CO<sub>2</sub> in a humidified incubator.

C6/36 and U4.4 (*Aedes albopictus*) and Aag2 (*Aedes aegypti*) cells were maintained in Leibovitz's L-15 medium (Gibco<sup>TM</sup> Thermo Scientific) supplemented with 10% FBS and 10%

Tryptose Phosphate Broth (TPB, Gibco<sup>TM</sup> Thermo Scientific). Cells were maintained at 28°C in an insect cells incubator.

Chikungunya virus (CHIKV) LR2006 strain from La Réunion and Sindbis virus (SINV) Toto 1101 strain infectious molecular clones were used to generate replicative viruses: from the plasmid clone, viral RNA (vRNA) was generated by *in vitro* transcription using mMessage, mMachine kit, Ambion. Then, 5.10<sup>6</sup> BHK-21 cells were trypsinized, washed in PBS 1 X, resuspend in Opti-MEM medium (Gibco<sup>TM</sup> Thermo Scientific) and electroporated with 10µg of vRNA in a 0.4 cm cuvette using a Gene Pulser Xcell electroporation system (program used: 1 pulse, 270V, 950µF). Electroporated cells were seeded in T-75 flask cultured with DMEM, 10% FBS for 16h at 37°C, 5% CO<sub>2</sub> until medium change. The supernatant was harvested 24 hours later, filtered at 0.2 µm and then mixed with 0.5M sucrose (MP Biomedicals) and 50mM HEPES (Gibco<sup>TM</sup> Thermo Scientific) for conservation at -80°C. Viral stocks were titrated by TCID50 and plaque assay on VeroE6.

## 2.2 Generation of TP53 CRISPR-mediated knockout LHCN-M2 cell line

To analyze the potential role of p53 during CHIKV infection in LHCN-M2 cells, Knock-Out (KO) cells were generated using CRISPR/Cas9 technology. The several steps from design of a single-guide RNA (sgRNA) specific for the gene of interest to the validation of KO cells are presented as following:

### 2.2.1 sgRNA design

The design of single guide RNA (sgRNA) was performed on CHOPCHOP website. The sgRNA must be next to the protospacer adjacent motif (PAM) sequence of gene of interest for Cas9 cleavage and length of 19-21 nucleotides with 50% of GC. Some parameters were chosen to select sgRNA with the highest efficiency score, the highest out-of-frame score, and the smallest risk of off-targets (Table 3). In order to increase the efficiency of KO we have decided to design two different sgRNA, if possible, at the beginning and in the middle of the gene of interest in order to generate two double-strand breaks.

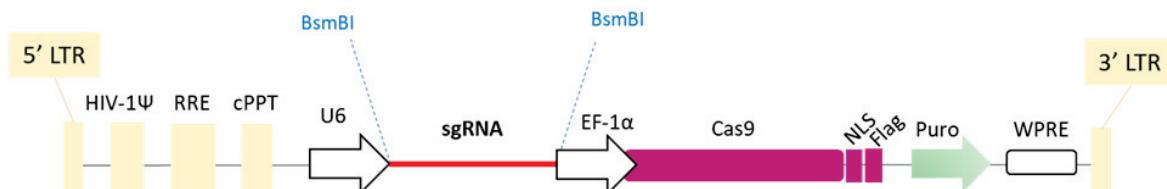
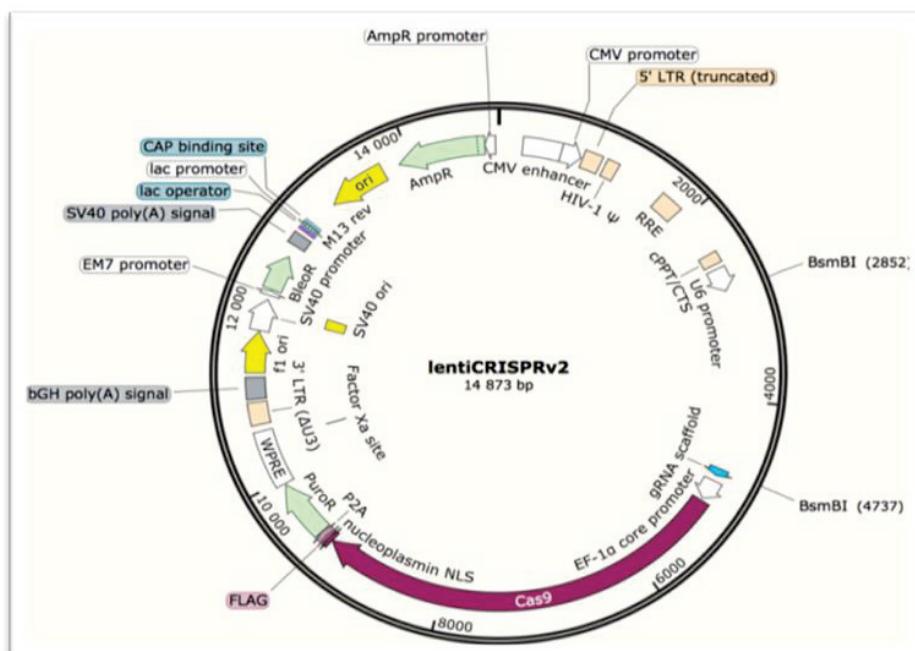
Then, sgRNA were cloned in Addgene lentiCRISPRv2 plasmid (Figure 26). Note that as the lentiCRISPRv2 contains a U6 promoter, a Guanine (in green) was added when primer started with another nucleotide during design of primer to increase transcription efficiency. To finish,

the BsmBI digestion generate an overhang on the plasmid. The sequences in red were added on forward and reverse primers for cloning plus a Guanine G / Cytosine C when the latter was not present on the primer.

The following primers were used for cloning sgRNA in lentiCRISPRv2:

sgRNA name	Sequence 5'-3'	
sgRNA_400_p53	Forward	<b>CACC</b> CCCCGGACGATATTGAACAA
	Reverse	<b>AAAC</b> TTGTTCAATATCGTCCGGGGC
sgRNA_402_p53	Forward	<b>CACC</b> CCCCCTTGCCTGCCAAGCAA
	Reverse	<b>AAAC</b> TTGCTTGGGACGGCAAGGGGC
sgRNA_luc	Forward	<b>CACC</b> GGGCATTCGCAGCCTACCG
	Reverse	<b>AAAC</b> CGGTAGGCTGCGAAATGCC

**Table 3: List of human primers used for cloning of sgRNA in lentiCRISPRv2 vector**



**Figure 26: The lentiCRISPRv2 cloning vector and zoom on the one system sgRNA Cas9.**

### **2.2.2 Cloning in lentiCRISPRv2 plasmid**

Firstly, the lentiCRISPRv2 vector was digested with BsmBI restriction enzyme (NEB) in NEB Buffer<sup>TM</sup> 3.1 for 1h at 55°C then 20minutes at 80°C for inactivate enzyme. Then, digested product was purified from agarose gel using Macherey-Nagel<sup>TM</sup> NucleoSpin<sup>TM</sup> Gel and PCR Clean-up Kit.

In parallel, each pair of primers (2 $\mu$ g) were annealed using an annealing buffer (10 mM Tris-HCl pH 8, 50 mM NaCl and 1 mM EDTA) for 3minutes at 90°C following by incubation of 15minutes at 37°C.

Ligation of annealed primers and digested lentiCRISPRv2 was performed using a ligation kit (Rapid DNA ligation kit, Roche) for 30minutes at Room Temperature (RT) and then transformed into DH5 $\alpha$  *E. coli* competent bacteria. On growth colonies, PCRs were realized to detect the insert DNA in lentiCRISPRv2 using the forward of primer of interest and reverse in the backbone. The positive clones were amplified and purified before being digested for verification.

### **2.2.3 Lentivirus production**

The generation of lentivirus allow to generate stable cell lines expressing the sgRNA of interest and the Cas9 nuclease. Day first, 2,5.10<sup>6</sup> HEK 293T cells were seeded in cell culture dish in 8 mL DMEM + 10% FBS. The next day, cells were co-transfected using calcium phosphate co-precipitation method by adding 8.3  $\mu$ g of HIV packaging construct with a CMV promoter (psPAX2, AddGene 12260), 8 $\mu$ g of the lentiCRISPRv2\_sgRNA of interest and 2.5 $\mu$ g of VSV glycoprotein-expressing construct with CMV promoter (pVSVg, AddGene 8454). Media were changed 16 hours after transfection, and 24 hours later supernatant were harvested, filtered through 0.45  $\mu$ m pore-sized membranes and stored at -80°C before transduction of target cells.

### **2.2.4 Stable cell line generation**

Target cells were seeded in P6-well plate at 2.10<sup>5</sup> density in 2mL of medium and transduced day after with lentivirus previously produced. Day after transduction, cells were treated with puromycin to select only cells where the lentiCRISPRv2\_sgRNA had been incorporated into DNA. This puromycin selection was maintained until all non-transduced control cells were

dead. After that, transduced cells were amplified during a week and their phenotype was analyzed.

#### **2.2.5 Verification of gene knock-out by Western blot**

To confirm TP53 gene knockout we used SDS-PAGE using different p53 antibodies described below (6. Western Blot).

### **2.3 Infection of knockout cell lines with CHIKV**

LHCN-M2 Wild Type (WT), sgRNA\_luc and sgRNA\_p53 were seeded whether in 48-well plate at 30 000 cells/well, or in 6-well plate at 250 000 cells/well or in 12-well plate at 140 000 cells/well and the day after recounted to adapt the multiplicity of infection (MOI). Cells were incubated with CHIKV MOI 0.1 for 1h30 at 37°C and then washed once with PBS 1X and culture in LHCN-M2 medium for 6, 8, 12, 16, 24, and 36 hours. At these different times, cells were collected for different analysis. Cells were seeded in a BioSafety level 2 (BSL2) laboratory and for CHIKV and SINV infections, cells were manipulated in BioSafety level 3 (BSL3) laboratory.

### **2.4 Transfection of plasmid Flag-RIG-I 2CARD**

LHCN-M2 immortalized cell lines WT, sgRNA\_luc and sgRNA\_p53 were cultured in complex LHCN-M2 medium and transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The cells were seeded in 6-well plate at 250 000 cells/well and the day after transfected with 10 or 100 ng of plasmid Flag-RIG-I CARD for 24 hours, kindly provided by Dr. Ratinier (Versteeg *et al.* 2013). The plasmid expresses the two CARD domains of retinoic acid inducible gene I (RIG-I) which activate MAVS and allow the induction of IFN production and signaling pathway.

### **2.5 Flow cytometry analysis**

After CHIKV infection in 48-well plate and at different times post-infection, cells were fixed in 2% paraformaldehyde for 15 minutes. Then, fixed cells were washed twice in PBS 1X and incubated with anti-viral capsid primary antibody (1/800) in permeabilization solution A (0.1% saponin, 10% FBS, PBS 1X) for 1 hour at 4°C. After primary antibody incubation, cells were

washed twice with solution A before being incubated with FITC conjugated anti-mouse IgG secondary antibody (1/200) (F0257, Sigma Aldrich) in solution A for 45 minutes at 4°C. After that, cells were washed twice with PBS 1X and immunostained cells were analyzed using flow cytometer FACS Calibur, BD Biosciences.

## 2.6 Western blot

After CHIKV infection in 6-well plate and at different times post-infection, cells were lysed in 200µl of cell Lysis Buffer 1X (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, Cell Signaling Technology®) supplemented with Phosphatase and Inhibitors Protease 1X (Sigma) and Dithiothreitol (DTT) at 1mM. Cell lysates were sonicated on ice and after 30 minutes of incubation on ice, centrifuged 10 minutes, 11 000g, 4°C. The supernatants were transferred in new tubes and a Bradford protein quantification was performed. 50µg of cell extracts were loaded on SDS-PAGE gel and transferred on nitrocellulose membrane as indicated on manufacturer's protocol (Biorad). Membranes were blocked in 10% milk, PBS 1X, 0.1% Tween20 for 2 hours at Room temperature (RT) and incubated with appropriated primary antibodies overnight in agitation at 4°C. Day after, membranes were washed three times for 5 minutes with PBS 1X, 0.1% Tween20 at RT and secondary antibodies were incubated 2h in agitation at RT. After three new washes, proteins were revealed with SuperSignal™ chemiluminescence substrate (Thermo Scientific) using ChemiDoc® imaging system (Biorad).

PRIMARY ANTIBODIES	PROVIDER - Reference	DILUTION, BUFFER
Mouse anti-p53 DO-1	Santa Cruz sc-126	1:1000, 5% milk PBS Tween20 0,1%
Mouse anti-p53 DO-11	BIO-RAD MCA1704	1:1000, 5% milk PBS Tween20 0,1%
Rabbit anti-p53 CM1	Biolegend 925401	1:1000, 5% milk PBS Tween20 0,1%
Rabbit anti-PARP	Cell Signaling Technology #9542	1:1000, 5% milk, PBS Tween20 0.1%
Rabbit anti-Bax	Cell Signaling Technology #2772	1:500, 5% BSA, PBS Tween20 0.1%
Mouse anti-Capsid alphavirus (SFV-C)	Hybridoma	1:300, 5% milk, PBS Tween20 0.1%
Rabbit anti-NF-κB p65 (D14E12)	Cell Signaling Technology #8242	1:1000, 5% milk, PBS Tween20 0.1%
Rabbit anti-α Tubulin	Abcam ab15246	1:1000, 5% milk, PBS Tween20 0.1%
Mouse anti-Lamin A	Abcam ab8980	1:1000, 5% milk, PBS Tween20 0.1%

Table 4: List of primary antibodies used for Western blot

SECONDARY ANTIBODIES	PROVIDER - Reference	DILUTION, BUFFER
Anti-rabbit IgG Peroxidase conjugate	Sigma Aldrich A6154	1:5000, 10% milk, PBS Tween20 0.1%
Anti-mouse IgG Peroxidase conjugate	Sigma Aldrich A5906	1:10 000, 10% milk, PBS Tween20 0.1%
Anti-β Actin Peroxidase conjugate	Sigma Aldrich A3854	1:75 000, 10% milk, PBS Tween20 0.1%

Table 5: List of secondary antibodies used for Western blot

## 2.7 RNA extraction and RT-qPCR

After CHIKV infection in 12-well plate and at different times post-infection cells were lysed in 350µl RA1 buffer from NucleoSpin® RNA kit (Macherey Nagel) supplemented with Beta-mercaptoethanol (1/100). The total RNA was extracted according to the manufacturer's protocol. RNA was reverse transcribed in complementary DNA (cDNA) using PrimeScript™ RT-PCR kit (Takara). Specifics cDNA were quantified by qPCR using SYBR Premix Ex TaqII (Takara) using AriaMx system (Agilent AriaMx).

List of oligos used for qPCR:

Oligo Name	Sequence 5'-3'	
h_p21	Forward	CCAGCATGACAGATTCTACCA
	Reverse	GAACCTCTCATCAACCGCC
h_Gadd45	Forward	TCTCCCTGAACGGTGTGGCA
	Reverse	ACTCACTCAGCCCCGGCA
h_Puma	Forward	GAGCAGGGCAGGAAGTAAC
	Reverse	CTCCCTGGGGCCACAAATC
h_Bax	Forward	TTTGCTTCAGGGTTCATCCA
	Reverse	GCAGCTCCATGTTACTGTCC
h_Bcl2	Forward	TCTTGAGTTGGTGGGTC
	Reverse	CAAATAACACAAGGGCATCCCAG
h_IFNβ	Forward	GTGTCTCCTCAAATTGCTC
	Reverse	TGTCAAAGTTCATCCTGTCC
h_Mx1	Forward	CAGGACCATCGGAATCTGAC
	Reverse	GGCACTTGACAATCATGTAACC
h_IFIT1	Forward	GGACAGGAAGCTGAAGGAG
	Reverse	AGTGGGTGTTCTGCAA
CHIKV_nsp2	Forward	CAAAGAAGACAAAGCATACTCACC
	Reverse	TCCGCGTAATACACAGACAC

h_RNA5S	Forward	GTCTACGGCCATACCACCCCTG
	Reverse	AAAGCCTACAGCACCCGGT
h_GusB	Forward	GATTGCCAATGAAACCAGGTATC
	Reverse	ACACGCAGGTGGTATCAGTCTT
h_RpL22	Forward	TCGCTCACCTCCCTTCTAA
	Reverse	TCACGGTGATCTGCTCTG
h_RpL27	Forward	ATCGCCAAGAGATCAAAGATAA
	Reverse	TCTGAAGACATCCTTATTGACG

Legend: h=*homo sapiens*

**Table 6: List of mammalian oligos used for the RT-qPCR**

## 2.8 Cell viability assays

In order to monitor the effect of viral infection on LHCN-M2 Wild Type (WT), LHCN-M2 sgRNA\_luc and sgRNA\_p53 viability, the ATP was quantified in cells and supernatants using CellTiter-Glo® luminescence cell viability assay kit (Promega). Cells were plated on 96-well plate day one at 10 000 cells/well in 100µl of LHCN-M2 medium. At 8, 24, 36, 48 and 72 hours, 100µl of cell titer was added on cells and ATP quantification was measured by luminescence using a plate reader (Victor<sup>2</sup> plate reader, Perkin Elmer).

## 2.9 Subcellular fractionation

To analyze the repartition of specific proteins between the cytoplasmic and nucleic fractions in CHIKV-infected cells, subcellular fractionation was carried out. Cells were seeded in T-150 flask at 5.10<sup>6</sup> cells/T-150. Day after, cells were infected with CHIKV at MOI 0.1 for 1 hour 30 at 37°C until medium change. At 8, 16 and 24 hours post-infection, cells were washed with PBS 1X, trypsinized and centrifuged 600g, 5 minutes at 4°C. Pellets were washed once with PBS 1X and centrifuged again 600g, 5 minutes at 4°C before separation of nucleus and cytoplasm using firstly solution A (10mM Hepes, 10mM NaCl, 1mM KH<sub>2</sub>PO<sub>4</sub>, 5mM NaHCO<sub>3</sub>, 1mM CaCl<sub>2</sub> and 0.5mM MgCl<sub>2</sub>) supplemented with 1mM Dithiothreitol (Sigma), 5mM EDTA (Sigma) and 1X Phosphatase and Inhibitors Protease (Sigma). The resuspended pellet in 500µl of solution A was placed into a tissue/cells grinder 1mL, Loose pestle (Wheaton) and 30 repetitions of moving the pestle up and down were applied. Then, the mixture was centrifuged 600g, 5 minutes at 4°C and supernatant (~420 µl - cytoplasmic fraction) was placed in a new 1.5mL tube

supplemented with 6X Laemmli and heated 10 minutes at 95°C for virus inactivation and Western blot analysis.

The pellet (nucleic fraction) was then resuspended in 500µl of solution B (10 mM TrisHCL pH 7.5, 300mM sucrose and 1mM EDTA pH 8) supplemented with 0.1 % of NP-40 (Sigma) and 1X Phosphatase and Inhibitors Protease (Sigma). Once again, resuspended pellet was place into tissue/cells grinder 1mL, Loose pestle (Wheaton) and 30 repetitions of moving the pestle up and down were applied. The exploded nucleus was centrifuged 600g, 5 minutes at 4°C and washed with complete solution B twice before being resuspended in 60µl of same solution supplemented with 6X Laemmli and heat 10 minutes 95°C. Cytoplasmic and nucleic fractions were stored at -20°C before analysis by SDS-PAGE using same protocol described in section 6. **Western blot.** The Tubulin and Lamin A were used to control the separation of cytoplasm and nucleus, respectively.

## 2.10 In vivo *Drosophila melanogaster*

Flies were reared on standard medium at 25°C. The following strains were used: w1118(wild-type), p53<sup>5A1-4</sup>null allele (Bloominutesgton Stock center, BL6815). These two strains were given by Professor B. Mollereau (ENS, Lyon, LBMC – Apoptosis and neurogenetics), (Rong *et al.* 2002). In this study we used only 3 to 6 days old adult male flies.

### 2.10.1 Detection of *Wolbachia* by PCR and tetracycline treatment

Flies were diagnosed for *Wolbachia* strains by PCR and positive *Wolbachia* flies were treated with Tetracycline into fly medium at final concentration of 0.25 mg/mL for at least two generations and tested again. Three independent flies per line were tested: flies were freeze 15 minutes at -20°C and then squashed in 50µl of squashing Buffer (10mM TrisHCL pH 8.2, 1mM EDTA, 25mM NaCl and 200µg/mL proteinase K) for 30 minutes at 37°C before proteinase K inactivation by heating 10 minutes at 95°C. PCR detection of *Wolbachia* strains was carried out using following primers: wsp81Fw 5'- TGGTCCAATAAGTGATGAAGAAC-3' and wsp691Rv 5'- AAAAATTAAACGCTACTCCA-3' (W. Zhou, Rousset, and O'Neil 1998).

### **2.10.2 Fly injection**

Three to six-day old male flies were injected intrathoracically with 46 nL of appropriate MOI of virus (200 PFU per *D. melanogaster*) using a nanoinjector (Nanoject II, Drummond Scientific) (Roy et al. 2020). CHIKV LR2006 strain and SINV Toto 1101 strain were collected in supernatants 24 hours post-infection of VeroE6 cells, filtered at 0.2µm and store at -80°C without sucrose and Hepes before TCID50 and plaque assays titrations.

After injections, at the indicated time points, different numbers of flies were pooled and harvested either for total RNA extraction using NuceoZOL reagent (Macherey Nagel) or for TCID50 using crushing medium (DMEM, 4% FBS, amphotericin 2.5 µg/mM, Nystatin 100U/mL, Gentamicin 50 µg/mL and penicillin/Streptomycin 50 g/mL).

### **2.10.3 Survival curve**

For survival curve, two independents experiments were carried out. First day, 15 flies of each strain (w1118 and p53-/-) were injected either with CHIKV virus or with Mock medium and incubated until we observed the death of all of one of the two strains (CHIKV vs. Mock). Each day, alive and dead flies were counted, and experiments were analyzed using “Survival” table of GraphPad Prism 6 software®.

### **2.10.4 RNA extraction of whole fly**

For total RNA extraction, two injected male flies were pooled in 500 µl of NuceoZOL reagent and crushed using a manual polypropylene pestle (Sigma-Aldrich). Crushed flies were centrifuged, 12 000g, 5 minutes at 4°C and supernatant was collected and stored at -80°C before RNA was extracted according to the manufacturer’s protocol NucleoSpin® RNA Set for NucleoZOL (Macherey Nagel).

Then, RNA was reverse transcribed in complementary DNA (cDNA) using PrimeScript™ RT-PCR kit (Takara). Specifics cDNA were quantified by qPCR using SYBR Premix Ex TaqII (Takara) using AriaMx system (Agilent).

List of oligos used for qPCR:

Oligo name	Sequence 5'-3'	
Dm_TBP	Forward	GTTTCCCTGCAAAGTTCCCTC
	Reverse	GCACCATACGATAGATTAAGCC
Dm_EF1	Forward	GCGTGGGTTTGATCAGTT
	Reverse	GATCTTCTCCTGCCCATCC
Dm_Actin	Forward	GCGTCGGTCAATTCAATCTT
	Reverse	AAGCTGCAACCTCTCGTCA
CHIKV_nsp2	Forward	CAAAGAAGACAAAGCATACTCACC
	Reverse	TCCCGCGTAATACACAGACAC
SINV_nsp3	Forward	AAAACGCCTACCATGCAGTG
	Reverse	TTTTCCGGCTGCGTAAATGC

**Table 7: List of *Drosophila melanogaster* oligos used for RT-qPCR**

Legend: Dm= *Drosophila melanogaster*; TBP=TAT-Box Binding protein; EF1=Elongation Factor 1. Primers targeting Actin mRNA (Ponton *et al.* 2011); Primers targeting SINV nsp3 (van Mierlo *et al.* 2014).

### 2.10.5 TCID50/mL

For TCID50 titration, 5 to 10 flies were drowned in 1 mL of PBS 1X supplemented with 50µL of dishwasher soap before being washed in PBS 1X twice. Then, each fly was placed in 1mL of crushing medium (DMEM, 4% FBS, amphotericin 2.5 µg/mM, Nystatin 100U/mL, Gentamicin 50 µg/mL and penicillin/Streptomycin 50 g/mL) to be crushed two times in a row using a TissueLyser II, Qiagen (program: 2 minutes, High 30). 900 µl of Supernatants were collected and stored at -80°C before TCID50 titration by dilution 10 to 10 on VeroE6 and reading of Cytopathic effects 4 days later.

### 2.10.6 Statistical analysis

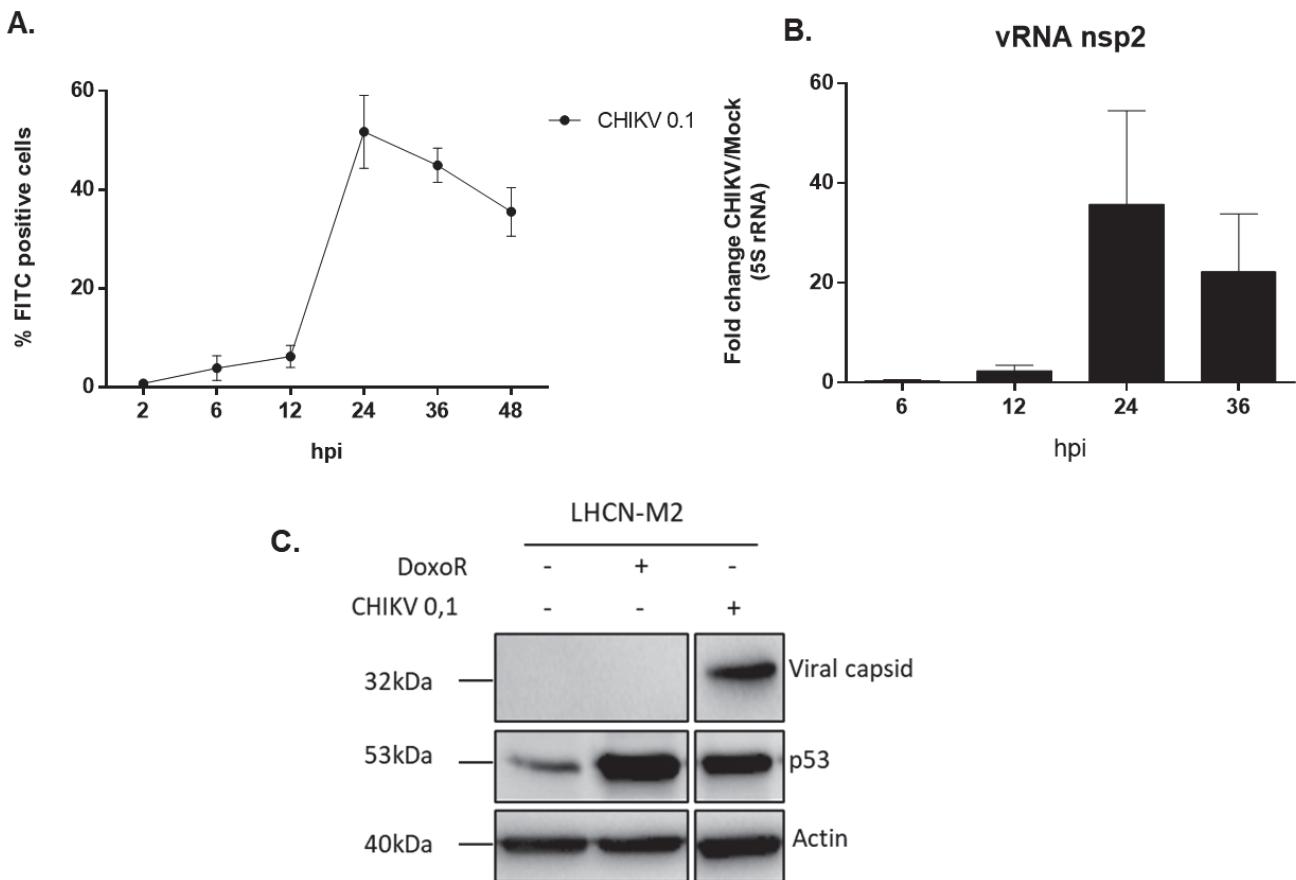
Statistical analyses were performed using GraphPad Prism software, version 6.01 for Windows. The unpaired t-test and survival test were used for statistical comparisons. When applicable (at least three independent experiments), data were represented as mean ± standard deviation (SD) and a p-value of 0.05 or less was considered as significant. ns: nonsignificant p-value > 0.05; \* p-value <0.05; \*\* p-value <0.01 and \*\*\* p-value <0.001.

### 3 Results

#### In mammals: innate immune antiviral activity of p53 in human skeletal muscle cells infected with CHIKV

##### 3.1 Infection of an LHCN-M2 cell line by CHIKV induces stabilization of p53 protein

As it has been reported that CHIKV infects satellite muscle cells, we decided to use the immortalized human skeletal muscle cell line LHCN-M2 (Zhu et al. 2007) which represents a relevant cellular model for CHIKV studies. First, we investigated the permissiveness of cells by detecting the percentage of infected cells over time by flow cytometry, targeting the viral capsid, and the efficiency of viral replication *via* quantitative reverse transcription-PCR (RT-qPCR), targeting a fragment of nsP2 (Figure 27 A. and B.). We observed that CHIKV (MOI=0.1) could infect and replicate efficiently in LHCN-M2 overtime with an exponential phase from 12 to 24 hours post-infection (hpi) and a decrease of CHIKV-positive cells from 24 to 48 hpi associated with cytopathic effects (CPE) and the complete destruction of an infected cell layer after 48 hours. Then, the stabilization of p53 protein was investigated in CHIKV-LHCN-M2 infected cells at 24 hpi (Figure 27 C.) and we observed that CHIKV infection led to p53 accumulation at 24 hpi. The positive control of p53 stabilization was obtained by the treatment of LHCN-M2 with 0.8  $\mu$ M of doxorubicin which induced DNA damage.



**Figure 27: The efficient infection and replication of CHIKV in LHCN-M2 cells was associated with p53 stabilization. The LHCN-M2 cells were infected with CHIKV at MOI 0.1. At different time points post infection, cells were either collected and fixed in 4% paraformaldehyde (PFA) for viral capsid immunostaining (A.) or collected for RT-qPCR assay (B.), or protein expression by western blot (C.).**

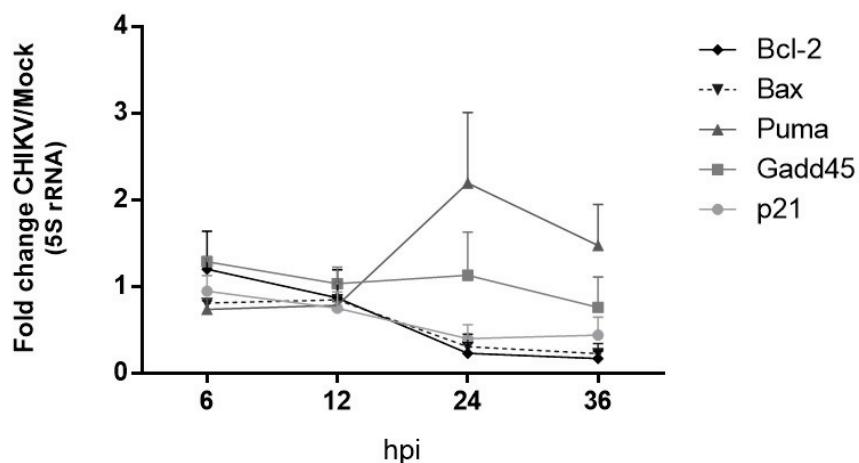
- CHIKV infection kinetics in LHCN-M2. Cells were infected with CHIKV, fixed in 4 % PFA at different time points before immunostaining with a primary antibody raised against SFV nucleocapsid protein (SFV-C antibody) which reacts with the CHIKV capsid protein, and then analyzed by flow cytometry analysis. Data are the mean  $\pm$  SD of three independent experiments.
- CHIKV replication analysis in LHCN-M2. Cells were infected with CHIKV, then harvested at different time points post infection with Buffer RA1 (Macherey Nagel) supplemented with  $\beta$ -mercaptoethanol, after which viral replication was analyzed by RT-qPCR. The quantification of CHIKV nsp2 RNA was measured and normalized on housekeeping gene 5S rRNA ( $n=2$ ).
- CHIKV capsid expression and p53 protein stabilization in LHCN-M2 at 24 hpi. SFV-C mouse antibody was used to detect CHIKV capsid and p53 DO-1 mouse antibody was used to detect p53 protein stabilization in CHIKV-infected LHCN-M2. The antibody raised against housekeeping protein  $\beta$ -actin was used as an internal control ( $n=3$ ).

### **3.2 Infection of LHCN-M2 cell line by CHIKV induces Type I interferon immune signaling response but not cell cycle arrest and not apoptotic p53 dependent**

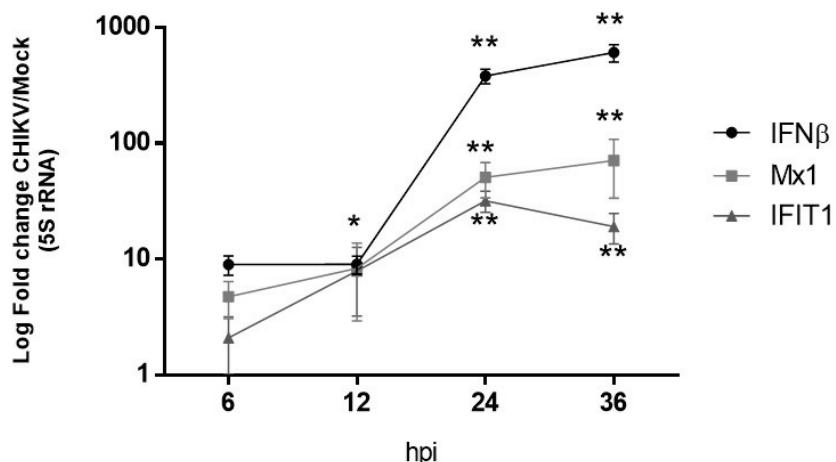
To examine the function of p53 stabilization during CHIKV infection, we examined p53 target gene mRNA expression involved in cell cycle arrest (P21 and GADD45) and apoptosis (PUMA, BAX and anti-apoptotic BCL-2), and the interferon beta (IFN $\beta$ ) expression and IFN Type-I signaling pathway by targeting Interferon-stimulated genes (ISGs: MX1 and IFIT1) over time (Figure 28 A. and B.). We observed no significant regulation of Bcl-2, p21, Gadd45 and Bax mRNA. However, the Puma mRNA seemed to be upregulated at 24 hours post infection but the results were not significant because of the variability of the data (Figure 28 A.). From 12 to 36 hours post infection, the targets genes tended to decrease during CHIKV infection. In parallel, IFN $\beta$  mRNA and Interferon stimulated genes Mx1 and IFIT1 increased drastically from 12 to 36 hours post infection (Figure 28 B.).

The infection of LHCN-M2 with CHIKV at MOI 0,1 induced the production of IFN $\beta$  and the Type-I interferon signaling pathway, whereas the cell cycle genes and apoptotic genes dependent on p53 transcription were not transcriptionally induced. Then, as we observed p53 accumulation, we generated p53 knockout LHCN-M2 cells using CRISPR/Cas9 technology to investigate its potential activity.

**A.**



**B.**



**Figure 28: The infection of LHCN-M2 with CHIKV induced Type-I interferon production and signaling pathway but no regulation of the p53 target genes between 6 to 36 hours post-infection.** Cells were infected with CHIKV at MOI 0.1 and then harvested at different time points post infection. The expression level of p53 target genes (A.) and Type I interferon pathway genes (B.) were monitored by RT-qPCR. The expression level of target genes was measured and normalized against housekeeping gene 5s rRNA. The values of infected WT cells were compared to mock cells. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $<0.01$ .

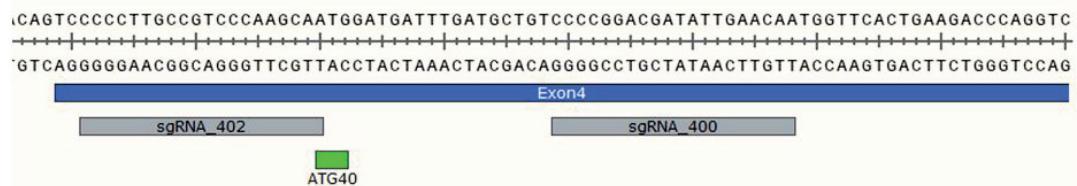
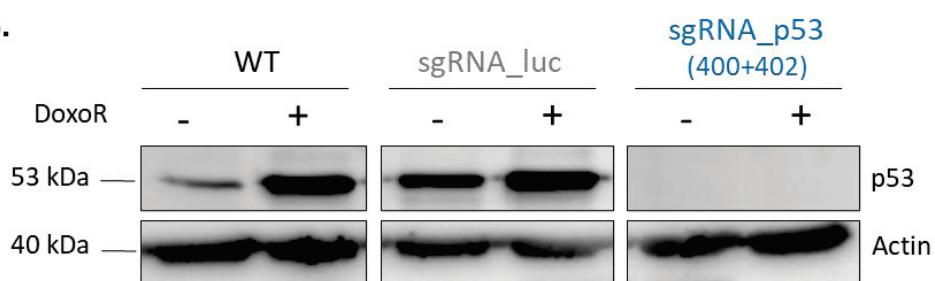
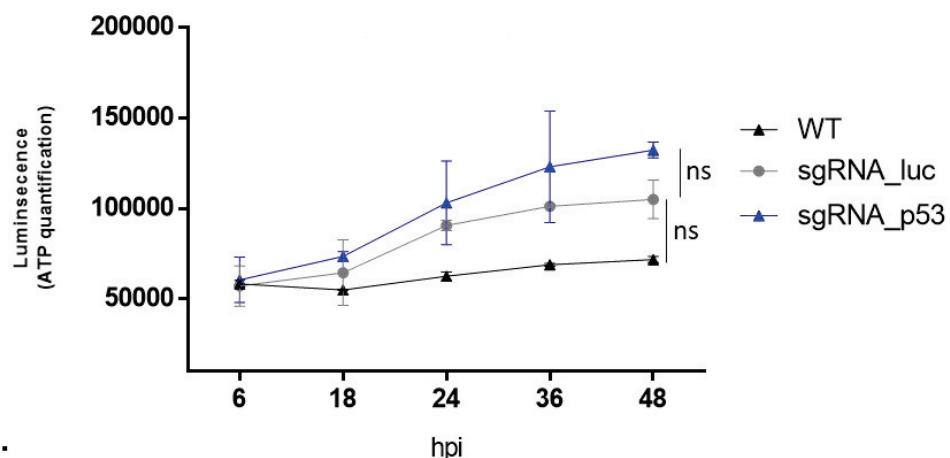
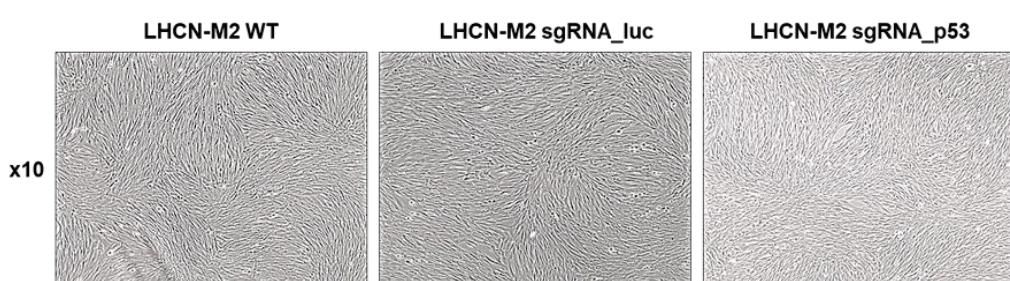
### 3.3 Effect of LHCN-M2 p53 deletion on CHIKV infection and cellular outcome

#### 3.3.1 Generation of p53 knockout LHCN-M2 (sgRNA\_p53) and luciferase (sgRNA\_luc) control cell line using CRISPR/Cas9 technology

To investigate the role of p53 protein in LHCN-M2 infected by CHIKV we generated p53 knockout LHCN-M2 cells using CRISPR/Cas9 technology. To avoid off-targets, sgRNAs were designed with the lowest risk of off-target score and the highest efficiency score. To start, we wanted to study the role of p53 without looking in detail at the different p53 isoforms. Thus, the induction of double-strand DNA damage was managed at the level of the first promoter of the p53 gene, upstream and downstream of ATG40 (Figure 29). We thought this would damage the promoter part of the coding transcript of full-length p53 and Δ40p53. We were not able to correctly target the short promoter region of Δ133p53, considering the necessary characteristics of single guide RNA design. Thus, we decided not to consider the Δ133p53 isoform. The transcript of the Δ133p53 isoform could theoretically be expressed in cells.

Next, each sgRNA was annealed and then cloned into the lentiCRISPRv2 vector and used for the generation of VSVg pseudo-particles which allow the insertion of the sgRNA-Cas9 sequence into the LHCN-M2 genome thanks to the flanked HIV-LTR. Adherent LHCN-M2s were transduced with pseudo-particles containing specific sgRNA-Cas9 and 24 hours later selected with puromycin antibiotic treatment. To increase the efficiency of p53 knockout, both sgRNAs (400 and 402) were co-transduced (Figure 29 A.).

The CRISPR/Cas9 control cell line was generated by transduction of the sgRNA targeting luciferase gene. Indeed, luciferase comes from the firefly and the sgRNA luciferase (sgRNA\_luc) is theoretically not able to target human genes. Thus, the LHCN-M2 sgRNA\_luc cell line underwent different steps for the generation of CRISPR-mediated knockout without presenting a specific protein knockout. After antibiotic selection, the cells were cultured for one week before the analysis of the p53 profile by Western blot.

**A.****B.****C.****D.**

**Figure 29: Generation and validation of the p53 knockout LHCN-M2 cell line using CRISPR/Cas9 technology.**

**A.** Positions of Single guide RNA 400 and 402 on p53 gene. **B.** Validation of full length p53 knockout LHCN-M2 using CRISPR/Cas9 technology. **C.** Effect of CRISPR-mediated p53 knockout on LHCN-M2 viability ( $n=3$ ). The values of WT cells were compared to sgRNA\_luc and the values of sgRNA\_p53 compared to sgRNA\_luc. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $>0.05$ . ns: nonsignificant. **D.** cellular morphology of LHCN-M2 wildtype, sgRNA\_luc and sgRNA\_p53 (Objectif x10).

In order to be sure of the knockout, the LHCN-M2 sgRNA\_p53 cells were treated with doxorubicin to detect possible p53 stabilization after DNA-damage (Figure 29 B.). These results showed an undetectable level of p53 protein and accumulation after doxorubicin treatment, suggesting a loss of p53 expression in the LHCN-M2 sgRNA\_p53 cell line. The generation of control CRISPR LHCN-M2 sgRNA\_luc cell line presented a higher quantity of p53 protein in untreated cells and a higher p53 stabilization after doxorubicin treatment, compared to treated LHCN-M2 WT. As the CRISPR/Cas9 system is based on double-strand DNA breaks, integrated in the host genome and constitutively active, it seemed that the double-strand breaks induced by Cas9 could activate the p53 signaling pathway after DNA-damage detection. Indeed, it has been recently demonstrated that CRISPR/Cas9 technology induces a p53-mediated cell cycle arrest through the p53-p21-pRb axis (Haapaniemi *et al.* 2018).

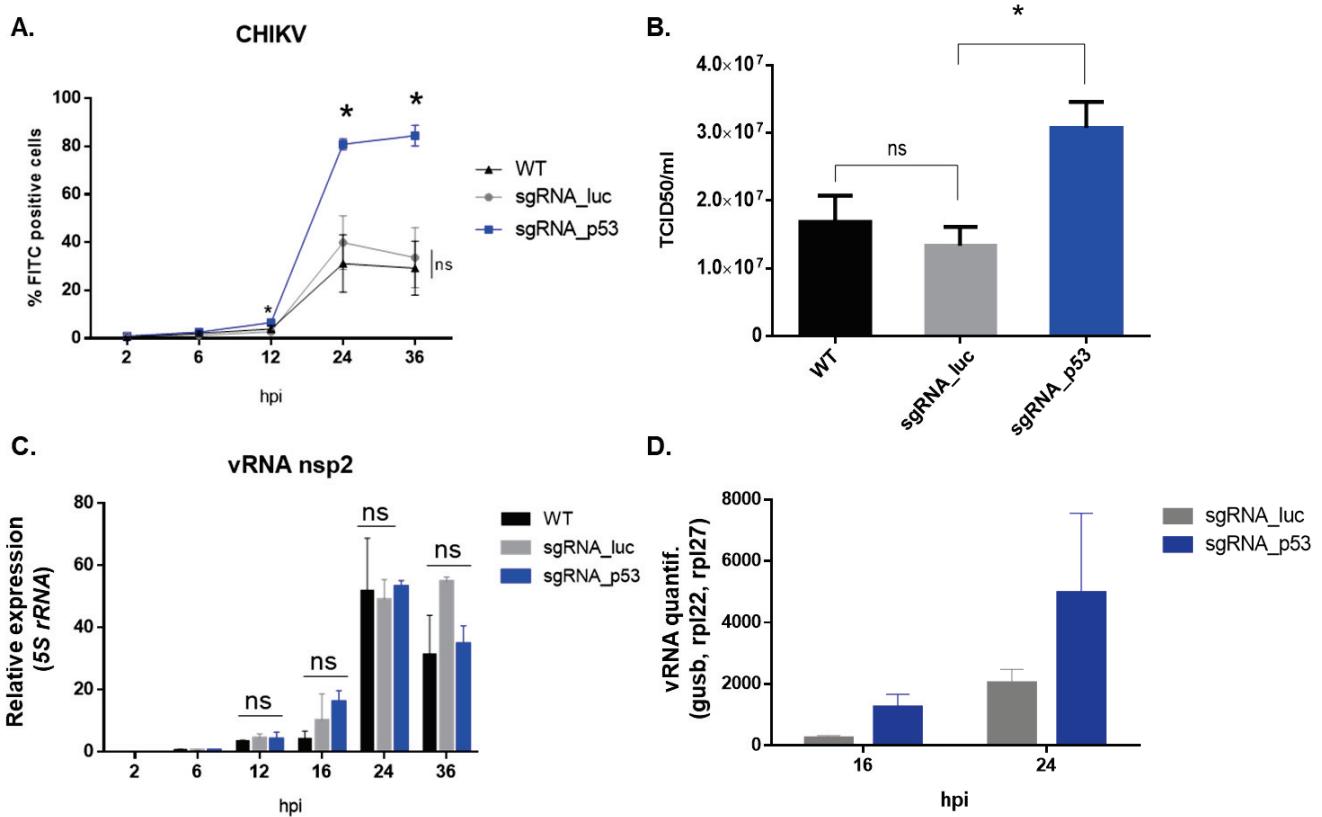
Moreover, we compared the cell viability of LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 by ATP quantification using the CellTiter-Glo® luminescent assay kit (Promega). We observed that cell viability and growth in sgRNA\_luc and sgRNA\_p53 were higher than LHCN-M2 WT but the differences were not significant (Figure 29 C.). Observation under the microscope of cellular morphology at 48 hours post-seeding showed a similar density of sgRNA\_luc and sgRNA\_p53 cells compared to WT cells (Figure 29 D.).

Taken together, these results first indicated that we generated a p53 knockout LHCN-M2 cell line without any detectable p53 protein and p53 protein isoforms, using two different antibodies which recognize all p53 isoforms as targeting a DNA-binding domain. These results also indicated that it seemed essential to have this CRISPR/Cas9 control sgRNA\_luc cell line to analyze the effect of specific protein knockout using CRISPR/Cas9 technology, especially for our investigation into the transcriptional factor p53 function during CHIKV infection.

After the generation and validation of p53 knockout LHCN-M2, the first objective was to study the effect of p53 knockout on CHIKV infection.

### 3.3.2 Infection of p53 knockout LHCN-M2 with CHIKV

LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 were infected with CHIKV at MOI 0.1 at several time points (from 2 hours to 48 hours) and the intracellular viral capsid was detected by immunostaining. The percentage of infected cells was analyzed by flow cytometry assay. The results showed that the susceptibility of LHCN-M2 sgRNA\_p53 was significantly higher than sgRNA\_luc and WT infected cell lines from 6 to 36 hours (Figure 30 A.). Viral production was assessed by TCID50/ml on VeroE6 and showed that more infectious particles were produced from sgRNA\_p53 than WT and control sgRNA\_luc cell lines (Figure 30 B.). Viral replication was evaluated by RT-qPCR targeting a fragment of nsP2 sequence. The viral fragment was normalized either on a gene transcribed by Polymerase III (5S rRNA) to take into account the cellular transcriptional shut-off mediated by viral nsP2 through the degradation of subunit RPB1 of Polymerase II (Figure 30 C.), or on housekeeping genes transcribed by RNA Polymerase II (the average expression of GUSB, RPL22 and RPL27- Figure 30 D.). The quantity of nsp2 RNA normalized on 5S rRNA mRNA showed no differences in viral replication comparing the three cell lines, whereas the normalization on the average expression of gusb, rpl22 and rpl27 mRNA demonstrated a higher viral replication in sgRNA\_p53 than in sgRNA\_luc. Given that CHIKV nsP2 induces the degradation of subunit RPB1 of RNA polymerase II, the housekeeping genes transcribed by RNA polymerase II decreased drastically from 12 hpi but not the 5S rRNA as transcribed by RNA polymerase III (data not shown). Using 5S rRNA transcribed by RNA polymerase III we obtained a constant expression level of the housekeeping gene between mock and infected cells. We suggest using housekeeping genes transcribed by polymerase II because it is representative of the state of the cell. However, we decided to normalize the viral and cellular RNA on housekeeping gene 5S rRNA for the following experiments.



**Figure 30: The CHIKV infection in p53 knockout LHCN-M2 cell lines enhances higher viral infection and production.**

- A.** *CHIKV infection kinetics in LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 cell lines:* cells were infected with CHIKV at MOI 0.1 and collected at different times. The harvested cells were fixed in PFA 4% before immunostaining with primary antibody targeting alphavirus capsid and reaction with the CHIKV capsid. The immunostained cells were analyzed by flow cytometry analysis. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $<0.05$ .
- B.** *CHIKV infectious particle production from LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53:* Cells were infected with CHIKV at MOI 0.1 for 24 hours. The supernatants were collected and titrated on VeroE6 by limiting dilution. The TCID<sub>50</sub>/ml was obtained using the Reed and Muench method. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $<0.05$ .
- C. and D.** *Expression level of specific sequences of CHIKV nsP2 in LHCN-M2 sgRNA\_luc and sgRNA\_p53 using different housekeeping genes for normalization:* Cells were infected with CHIKV at MOI 0.1 and collected at several different time points for RT-qPCR analysis targeting CHIKV nsP2 sequence. **C.** The expression level of target genes was measured and normalized on housekeeping gene 5S rRNA. The values of WT cells were compared to sgRNA\_luc and the values of sgRNA\_p53 compared to sgRNA\_luc. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $>0.05$  ns: nonsignificant. **D.** The expression level of the target gene was measured and normalized on the average of housekeeping genes gusb, rpl22 and rpl27 (n=2).

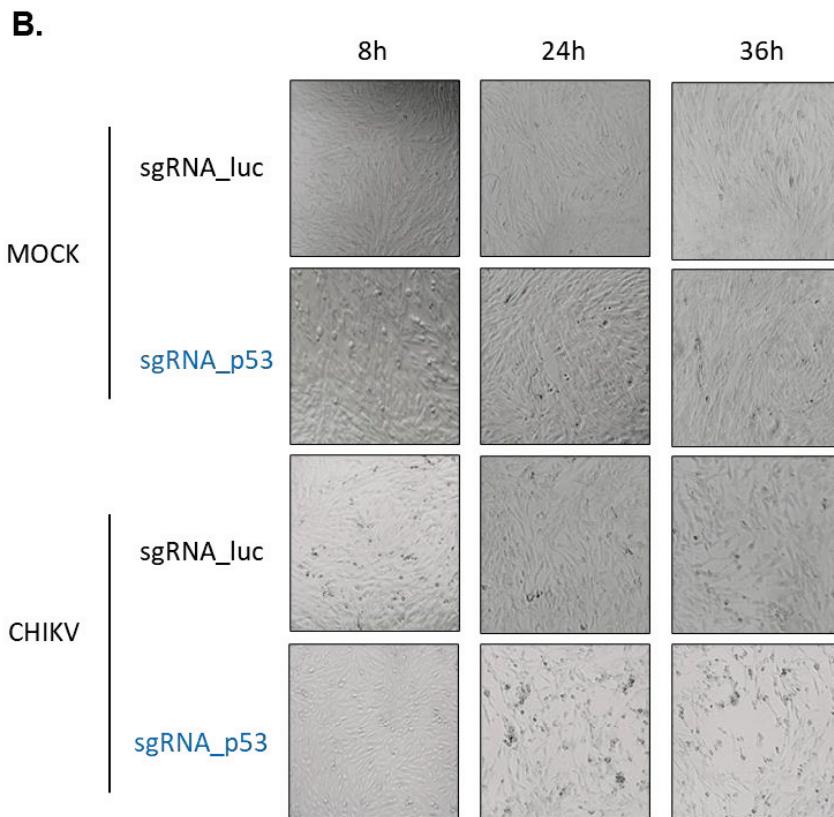
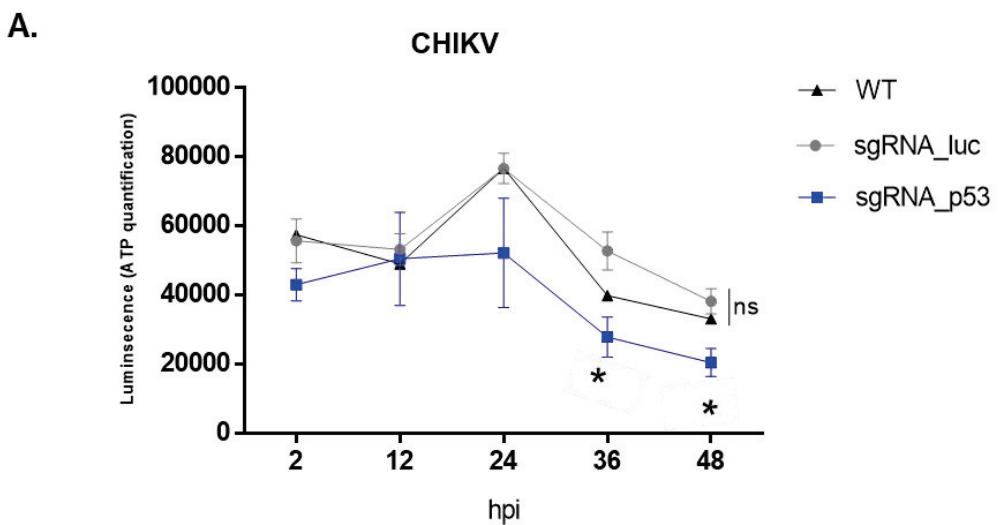
### 3.3.3 Effect of p53 knockout on cell viability and p53-target genes during CHIKV infection

To investigate the impact of p53 knockout on cell viability and cytopathicity during CHIKV infection, we examined cell viability using the CellTiter-Glo® luminescent assay kit (Promega) and we observed cellular morphology and cytopathic effects under the microscope (Figure 31 A. and B.).

The cell growth of CHIKV-infected LHCN-M2 sgRNA\_p53 decreased significantly from 24 to 48 hpi compared to LHCN-M2 WT and sgRNA\_luc (Figure 31 A.) and this result is associated with larger observable CPE in CHIKV-infected LHCN-M2 sgRNA\_p53 from 24 hpi (Figure 31 B.). Taken together, these results showed that p53 knockout enhances CHIKV infection and impairs cell viability, suggesting an antiviral effect of p53 protein during CHIKV infection.

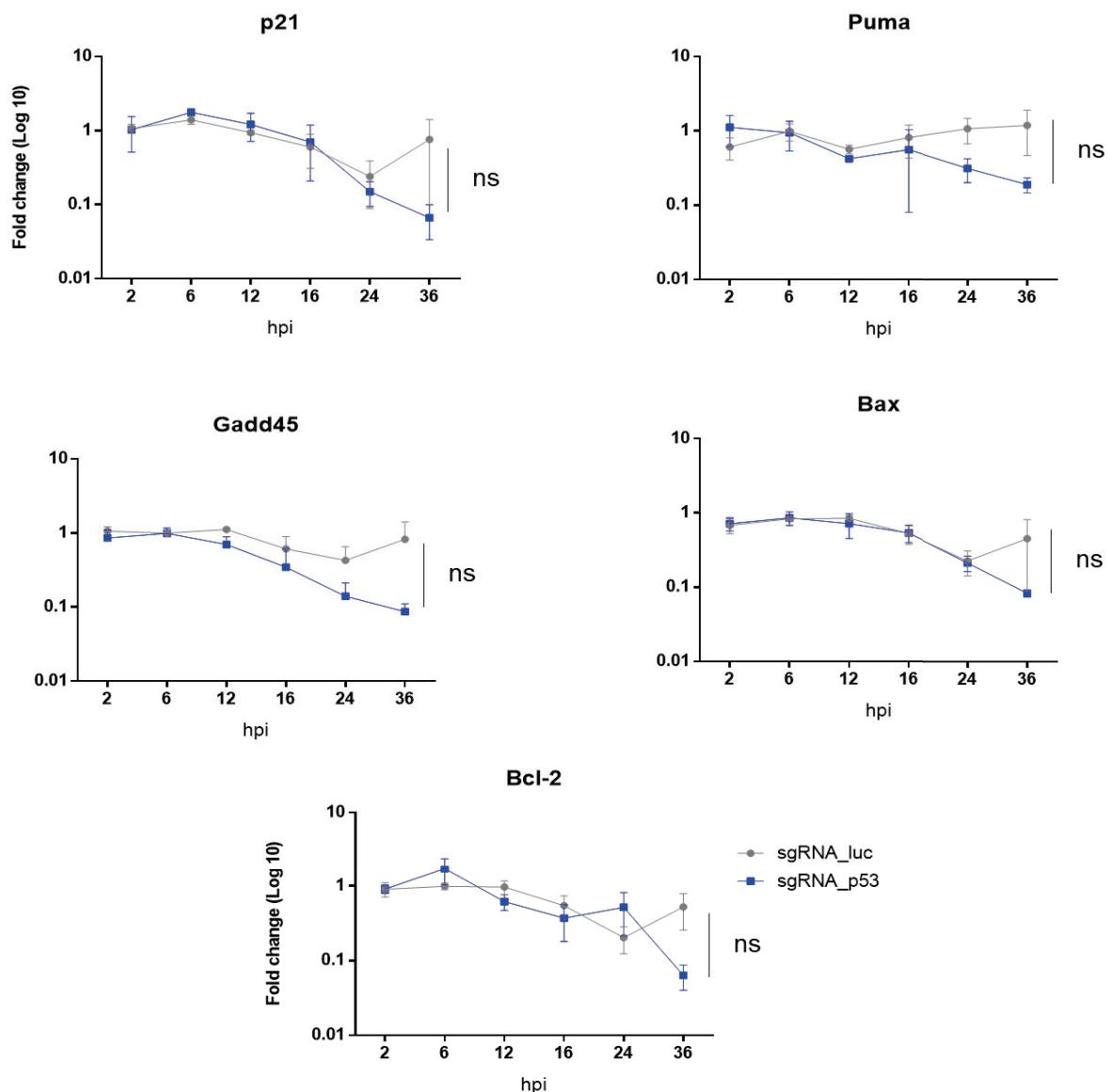
In parallel, we investigated the regulation of p53-target genes involved either in cell cycle arrest (P21, GADD45) or in pro- or anti-apoptotic response (Bax, PUMA and Bcl-2). Our data (Figure 32) showed no significant differences in the regulation of the five target genes in infected sgRNA\_p53 compared to sgRNA\_luc.

In conclusion, the increase of cellular mortality in p53 knockout infected cells and the absence of specific p53-target gene regulation, suggests a protective role of p53 and that CHIKV-mediated cell death is p53-independent or engages other p53-target genes.



**Figure 31: CHIKV infection in p53 knockout LHCN-M2 cell line impairs cell viability overtime.**

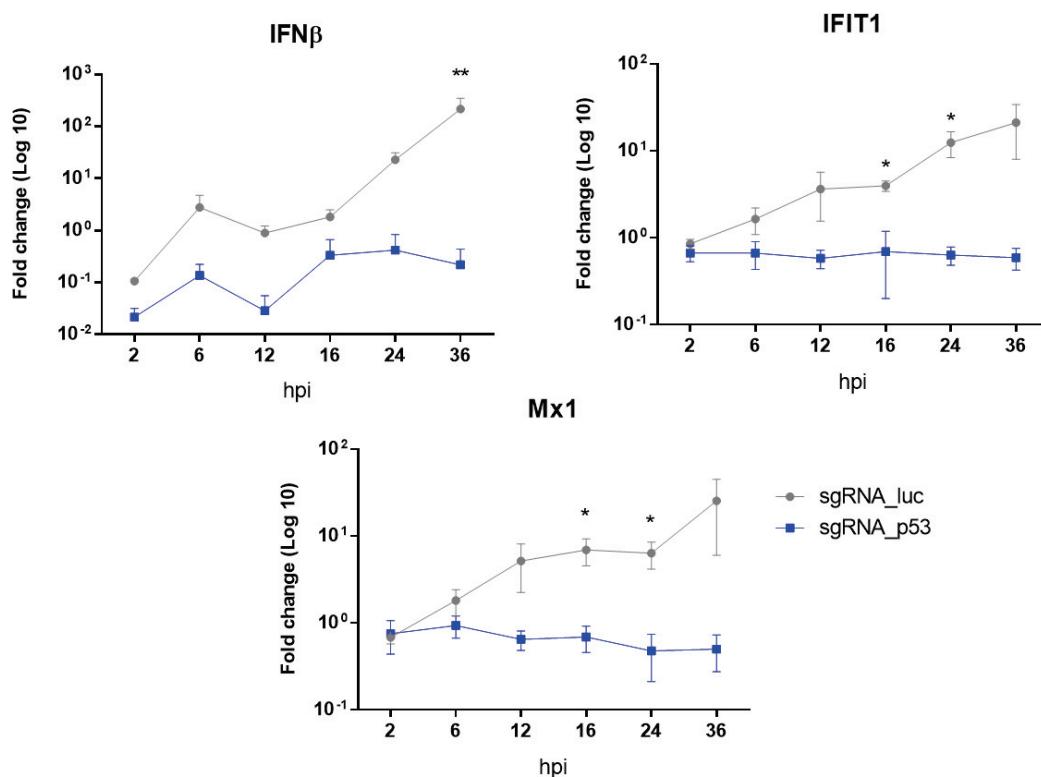
- A.** Cells were infected with CHIKV at MOI 0.1 and cell viability was measured at different times after infection using the CellTiter-Glo® luminescent cell viability assay kit (Promega). Luminescence is representative of ATP quantification in the supernatant and the cells. The values of WT cells were compared to sgRNA\_luc and the values of sgRNA\_p53 compared to sgRNA\_luc. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $<0.05$ . ns: nonsignificant.
- B.** Cellular morphology of LHCN-M2 sgRNA\_luc and sgRNA\_p53 uninfected (up panel) and infected (bottom panel) with CHIKV at MOI 0.1 at 8, 24- and 36-hours post-infection. The pictures were obtained with an objective x10.



**Figure 32: Expression level of pro- and anti-apoptotic p53 target genes in LHCN-M2 sgRNA\_luc and sgRNA\_p53 infected with CHIKV.** Cells were infected with CHIKV at MOI 0.1 and then harvested at different time points post-infection. The expression level of p53 target genes p21, puma, gadd45, bax and bcl-2 were monitored by RT-qPCR. The expression level of the target genes was measured and normalized against housekeeping gene 5s rRNA. The mRNA level of mock cells was established at 1 and data are presented as (Log10) Fold change. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $>0.05$ . ns: nonsignificant.

### 3.3.4 Effect of p53 knockout on interferon Type-I signaling during CHIKV infection

We investigated IFN $\beta$  mRNA expression and ISGs genes Mx1 and IFIT1 (Figure 33). We observed a shutdown of IFN $\beta$  mRNA expression and ISGs Mx1 and IFIT1 genes in CHIKV-infected LHCN-M2 sgRNA\_p53 compared to sgRNA\_luc infected cells. In parallel, in CHIKV-infected LHCN-M2 sgRNA\_luc, the expression levels of IFN $\beta$ , Mx1 and IFIT1 mRNA were significantly upregulated from 6 to 36 hours postinfection compared to uninfected sgRNA\_luc cells.



**Figure 33: p53 knockout in LHCN-M2 impairs Type I interferon production and signaling pathway during CHIKV infection.**

LHCN-M2 sgRNA\_luc and sgRNA\_p53 cells were infected with CHIKV at MOI 0.1 and then harvested at different times points post-infection. The expression level of Type I interferon pathway genes was monitored by RT-qPCR. The expression level of target genes was measured and normalized against housekeeping gene 5s rRNA. The mRNA level of mock cells was established at 1 and data are presented as (Log10) Fold change. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $<0.05$ .

Thus, the p53 knockout of infected cells impaired the production of interferon and the induction of Type-I signaling pathway after CHIKV infection. However, it was necessary to investigate the capability of LHCN-M2 sgRNA\_p53 to produce and respond to interferon to understand which step of the p53-dependent immune response was blocked in our cellular model during CHIKV infection.

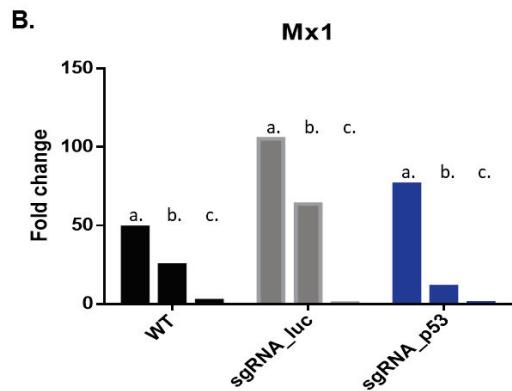
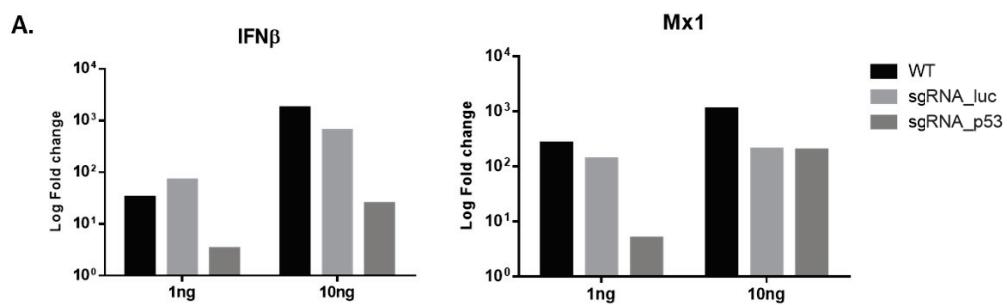
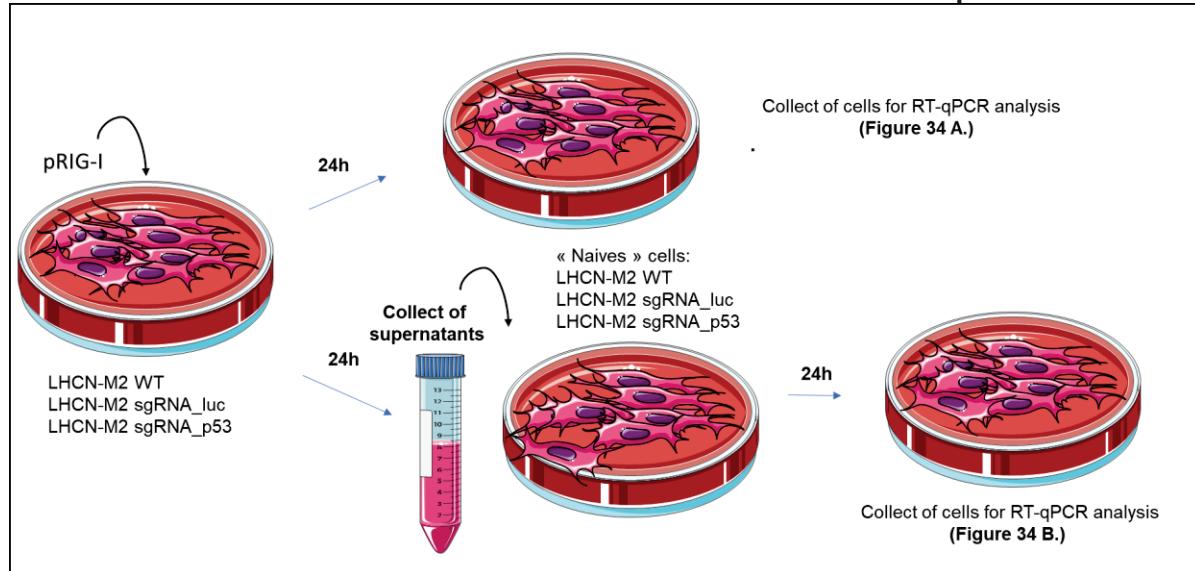
### **3.3.5 Capacity of p53 knockout LHCN-M2 cells to induce the Type-I interferon signaling pathway**

We next explored the capacity of p53 knockout LHCN-M2 to induce Type-I interferon expression and signaling pathway. To do this, LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 were transfected with an RIG-I CARD plasmid expressing only two CARDs domains, allowing the activation of the signaling pathway for IFN $\beta$  production. Indeed, RIG-I includes two caspase activation and recruitment domains (CARDs), which are essential for interaction with mitochondrial antiviral signaling protein MAVS and subsequently the production of IFN $\beta$ . We analyzed the mRNA expression of IFN $\beta$ , Mx1 and IFIT1, 24 hpi (Figure 34 A.). The box above figure 34 shows the workflow of the experiment schematically. Our data showed that the expression of the RIG-I CARD domain induced Type-I interferon response only in LHCN-M2 WT and LHCN-M2 sgRNA\_luc. In transfected LHCN-M2 sgRNA\_p53, the expression of IFN $\beta$  mRNA was upregulated at a weaker level compared to sgRNA\_luc. However, the level of expression of Mx1 in sgRNA\_p53 was equivalent to sgRNA\_luc after the transfection of 10 ng of plasmid RIG-I.

Then, we transfected cells with plasmid RIG-I CARDs, collected the supernatants of cells 24 hours post transfection (Figure 34 B.) and challenged the three cell lines. The three cell lines challenged with supernatants of WT and sgRNA\_luc transfected cells, were able to express Mx1, whereas the three cell lines challenged with supernatant of sgRNA\_p53 transfected cells, were not able to induce the Type-I interferon signaling pathway.

These results suggest that the level of IFN $\beta$  in the supernatant of p53 knockout LHCN-M2 cells was insufficient to induce Mx1 expression. Other repetitions are required; however, this experiment suggests that LHCN-M2 sgRNA\_p53 can induce interferon signaling after RIG-I CARD domain expression but, curiously, it produces weaker and not sufficient IFN $\beta$  to induce Type-I interferon signaling pathway.

### Schematic workflow of the transfection of LHCN-M2 cells lines with pRIG-I



**Figure 34: p53 knockout LHCN-M2s are less competent to produce IFNβ but are able to induce the Type-I interferon signaling pathway (n=1).**

- LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 were transfected with 1 ng or 10 ng of 2CARDs-RIG-I plasmid for 24 hours and the expression levels of IFNβ and Mx1 were monitored by RT-qPCR. The expression level of the target genes was measured and normalized against housekeeping genes gusb, rpl22 and rpl27. The mRNA level of non-transfected cells was established at 1 and data are represented as (Log10) Fold change. Data represent values of one experiment.*
- LHCN-M2 wildtype, sgRNA\_luc and sgRNA\_p53 were challenged with the supernatants of previously transfected cells with 100 ng of 2CARDs-RIG-I plasmid for 24 hours and the expression level of Mx1 was monitored by RT-qPCR. The expression level of target*

genes was measured and normalized against housekeeping genes *gusb*, *rpl22* and *rpl27*. The mRNA level of mock cells was established at 1 and data are presented as Fold change. Data represent values of one experiment.

**a.** = cells challenged with supernatant of LHCN-M2 wildtype transfected with 100 ng of 2CARDs-RIG-I plasmid.; **b.** = cells challenged with the supernatant of LHCN-M2 sgRNA\_luc transfected with 100 ng of 2CARDs-RIG-I plasmid.; **c.** = cells challenged with the supernatant of LHCN-M2 sgRNA\_p53 transfected with 100 ng of 2CARDs-RIG-I plasmid.

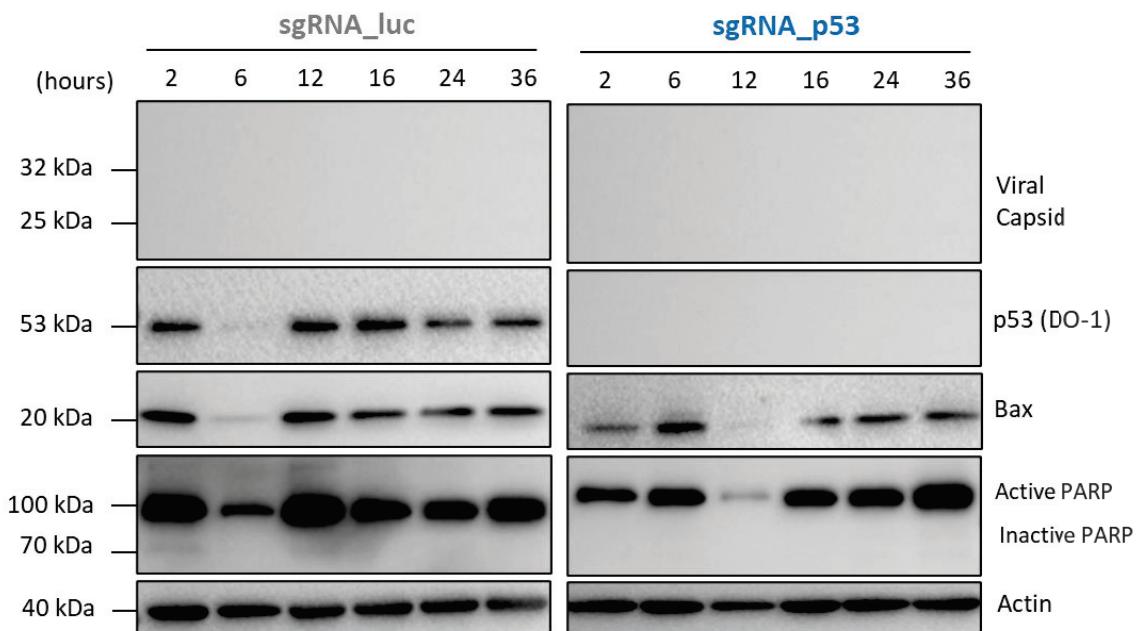
In conclusion, the antiviral effect of p53 in LHCN-M2 during CHIKV infection seems to be correlated to Type-I interferon response independently of its antiviral apoptotic function. The impairment of p53 knockout cells to induce antiviral response seems to be dependent on IFN $\beta$  production and not on the expression of ISGs genes Mx1 and IFIT1.

### 3.3.6 Effect of p53 knockout on CHIKV-induced cell death

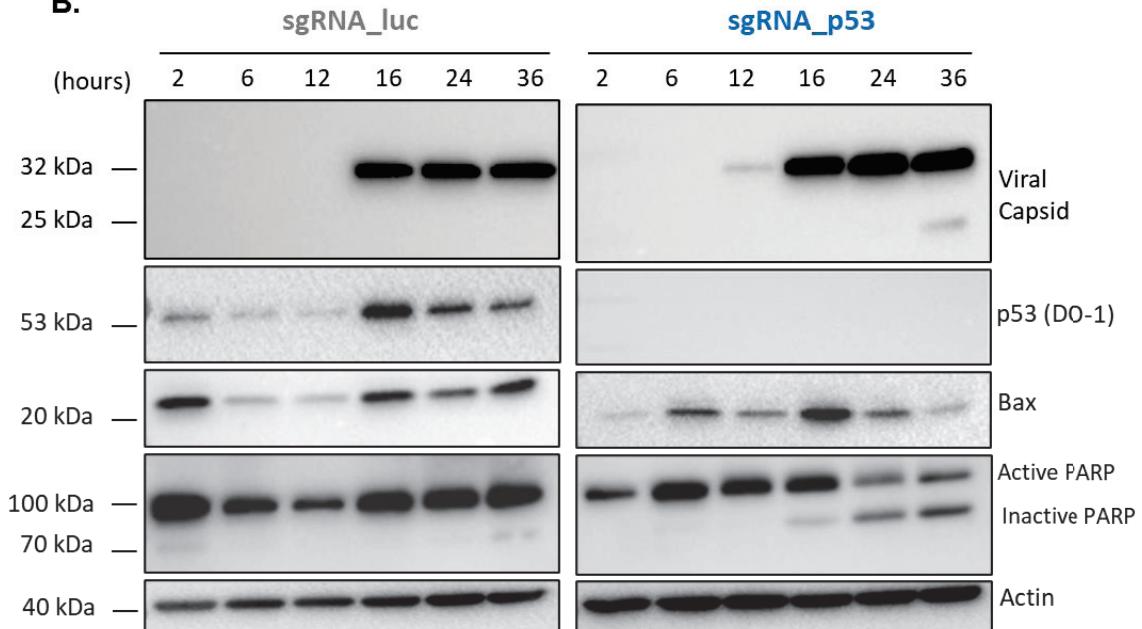
We have shown that more p53 knockout cells are infected by CHIKV compared to sgRNA\_luc cells. Moreover, more p53 knockout cells undergo cell death and cannot induce Type-I interferon response after CHIKV infection. Thus, we suggest that in infected control sgRNA\_luc cells, the uninfected neighboring cells were protected against CHIKV infection and virus-induced cell death, possibly due to the activation of the interferon Type-I signaling pathway.

As the infected p53 knockout cells presented earlier cytopathic effects (CPE) compared to the control sgRNA\_luc infected cells, we detected the cleavage of PARP (an indicator of late apoptosis and activation of caspase-3) and the expression of Bax (an indicator of mitochondrial apoptosis). In addition, the expression of the viral capsid of CHIKV and p53 accumulation were obtained (Figure 35). The western blot presented below is representative of what we observed in three separate experiments for the viral capsid, p53 and PARP but not the expression of Bax (n=1).

**A.**

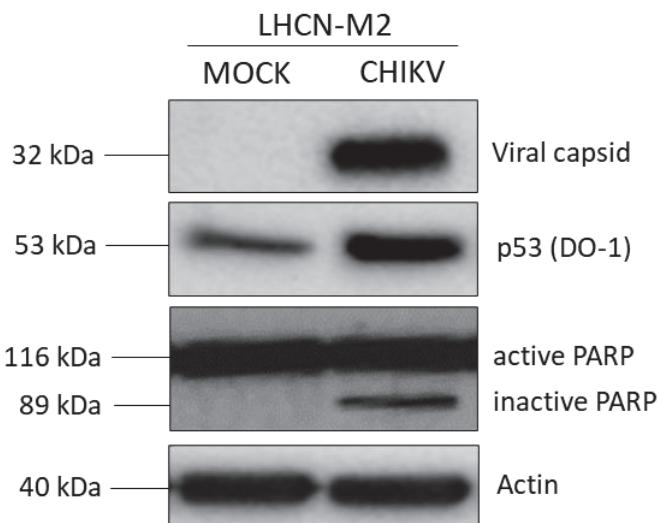


**B.**



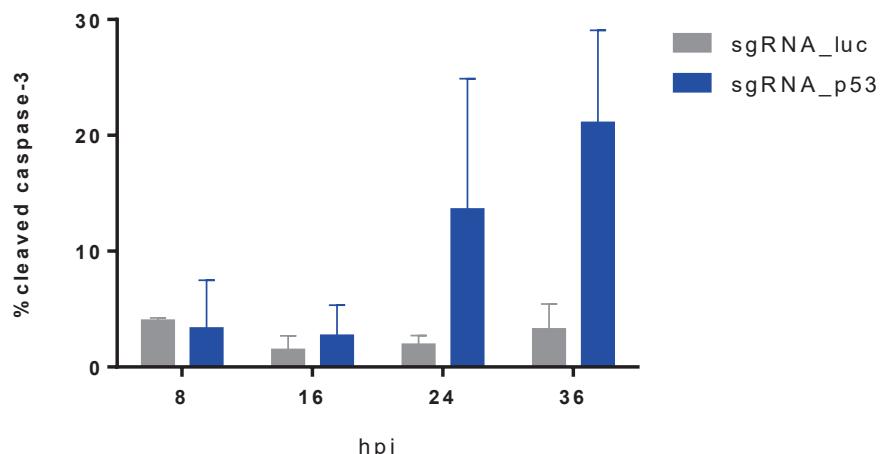
**Figure 35: Viral capsid, p53, Bax and PARP protein expression in p53 knockout LHCN-M2 cell lysate after 24 hours of CHIKV infection.**

Cells were infected with CHIKV at MOI 0.1 for 24 hours. The cells were collected, and 50  $\mu$ g of proteins were loaded on SDS-PAGE for Western blot assay. Viral capsid SFV-C antibody was used to detect CHIKV, anti-p53 DO-1 to detect full-length p53, anti-Bax and anti-PARP. The antibody raised to housekeeping protein  $\beta$ -actin was used as loading control. **A.** Uninfected sgRNA\_luc and sgRNA\_p53 cell lines. **B.** Infected sgRNA\_luc and sgRNA\_p53 cell lines.



**Figure 36: Viral capsid, p53 and PARP protein expression in LHCN-M2 wildtype cell lysate after 48 hours of CHIKV infection.**

Cells were infected with CHIKV at MOI 0.1 for 24 hours. The cells were collected, and 50  $\mu$ g of proteins were loaded on SDS-PAGE for Western blot assay. Viral capsid SFV-C antibody was used to detect CHIKV, anti-p53 DO-1 to detect full-length p53 and anti-PARP. The antibody raised to housekeeping protein  $\beta$ -actin was used as loading control.



**Figure 37: Immunostaining of cleaved caspase-3 in CHIKV-infected LHCN-M2 sgRNA\_luc and sgRNA\_p53 and flow cytometry analysis (n=1).**

LHCN-M2 sgRNA\_luc and sgRNA\_p53 cells were infected with CHIKV at MOI 0.1 and fixed at different times post infection for immunostaining, targeting cleaved caspase-3 (anti-cleaved caspase-3 Asp175, #9664 CST). The percentage of positive cells was detected by flow cytometry.

First, the viral capsid expression in CHIKV-infected LHCN-M2 sgRNA\_luc and sgRNA\_p53 showed a higher quantity of capsid in sgRNA\_p53 cells from 12-16 hours post infection compared to sgRNA\_luc. The result of the Western blot assays was correlated to that of the flow cytometry

assay on the percentage of infected cells (*cf.* Figure 30), with a higher quantity of CHIKV capsid and earlier detection in sgRNA\_p53 compared to sgRNA\_luc. Moreover, we observed an additional 25 kDa capsid form, from 16 hpi, only in the infected p53 knockout cells. This 25 kDa capsid form was not detectable in infected LHCN-M2 sgRNA\_luc from 16 to 36 hpi.

Secondly, the stabilization of p53 was analyzed using the DO-1 antibody which recognizes only full-length p53 as the epitope is localized in the first TAD domain. On looking at the three independent experiments, the profile of p53 stabilization in uninfected and infected LHCN-M2 sgRNA\_luc presented a general trend with less accumulation in infected cells at early infection (from 2 to 12 hpi) and then higher accumulation in infected cells (from 12 to 36 hpi), compared to uninfected cells.

In addition, the inactive form of PARP (89 kDa) in CHIKV-infected LHCN-M2 sgRNA\_p53 was detectable much earlier, at 16 hpi, and increased overtime until 36 hpi, compared to sgRNA\_luc where we observed the cleaved form at 36 hpi only once. Previously, the infection of LHCN-M2 WT cells with CHIKV at MOI 1 showed the inactive form of PARP at 48 hours post infection (Figure 36). Maybe that in CHIKV-infected sgRNA\_luc the cleavage of PARP will be detectable after 36 hours of infection and thus correlated to the percentage of infected cells.

Moreover, the expression of Bax was analyzed only once because we observed several confusing regulations overtime. Bax is found in the cytosol and, upon the initiation of apoptotic signaling, it undergoes a conformational shift and is integrated in the membrane of the mitochondria, forming channels and leading to the release of cytochrome c. Thus, the expression of Bax seems difficult to analyze, which is why we detected the release of the cytochrome from the mitochondria by fluorescent microscopy assay.

Finally, PARP can be cleaved by caspase-3 during apoptosis. Thus, we analyzed the cleavage of caspase-3 by immunostaining and flow cytometry assay (Figure 37). Our data showed that no cleavage of caspase-3 was detected in sgRNA\_luc infected cells, whereas the cleavage of caspase-3 was detected in sgRNA\_p53 infected from 24 to 36 hpi with around 20% of the total population positive.

Taken together, these results first suggests that the stabilization of p53 seems regulated over time (degraded in the early stage of infection and then stabilized in the late stage of infection), maybe by the infection and second, p53 depletion induces an earlier inactivation of PARP

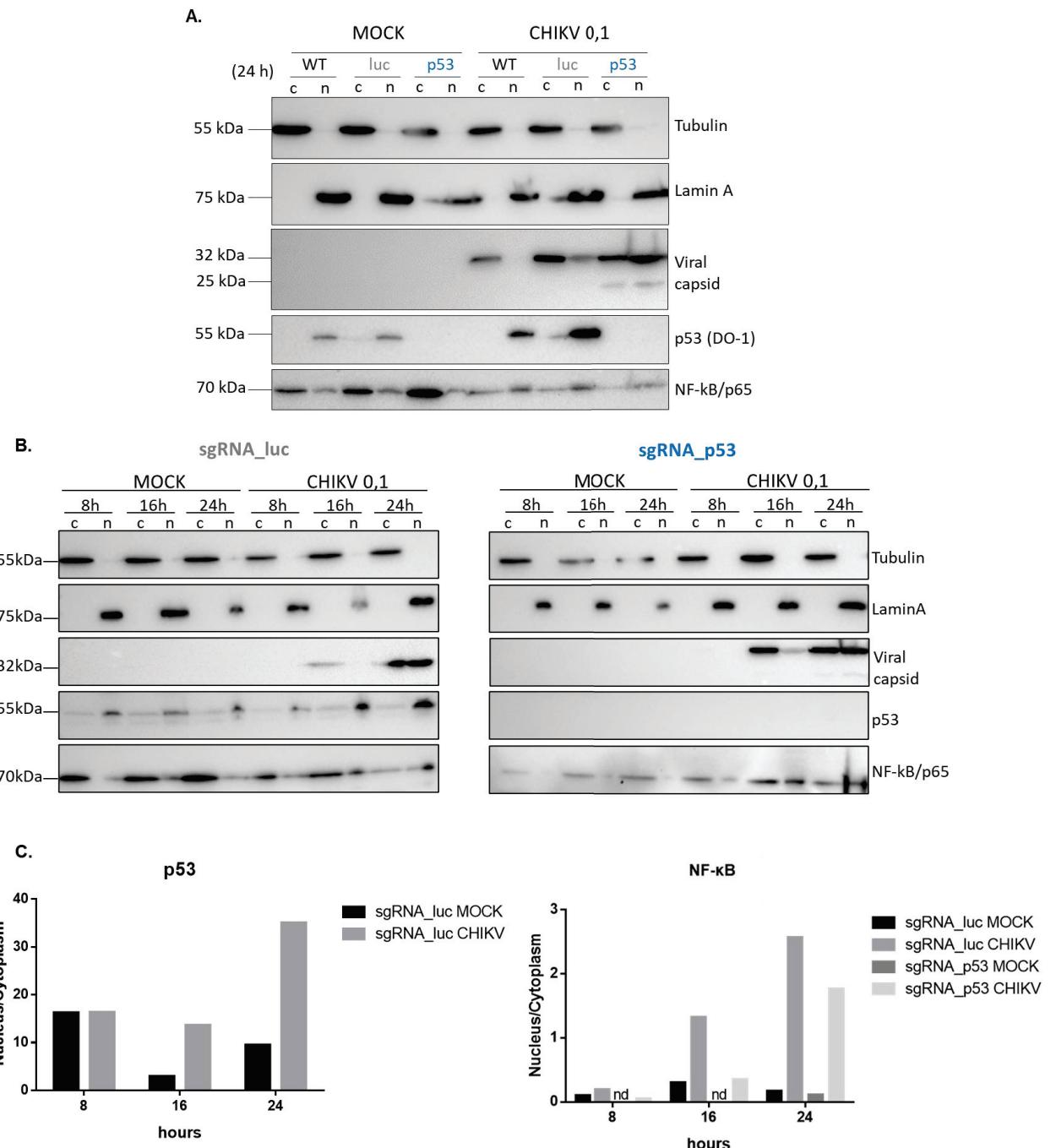
maybe cleaved by caspase-3 during CHIKV infection, supporting the previous results on cell viability and cellular morphology, thereby indicating an antiviral role of the p53 protein allowing the delay of CHIKV-induced cell death.

### 3.3.7 Nuclear translocation of p53 and NF-κB during CHIKV infection

As p53 is a transcription factor and can be subjected to several different post-translational modifications guiding its response and nuclear translocation, it seems important to analyze if its stabilization during CHIKV infection around 16 and 24 hpi promotes its nuclear localization. To this end, we decided to test the nuclear translocation of p53 using the subcellular fractionation technique and SDS-PAGE analysis (Figure 38). The aim was to separate the nucleus and cytoplasm of uninfected and infected LHCN-M2 cells and first detect the viral capsid and second the distribution and accumulation of p53 between the cytoplasm and the nucleus. Moreover, using this technique, we investigated the nuclear translocation of transcription factor NF-κB which is involved in interferon Type-I production after the detection of viral RNA through RLRs and TLRs. In parallel, we tried to detect p53 using the immunostaining method and confocal microscopy analysis to have a second and more qualitative method of detection. Unfortunately, in the preliminary data, the detection of p53 in LHCN-M2 was not a success (data not shown) and different protocols are now being formulated.

Figure 38 A. shows the subcellular fractionation of LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 infected with CHIKV with MOI 0.1 at 24 hpi. Figure 38 B. presents the subcellular fractionation of sgRNA\_luc and sgRNA\_p53 cells infected with CHIKV at MOI 0.1 and cell lysates collected at 8, 16 and 24 hpi. The complete separation of fractions was confirmed for targeting tubulin and lamin A for the identification of cytoplasm and nucleus fractions, respectively. Figure 38 C. shows the nucleus/cytoplasm ratio of p53 and NF-κB.

Targeting the CHIKV viral capsid, we could clearly observe a dissimilarity between LHCN-M2 WT and LHCN-M2 sgRNA\_luc. We observed less p53 accumulation in infected LHCN-M2 WT compared to sgRNA\_luc. Moreover, we observed p53 in the nucleus of infected sgRNA\_luc but not in WT cells. Then, we observed a higher quantity of capsid in the nucleic fraction of infected p53 knockout cells compared to the nucleic fraction of sgRNA\_luc. Finally, the 25 kDa capsid form was also detected only in infected sgRNA\_p53, in both fractions.



**Figure 38: Analysis of viral capsid, p53 and NF-κB distribution between the cytoplasm and nucleus during infection of LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 with CHIKV.**

**A.** LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53 cells were infected with CHIKV at MOI 0,1 for 24 hours. c=cytoplasm, n=nucleus.

**B.** LHCN-M2 sgRNA\_luc and sgRNA\_p53 cells were infected with CHIKV at MOI 0,1 for 8, 16 and 24 hours. c=cytoplasm, n=nucleus.

**C.** Report of nucleus/cytoplasm of p53 in sgRNA\_luc and NF-κB expression in CHIKV-infected sgRNA\_luc and sgRNA\_p53. Densitometry monitored on ImageJ software.

The cytoplasmic and nucleic fractions were separated as described in materials and method and then the same volume of different fractions was loaded on SDS-PAGE for Western blot assay. Viral capsid SFV-C antibody was used to detect CHIKV, anti-p53 DO-1 to detect full-length p53 and anti-NF-κB/p65 to detect NF-κB. The antibodies raised against housekeeping proteins Tubulin and Lamin A were used as loading and separating fraction controls.

The detection of p53 showed a higher accumulation and nuclear translocation in infected LHCN-M2 WT and sgRNA\_luc compared to uninfected cell lines at 24 hpi. Moreover, the accumulation in infected sgRNA\_luc was higher than in WT cells. These observations suggest that p53 is stabilized and translocated to the nucleus of CHIKV-infected sgRNA\_luc.

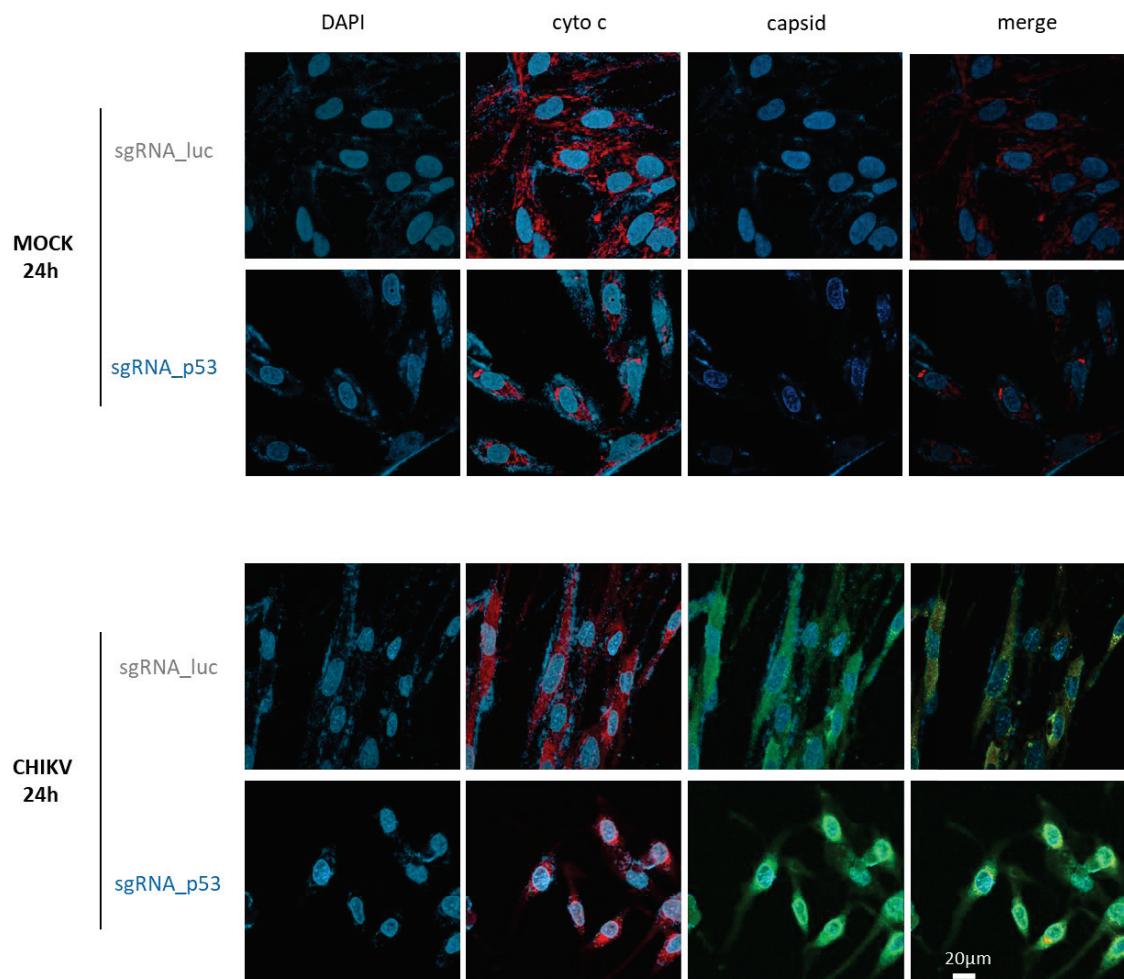
The detection of the transcription factor NF-κB first showed a higher quantity in the cytoplasm of uninfected cells than in the nucleus compared to infected cells. Then, we observed that in sgRNA\_luc and sgRNA\_p53 infected cells, the quantity of NF-κB was higher in the nucleus than in the cytoplasm. Moreover, the densitometry analysis of the second experiment (Figure 38 C.) showed a higher increase of NF-κB detection in the nucleus of infected sgRNA\_luc compared to the nuclear fraction of infected sgRNA\_p53.

These results suggest that NF-κB is translocated to the nucleus of both cell lines during CHIKV infection, but the depletion of p53 impairs its nuclear translocation. This experiment must be repeated to support this observation.

### **3.3.8 Effect of p53 knockout on the release of cytochrome c during CHIKV infection**

In order to strengthen the subcellular fractionation results on viral capsid distribution in both fractions (cytoplasm and nucleus) and to obtain an image of individual infected cells, the distribution of CHIKV capsid in LHCN-M2 sgRNA\_luc and sgRNA\_p53 cells was studied by fluorescence imaging microscopy. Moreover, in order to investigate the involvement of mitochondria during CHIKV-induced cell death we set-up the detection of cytochrome c release (Figure 39).

In sgRNA\_luc and sgRNA\_p53 uninfected cells, we observed punctuated staining of cytochrome c in the cytoplasm and perinuclear zone, suggesting a mitochondrial localization of cytochrome c. In contrast, the two infected cells showed diffuse staining of cytochrome c, indicating its release in the cytoplasm at 24 hpi. In addition, by analyzing the DAPI fluorescence, we observed that in the sgRNA\_luc cells, the nucleus was smooth in mock conditions while the chromatin was condensed in infected conditions. Finally, in infected sgRNA\_p53 cells, we detected higher chromatin condensation compared to infected sgRNA\_luc cells, visualized by DAPI staining.



**Figure 39: Analysis of CHIKV capsid and cytochrome c distribution in LHCN-M2 sgRNA\_luc and sgRNA\_p53 by fluorescence imaging microscopy.** Cells were infected with CHIKV at MOI 0.1 for 24 hours and then fixed in 4% paraformaldehyde. The viral capsid and cytochrome c were co-immunostained and the nucleus counterstained with DAPI. The targeted proteins and DAPI were visualized by fluorescent microscopy assay (Obj. x40), (n=1).

The staining of the nucleus in infected sgRNA\_luc and sgRNA\_p53 cell lines showed fewer sgRNA\_p53 cells on several panels during acquisition compared to sgRNA\_luc, due to more dead cells. The flow cytometry results showed a higher percentage of infected sgRNA\_p53 cells than sgRNA\_luc cells and the microscopy analysis showed diffuse staining of capsid in the cytoplasm of infected sgRNA\_luc and greater intensity for stained capsid in sgRNA\_p53 around the nucleus. Thus, the microscopy analysis did not confirm the results obtained by flow cytometry, perhaps due to the difference in the technique used. Thus, the experiment should be repeated. In conclusion, this result suggests that during CHIKV infection the mitochondria seemed to be involved in virus-induced cell death.

### 3.3.9 Discussion

The role of p53 protein during CHIKV infection in human LHCN-M2 cells has never been investigated. However, it has been shown that p53 can be involved in several different viral replication cycles as mentioned in the introduction of the manuscript. In our study, we investigated and showed an antiviral role of p53 in CHIKV infection in a human immortalized LHCN-M2 muscle cell line. Then, we studied the signaling pathway mediated by p53 during CHIKV infection and showed its influence on Type-I interferon production and/or signaling pathway independently of its apoptotic response.

We have initially showed that LHCN-M2 WT cells were permissive to CHIKV which led to considerable viral replication and cytopathic effects until the complete destruction of the cell layer. Moreover, we demonstrated the stabilization of full-length p53 in CHIKV-infected LHCN-M2 WT cells and its nuclear translocation. In addition, we demonstrated an increase of Type I interferon production and signaling pathway. In parallel, we did not observe the induction of two specific p53 target genes that are involved in the induction of apoptosis. This result is in accordance with a publication that showed that infection with Semliki Forest virus (SFV) induced p53-independent apoptosis (Glasgow *et al.* 1998).

Then, to go further we generated p53 knockout LHCN-M2 cells using CRISPR/Cas9 technology. We demonstrated a higher percentage of CHIKV-infected sgRNA\_p53 cells compared to sgRNA\_luc control cells. The increase in the viral infection of sgRNA\_p53 was linked to more significant cytopathic effects and a decrease in cell viability. The CHIKV-infected sgRNA\_p53 was not able to express the IFN $\beta$  transcript and the ISGs genes, Mx1 and IFIT1. Interestingly, the analysis of five p53 target genes (P21, GADD45, PUMA, Bax, Bcl-2) showed no activations during infection. In conclusion, during CHIKV infection of a human immortalized LHCN-M2 skeletal muscle cell line, the p53 protein plays an antiviral role influencing antiviral immunity independently of its antiviral apoptotic response. To confirm this hypothesis, we must first know if the p53 knockout LHCN-M2 cell line can induce an efficient Type-I interferon signalling pathway. To test the capacity of the sgRNA\_p53 cell line to produce IFN $\beta$  and activate the Type I interferon signalling pathway and respond to IFN $\beta$  detection on receptor IFNARs, we transfected cells with plasmid expressing only the two CARDs of RIG-I, allowing the constant

activation of IRF3/IRF7 and the subsequent expression of IFN $\beta$ . Interestingly, it seems that sgRNA\_p53 cells express less IFN $\beta$  mRNA than sgRNA\_luc and WT transfected cell lines. However, all the three different cell lines expressed Mx1 at the same level.

We tested the capacity of p53 knockout cells to produce IFN $\beta$  in their supernatant and activated the Type I interferon signalling pathway to neighbouring cells. It seemed that p53 knockout cells could not produce IFN $\beta$  in their supernatant, however, they could respond to the IFN $\beta$  and induce the activation of the Type I interferon signalling pathway.

Considering that LHCN cell lines are hard-to-transfect, it could be interesting to use Polyinosinic-polycytidylic acid (Poly(I:C)). Indeed, poly(I-C) is a synthetic analogue of double strand RNA (dsRNA) recognized by TLR3, leading to the phosphorylation and activation of transcription factors IRF3/7 and NF- $\kappa$ B.

p53 could be involved in the expression of one or several factors of the TLR3-TRIF pathway or RIG-I/MDA5-MAVS pathway during CHIKV infection, thus explaining the inability of p53 knockout cells to produce IFN $\beta$ . In the absence of IFN $\beta$  in the supernatant of CHIKV-infected LHCN-M2 sgRNA\_p53 cells, the signal to protect nearby uninfected cells is absent, possibly explaining why more p53 knockout cells are infected. To test this hypothesis, we are analysing the expression level of different p53 target genes such as TLR3 or IRF9 and the activation of transcription factor IRF3 by phosphorylation detection. In addition, we are monitoring the capacity of IFN $\beta$  in the supernatant of sgRNA\_luc control cells mediated by p53 during CHIKV to protect the neighbouring cells from infection. To do this, the supernatant of infected sgRNA\_luc cells will be collected and filtered to retain the CHIKV particles and recover the supernatant with IFN $\beta$  secreted on the uninfected sgRNA\_p53 cells which are able to induce the expression of ISGs. After being challenged with this supernatant, the p53 knockout cells will be infected with CHIKV and the rate of viral replication and interferon Type-I signalling pathway will be compared to sgRNA\_luc cells. We assume that the IFN $\beta$  secreted by infected cells on nearby uninfected cells could delay the cell death observed in p53 knockout infected cells.

It has been demonstrated that TLR3 can be regulated by p53 in HCT116 epithelial cells by binding to the p53 consensus site in the TLR3 promoter in response to poly(I-C) (Taura *et al.* 2008). Furthermore, TLR3 regulates antiviral immunity in CHIKV infection in primary fibroblasts in humans and mice (Her *et al.* 2015). These data allow us to propose for our analysis the

influence of p53 for the expression of TLR3, leading to the recognition of CHIKV in early endosomes, and subsequently the expression of IFN $\beta$ . To test this hypothesis, it would be interesting to explore the expression level of TLR3 mRNA and the status of IRF3 phosphorylation in CHIKV-infected LHCN-M2 sgRNA\_luc cells and sgRNA\_p53 cells.

The comparison of NF- $\kappa$ B translocation to the nucleus in sgRNA\_luc and sgRNA\_p53 cells showed less NF- $\kappa$ B in the nucleus of p53 knockout infected cells. The p53 and NF- $\kappa$ B pathways can crosstalk and in the absence of p53, the activation and translocation of NF- $\kappa$ B is reduced during CHIKV infection. NF- $\kappa$ B can protect against or contribute to apoptosis and our data suggest that the reduction of NF- $\kappa$ B is correlated to an increase in viral replication and virus-induced cell death.

Using fluorescent microscopy analysis, it would be interesting to double stain the viral capsid of CHIKV with TLR3, RIG-I or transcription factors (IRF3 and NF- $\kappa$ B) and thus examine if p53 stabilization occurs in infected cells and leads to the induction of TLR3 pathways and IFN $\beta$  expression.

We observed a decrease in p53 stabilization from 2 to almost 12 hours in CHIKV-infected sgRNA\_luc cells compared to mock cells, suggesting that viral infection induced a degradation of p53. CHIKV could control the immune response dependent on p53 at the early stage of infection and perform efficient viral replication to produce new particles. The viral proteins involved in the degradation of p53 could be investigated to ascertain whether nsP2 or capsid are responsible, because of their catalytic activity. To do this, we have already generated an inducible cell line overexpressing CHIKV capsid and nsP2.

During the replicative cycle of alphaviruses, viral RNA is released and synthesized into the cytoplasm of the infected cells. To study the replication of CHIKV we analysed the viral capsid of CHIKV and showed a nuclear localization of the CHIKV capsid with a higher quantity of capsid in the cytoplasm and nucleus of p53 knockout infected cells compared to sgRNA\_luc cells. The viral capsid is expressed in the cytoplasm and possesses a nuclear import signal NLS and two nuclear export signals NESs (Thomas *et al.* 2013). The function of the Old-World arthritogenic alphavirus (CHIKV, SINV, SFV or O'NNV) capsid and the importance of its nuclear transport are not well studied. However, the investigations on New-World alphaviruses (VEEV and EEEV) have revealed that the capsid induces the transcriptional shut-off of infected cells by stopping

nuclear transport (Lundberg *et al.* 2017). The function of transcriptional shutoff is ensured by the nuclear localization of the non-structural protein nsP2 in the replicative cycle of Old-World alphaviruses.

Consequently, we could suggest that the higher quantity of viral capsid is a consequence of the increase in viral infection or we could hypothesize that p53 directly or indirectly controls the translocation of the CHIKV capsid to the nucleus. It could be interesting to monitor the interaction between p53 and the CHIKV capsid by Immunoprecipitation. Moreover, during the infection of p53 knockout cells, we observed a 25 kDa form of the viral capsid protein, in addition to the 32 kDa full-length viral capsid, suggesting a degradative process of the viral capsid. In parallel, we showed previously that the inducible overexpression of the capsid alone in LHCN-M2 cells was principally associated with the detection of the 25 kDa form (data not shown). To date, this is the first study to report such a cleaved form of the CHIKV capsid protein.

Concerning the type of cell death pathway engaged in CHIKV-infected LHCN-M2, we could not conclude on which pathway is activated during CHIKV infection of LHCN-M2. We observed the cleavage of PARP1 in LHCN-M2 WT cells at 48 hpi and in p53 knockout cells from 16 hpi. We analysed the presence of cleaved caspase-3 by flow cytometry assay in sgRNA\_luc and sgRNA\_p53 infected cells. Our data indicated the cleavage of caspase-3 in sgRNA\_p53 infected cells but not in sgRNA\_luc cells. These data need to be confirmed and it would be interesting, in parallel, to pre-treat the three LHCN-M2 cell lines with a caspase inhibitor. For example, QVD is a broad-spectrum caspase inhibitor that blocks caspases -8, -9, -10, -12, -3 and -7 and subsequently inhibits apoptosis. It might be informative if the cell death induced in CHIKV-infected cells is delayed and if the cleaved-PARP detected from 16 hours in p53 knockout infected cells is still detectable from 16 hpi or delayed. In parallel, it might be interesting to investigate the role of Inhibitors of Apoptosis proteins (IAP) in mammalian cells for their impact on caspase inhibition. The mammalian IAP could be overexpressed and the effect on CHIKV replication and infection analysed.

In brief, we showed that full-length p53 induces an antiviral response by influencing the Type-I interferon production and signalling pathway of the immortalized human muscle cell line LHCN-M2 infected with CHIKV. It seems that the virus-induced cell death is independent of p53 or

that other genes not targeted could be involved. Moreover, the influence of p53 in antiviral response seems to be correlated to the production of IFN $\beta$ , maybe by transcription of important factor(s) involved in the pathway from the detection of the viral RNA to the activation of transcription factors such as IRF3/IRF7 or NF- $\kappa$ B. The exact mechanism by which p53 influences the Type-I interferon production and signalling pathway needs to be studied further.

## In insects: in *Drosophila melanogaster* p53 expression impacts the viral replication of CHIKV and SINV

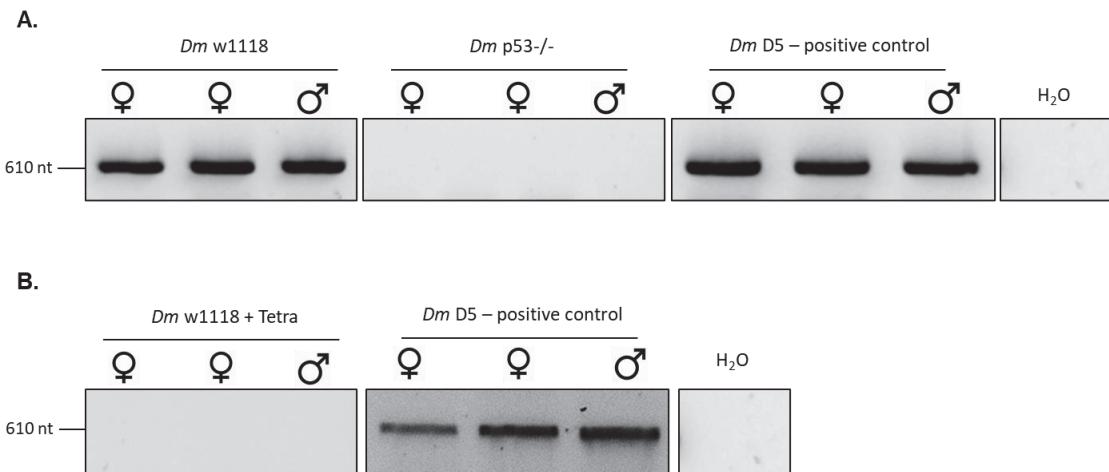
Studies of the p53 protein function in the natural vector *Aedes albopictus* mosquito infected by alphavirus and other arboviruses are scarce. The incomplete annotation of *Ae. albopictus* genome makes the study of specific protein functions very difficult. Quite recently, two p53 paralogues in the *Aedes* mosquito species, named p53-1 and p53-2, were discovered using a phylogenetic approach. Thanks to this discovery, it has been shown that infection of the mosquito C636 cell line by Dengue virus induced the upregulation of p53-2 mRNA expression. The upregulation of p53-2 mRNA was associated with antioxidant response during Dengue virus infection and it has been hypothesized that p53-2 participated in the decrease of virus-induced stress.

We decided to investigate the potential function of p53 protein during *alphavirus* infections *in vivo* in the *Drosophila melanogaster* insect model because of the availability of genetic mutants and better genetic characterization compared to the mosquito *Aedes albopictus*. Thanks to collaboration with Professor Bertrand Mollereau (ENS, Lyon – LBMC, Apoptosis and neurogenetics) we obtained two strains of drosophila: *Drosophila melanogaster* w1118 wildtype strain and a mutant p53-/- strain which presents a deletion on the p53 gene generated by CRISPR/Cas9 technology.

Our first goal was to investigate the potential function of p53 in the CHIKV-infected *Drosophila melanogaster* WT strains w1118 and p53-/- without considering p53 isoforms. The data in this part present the effect of p53 deletion on the viral replication of CHIKV (and Sindbis virus), the survival of flies injected with CHIKV, and the viral production of CHIKV.

### 3.4 Detection of *Wolbachia* spp. in *Drosophila* w1118 and p53-/- strains

We first investigated the absence or presence of the bacteria *Wolbachia* spp. in the *Drosophila* strains w1118 and p53-/- as it has been shown that the presence of the bacteria can interfere with viral replication (Aliota et al. 2016). To do this, we carried out PCR on two females and one male, using published primers as described in Material and methods which recognize several different bacterial strains. The w1118 strain was positive but not the p53-/- strain for *Wolbachia* spp. Thus, w1118 was treated with 5mg/ml of Tetracycline for two generations (Figure 40 A.) and we confirmed the elimination of bacteria with the same PCR (Figure 40 B.). The detection of bacteria was carried out regularly as it has been shown that flies can be recolonized by *Wolbachia* via the external environment.

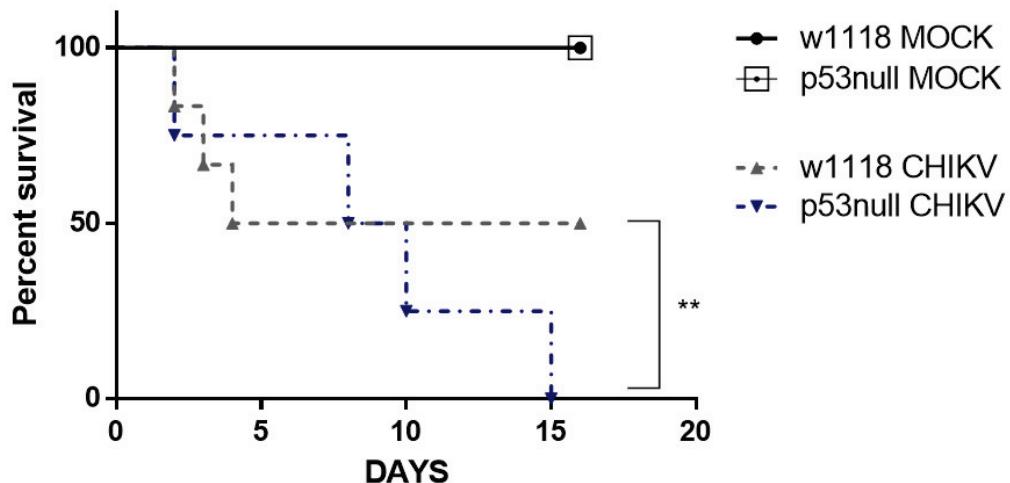


**Figure 40: Detection of *Wolbachia* strains by PCR in *Drosophila melanogaster* w1118 and p53-/- mutant strains (A.) and tetracycline treatment (B.).** Two females and one male were selected randomly and crushed to monitor specific PCR targeting several different *Wolbachia* strains using wsp81Fw 5'- TGGTCCAATAAGTGATGAAGAAC-3' and wsp691Rv 5'- AAAAATTAAACGCTACTCCA-3' (W. Zhou et al. 1998).

#### 3.4.1 Survival curve of w1118 and p53-/- flies injected with CHIKV

The survival of flies injected with 200 pfu of CHIKV solution or mock solution was investigated to analyze the impact of p53 deletion on virus-induced stress (Figure 41). First, the injection of the mock solution in both strains did not impact the survival of flies. Interestingly, five days after the injection of w1118 with 200 pfu of CHIKV, fifty percent of the flies died, and the other fifty percent survived till the end of the experiment (day 20). In parallel, the survival of p53-/-

injected with CHIKV decreased drastically from 6 to 15 days and all the injected flies were dead at day 20. In conclusion, the p53 deletion impaired the survival of flies infected with CHIKV. Next, we investigated the viral replication of CHIKV and SINV and the viral production of CHIKV to know if the death of p53<sup>-/-</sup> flies was due to a higher viral replication during the first day. Thus, we focused our analysis on 2 to 4 days post-injection, when the survival was comparable between the two strains (Figure 41).



**Figure 41: Survival curve of *Drosophila melanogaster* w1118 and p53<sup>-/-</sup> injected with CHIKV.** For each time, 10 flies were injected with CHIKV and survival was monitored by counting surviving flies. Data are the mean  $\pm$  SD of three independent experiments. The survival rate was drawn with GraphPad Prism 6 software (Kaplan-Meier survival plot).

### 3.4.2 Viral replication of SINV and CHIKV and CHIKV production in w1118 and p53<sup>-/-</sup> strains

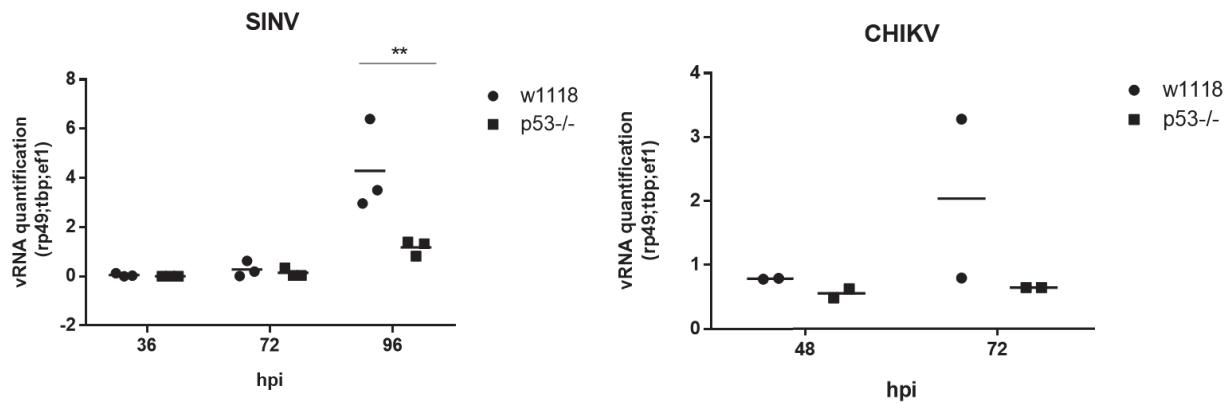
About 15 male flies aged 3 to 6 days were collected in culture medium tubes and injected with 200 pfu of SINV or CHIKV.

First, we did not detect significant differences in SINV replication, using RT-qPCR analysis, in the w1118 strain compared to the p53<sup>-/-</sup> strain (from 36 to 72 hours post-injections). Interestingly, from 72 to 96 hours post-injection the SINV replication in w1118 strain increased 4-fold compared to the mock w1118 strain and in p53<sup>-/-</sup> only once leading to a significant difference in SINV replication between w1118 and p53<sup>-/-</sup> at 96 hours. This first result suggested a negative impact of p53 deletion on SINV replication.

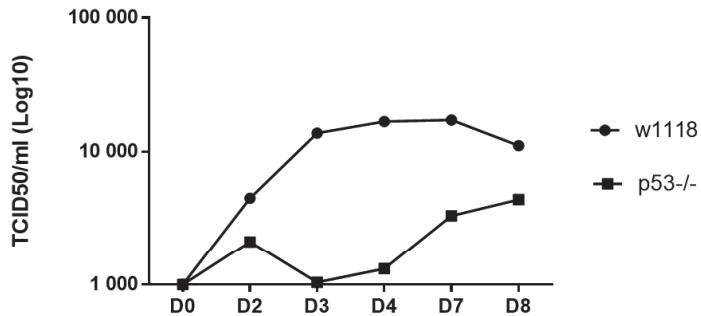
Next, the same experiment been performed twice with CHIKV but for a shorter time course (72) for practical reasons (Figure 42 A.). At 72 hours post-injection, we obtained two contradictory results with one experiment showing higher CHIKV replication in the wildtype w1118 strain compared to the p53-/- strain and another experiment indicating no differences between the two strains. These two experiments suggested that we should analyze this viral replication over a longer kinetic (96h) to consolidate the difference between the two strains.

In addition, we investigated the capacity of producing new infectious particles for the dissemination of CHIKV by the two different strains. To do this, a TCID50/mL analysis was carried out (Figure 42 B.). We observed considerable production of new infectious particles from day 2 to day 4 in the w1118 strain. In the p53-/- strain, it seemed that between day 1 to day 4, there was a production of new CHIKV infectious particles after which production increased from day 4 to day 8, but at a lower rate in the p53-/- strain compared to the w1118 strain. As the experiment of TCID50 was monitored only once, it must be repeated to consolidate this data. Taken all together, these results suggest that p53 is necessary for the survival of infected flies and for the viral replication of SINV and CHIKV.

A.



B.



**Figure 42: SINV and CHIKV virus replication (A.) and CHIKV production (B.) in *Drosophila melanogaster* w1118 and p53-/- strains.**

A. For each time course, a group of 10 flies were injected with Sindbis virus or CHIKV and whole flies were collected for RT-qPCR analysis. The quantifications of viral RNA using SINV nsp3 and CHIKV nsp2 were measured and normalized on housekeeping genes rp49, tbp and ef1. One dot groups 10 *Drosophila melanogaster* flies. SINV n=3: Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value<0.01. CHIKV n=2.

B. For each time, 10 flies were injected with CHIKV and the production of infectious particles was monitored by TCID50/ml. Data represent values of only one experiment (one dot groups 10 flies).

### 3.4.3 Discussion

The CHIKV and SINV replicated more efficiently in the w1118 strain than in the p53-/- strain. In addition, the CHIKV-infected w1118 strain produced more infectious particles compared to the p53-/- strain. The deletion of p53 seemed to impair the start of viral replication and production but did not definitively inhibit viral replication over time. To be complete, this study with CHIKV

should be repeated at least twice, adding the time point 96 hours post-injection for viral replication analysis. Nevertheless, all the infected p53-/- flies died at 15 days, while the w1118 flies presented 50 percent of survivors. Based on these data, we proposed different hypotheses based on the different roles of p53 and p53 isoforms on signaling pathways.

The first is based on the demonstration of antioxidant response induced by a p53 isoform in mosquito cells infected with Dengue virus (Chen et al. 2018). Indeed, Chen and colleagues have shown that only one of the two isoforms discovered in mosquito, called p53-2, was upregulated during Dengue virus infection and involved in the transcription of an antioxidant enzyme, catalase, allowing the control of cellular stress induced by the viral infection. Moreover, the p53-2 knockdown in infected C6/36 cells increased the cell death rate and decreased the expression of catalase. In addition, the over-expression of p53-2 decreased the cell death rate in infected C6/36 cells.

The second is based on the induction of autophagy by the p53 isoform in *Drosophila*. In *Drosophila*, among the four p53 isoforms described, two were studied in greater depth, i.e. p53 A and B. It was shown that only isoform A is involved in mediating the apoptotic response to DNA damage (Zhang et al. 2015). The p53 isoforms A and B could regulate opposite signaling pathways, apoptosis, and autophagy, differentially (Robin et al. 2019). The treatment of *Drosophila* p53-/- with ROS inducer (paraquat) was associated with a higher rate of mortality, higher caspase activation and impaired autophagic flux. Whereas isoform A inhibited autophagic flux, isoform B presented a protective response with a functional autophagic flux.

The *Drosophila* p53-/- strain did not express isoform A or B. The mortality rate of the p53-/- infected strain was higher and viral replication/production was impaired compared to the w1118 infected strain.

Firstly, we hypothesize that in the absence of one or two p53 isoforms, the antioxidant response induced during CHIKV infection could not be activated. In this way, the virus-induced stress increased and could not be controlled, inducing the impairment of viral replication, the accumulation of ROS, and then the death of the flies.

Secondly, we suggest that during SINV and CHIKV infection of *Drosophila*, autophagy could impact viral replication positively and be influenced by the activity of the p53 isoform. Given

that following stress induction (comparable to viral infection), the p53 B isoform presented a protective response and a functional autophagic flux, we hypothesize that during SINV and CHIKV infection, the p53 B-dependent autophagy participated in efficient viral replication and increased the survival of the flies. It could be interesting to test this hypothesis, first by analyzing the survival of the flies after the infection of different p53 isoform mutants strains: *Drosophila p53 A* -/- (express the isoform B) and *Drosophila p53 B* -/- (express the isoform A). In this case, it would be expected that the survival of the *D. melanogaster* p53 B-/- strain would decrease compared to the w1118 strain and that of the p53 A-/- strain would be comparable to the w1118 strain. Then, the analysis of viral replication by RT-qPCR might indicate whether the expression of p53 B isoform is correlated to a higher viral replication compared to the viral replication rate in p53 -/- and p53 B -/- strains.

As *Drosophila melanogaster* is not the natural vector of CHIKV, the question of resistance and permissiveness is interesting to discuss because it has recently been proposed that a rapid apoptotic response mediated by p53 could be associated with the resistance of the vector by the inhibition of viral propagation, whereas the inhibition of apoptosis and the engagement of innate immune response following necrotic cell death could be linked to permissiveness (Liu *et al.* 2013). Thus, based on our hypothesis, p53 seems important for the efficient infection of *Drosophila*, rendering this *in vivo* model interesting and useful for genetic investigations. However, thanks to the development and requirement of effective tools for genome editing in the mosquito *Aedes aegypti*, it seems that the next important investigation will be on *in vivo* mosquitoes infected with *alphaviruses* by blood feeding.

## In mosquito cells: Infection of mosquito *Aedes albopictus* and *Aedes aegypti* cell lines with CHIKV

### Results and discussion

#### 3.5 Effect of the origin of CHIKV production on the permissiveness and pro-apoptotic response of mosquito cell lines

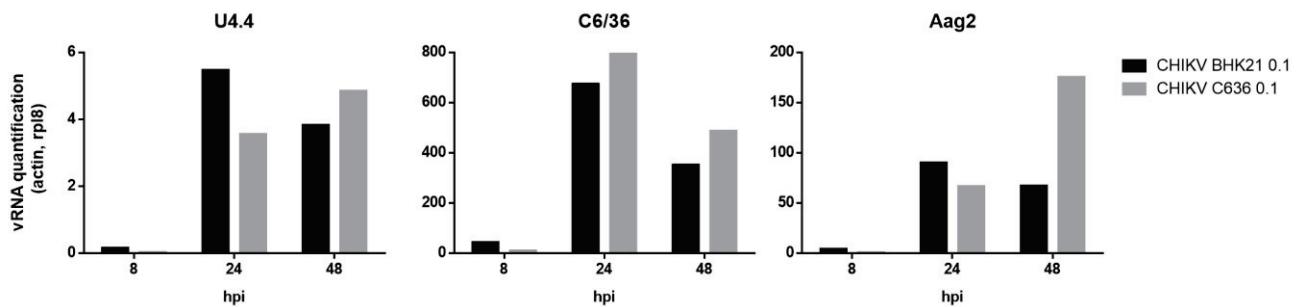
As presented in the introduction of the manuscript, the functional role of apoptosis in mediating insect immunity and/or participating in viral infection has been a topic of debate. Moreover, although insects present an immunological memory (Flemming 2017), apoptosis plays an important role in antiviral defense, maybe more effective than the antiviral effect in vertebrate hosts. Due to the difficulties of getting genetic tools to manipulate apoptotic response in mosquitoes, we decided to investigate the cellular response of mosquito cell lines infected with CHIKV, targeting the pro-apoptotic gene *Michelob\_mx* identified with a bioinformatics approach and verified as an orthologue of *Drosophila* IAP-antagonist genes (Zhou *et al.* 2005). We also designed primers targeting the p53 transcript and verified their specificity using the blast prediction tool and by obtaining a standard curve.

In addition, to “mimic” the primary infection of a mosquito cell following a blood meal taken from a vertebrate host, we infected mosquito cells with chikungunya virus produced either in mammalian cells (BHK21) or in mosquito cells (C636). Indeed, posttranslational modifications of proteins in mosquito cells are different from those in mammalian cells. These post-translational modifications occur in the Endoplasmic Reticulum (ER) and Golgi apparatus involving N-glycosylation. Compared to mammals, whose glycosylation has been studied extensively, the investigation of invertebrate glycosylation is limited. N-glycosylation refers to the attachment of a glycan to a specific asparagine of proteins, producing glycoproteins with variable extended glycan structures. The majority of secreted proteins and membrane are cotranslationally N-glycosylated (Zhu, Li, and Chen 2019). As the *alphavirus*'s glycoproteins are transported in the ER and the Golgi apparatus of mammalian and mosquito cells and then anchored in the plasma membrane of the two cells, the particles produced present different glycoprotein structures and different lipidic membrane compositions. These differences could have an impact on the entry process and replication and also on the cellular response after the

detection of the viral genome. Thus, when mosquito cells are infected with a particle produced in a mammalian cell, viral replication and /or the cellular response could be different to the cell response obtained after infection with a particle produced in a mosquito cell.

Considering that during the infection of a mosquito, the first ingested viral particles are derived from a mammal host and then by new particles produced in its own cells, we infected three different mosquito cell lines (C6/36, U4.4 and Aag2) with CHIKV produced either in the mammalian BHK-21 cell line or in the C6/36 cell line at MOI 0.1. At 8, 24 and 48 hours post-infection, the infected cells were collected and CHIKV replication was analyzed by RT-qPCR (Figure 43).

We observed an increase of CHIKV replication according to the relative expression of CHIKV nsp2 in the three cell lines tested (*Aedes albopictus* C6/36, *Aedes albopictus* U4.4, *Aedes aegypti* Aag2) from 8 to 48 hours post infection, and this increase was the same for both viruses tested. However, analyzing the quantity of viral RNA in the three cell lines, we observed that at 24 hours post infection, the viral RNA reached a value between 600 -800 in C6/36, a value of 100 in Aag2 and a very low level of expression in U4.4 with 4-6. Thus, the three cell lines seemed to be permissive to CHIKV, however they did not allow the viral replication at the same level. The differences between C6/36 and U4.4 could be explained by the dysfunctional antiviral RNAi pathway in C6/36 at the origin of a premature stop codon in the *dcr-2* gene (Brackney *et al.* 2010). Interestingly, in Aag2, which are Dcr-2 competent cells, the amount of CHIKV nsp2 RNA was higher than that of U4.4 (Dcr-2 competent) cells and less than that of C6/36 cells. Given that *Aedes aegypti* is the major vector of CHIKV, we suggest that the CHIKV is well adapted to Aag2 cells. However, this experiment was conducted only once, so it must be repeated to confirm the trend observed.

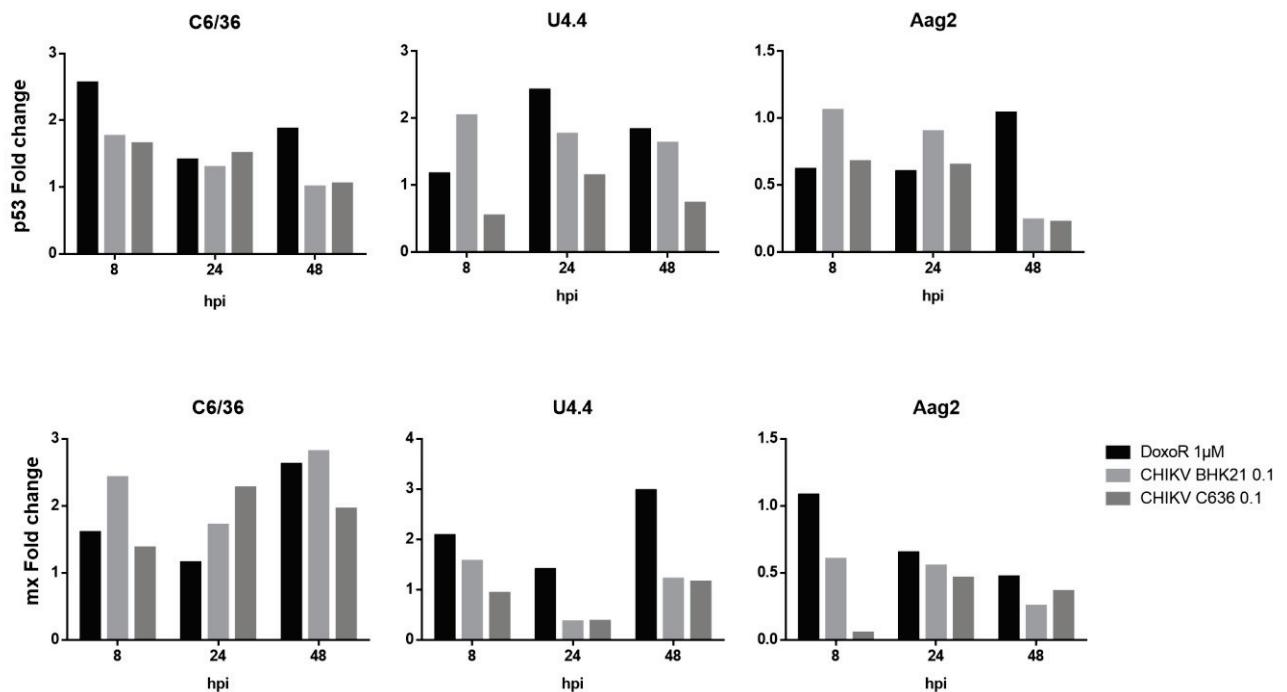


**Figure 43: Comparison of CHIKV replication in *Aedes albopictus* (U4.4 and C6/36) and *Aedes aegypti* (Aag2) mosquito cells lines with CHIKV produced in mammalian BHK-21 and mosquito C6/36 cells. The mosquito cell lines were infected with CHIKV produced in BHK-21 and C6/36 cells at MOI 0.1 and collected for RT-qPCR analysis at 8, 24- and 48-hours post infection. The expression level of CHIKV nsp2 was measured by RT-qPCR and normalized on the housekeeping genes actin and rp18 (n=1).**

Then, using the three cell lines we investigated the effect of CHIKV-BHK21 and CHIKV-C6/36 infection on p53 expression and the p53-target gene Michelob\_mx. First, we tried to detect *Aedes* p53 protein by Western blot using several antibodies (DO-1, CM1 and DO-11 cf. “List of primary antibodies used for Western blot” in Material and methods) but unfortunately none of them worked (data not shown).

Next, the regulation of p53 and mx mRNA was carried out by RT-qPCR. Since the genome of *Aedes albopictus* remains sparsely annotated the primers targeting p53 mRNA were validated by prediction and mx mRNA discovered by bioinformatics analysis (Zhou et al. 2005). We treated the cells with doxorubicin which induces DNA damage in mammal cells leading to the stabilization of the p53 protein and increasing the expression of p53-target genes, orthologues of mx. We treated cells with doxorubicin to obtain a positive control for mx upregulation and ensure confidence in the primers used. The results showed that treatment with doxorubicin induced different profiles of p53 and mx mRNA regulation over time in the three cell lines (Figure 44). In the C6/36 cell line, treatment with doxorubicin induced a one-fold increase of p53 at 8 hours and an increase in mx expression at 48 hours. In U4.4, the expression of p53 mRNA increased at 24 and 48 hours and mx mRNA increased at 8 and 48 hours. In Aag2, the doxorubicin treatment tended to decrease p53 and mx mRNA expression. This experiment was conducted only once, so it must be repeated to confirm the trend observed and conclude on the efficiency of doxorubicin as a positive control in mosquito cell lines.

Then, we analyzed the cellular response following the infection of cells either with CHIKV-BHK21 or CHIKV-C6/36. The infection of C6/36 with CHIKV-BHK21 led to an increase of *mx* expression at 8 hpi and 48 hpi with a decrease in the middle. Infection with CHIKV-C6/36 induced an increase in *mx* expression at 24 and 48 hpi. In parallel, *p53* gene expression increased slightly at 8 hpi for both viruses and at a later time point the amounts were comparable to those of mock cells.



**Figure 44: Analysis of the expression level of *p53* and *mx* in C6/36, U4.4 and Aag2 mosquito cell lines infected by CHIKV produced in mammalian (BHK-21) and mosquito (C6/36) cell lines (one experiment).**

The mosquito cell lines were infected with CHIKV produced in BHK-21 and C6/36 cells at MOI 0.1 and collected for RT-qPCR analysis at 8, 24- and 48-hours post-infection. The expression level of *p53* and *mx* were measured by RT-qPCR and normalized against the housekeeping genes *actin* and *rpl8* before calculating the fold change, i.e. the ratio of the target gene in CHIKV cells vs. mock cells. The mRNA level of mock cells was established at 1.

The infection of *Aedes albopictus* U4.4, dcr-2 competent cells with CHIKV-BHK21 led to the upregulation of *p53* mRNA, while infection with CHIKV-C6/36 induced the downregulation of *p53* mRNA. At 24 and 48 hpi, the amount of *p53* mRNA remained higher in cells infected with CHIKV- BHK21 than with CHIKV-C6/36. In parallel, the upregulation of *p53* mRNA in CHIKV-BHK21-infected cells was not associated with an increase of *mx* transcript. In addition, the

expression of *mx* mRNA decreased drastically from 8 to 24 hours for both viruses and then returned to a value close to the mock condition.

Regarding the infection of the *Aedes aegypti* Aag2 cell line, we observed a downwards trend in the p53 transcript from 8 to 48 hpi in CHIKV-BHK21- and CHIKV C6/36-infected cells. In parallel, we also observed a downregulation of *mx* transcript in CHIKV BHK21-infected cells from 8 to 48 hpi. The infection with CHIKV-C6/36 led to a drastic decrease of *mx* at 8 hpi and then an increase from 8 to 24- 48- hpi.

These data gave interesting results such as significant differences in the expression of the apoptotic *mx* gene after infection of cells presenting a functional or dysfunctional antiviral RNAi pathway and differences depending on viral particle composition. They will need to be repeated to confirm these elements.

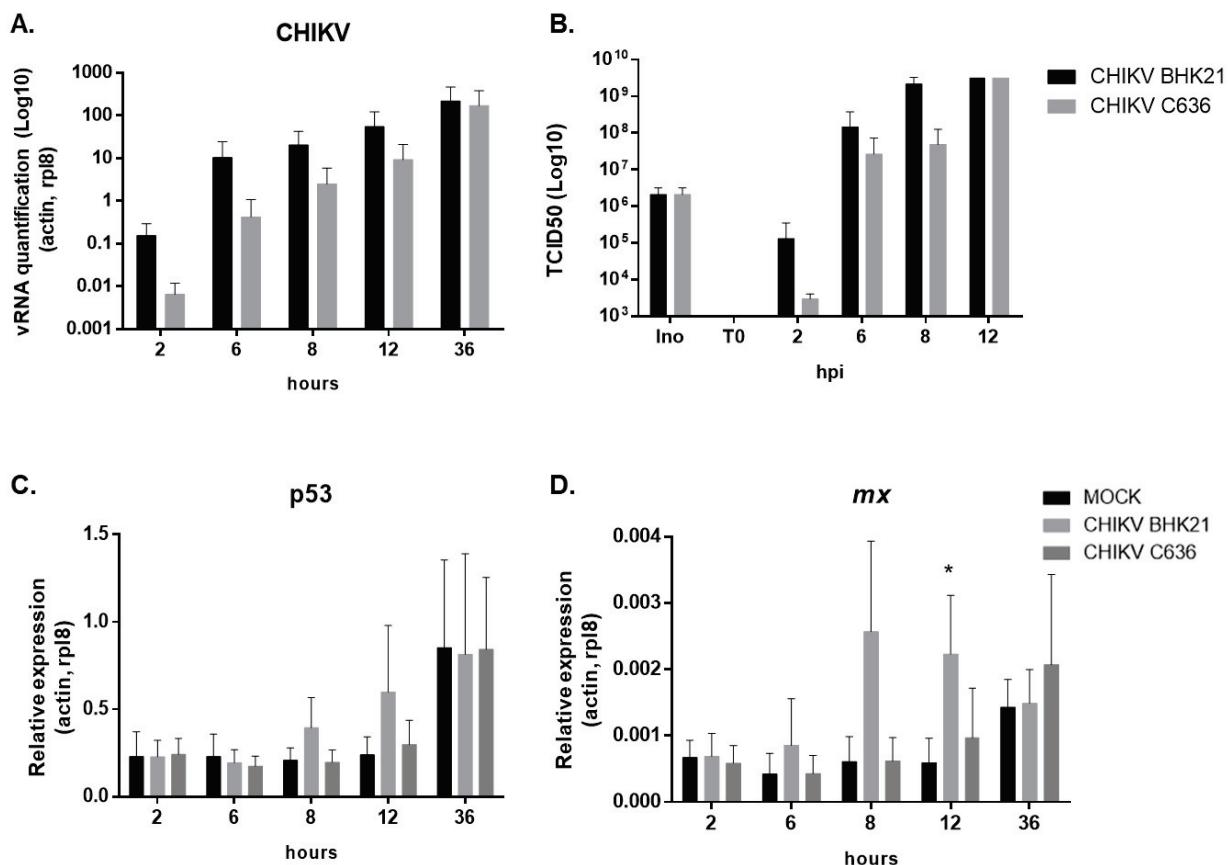
Indeed, it seems that infection of C6/36 induced an increase of *mx* transcript without cytopathic effects (CPE) observable under the microscope (data not shown), which is intriguing. We could not investigate the *Aedes* caspase activity in parallel due to lack of time and tools. In any case, it seems that whether there was an RNAi innate immune response or not, the expression of the p53 pro-apoptotic target gene was not accompanied by CPE. Was this response inhibited or is it present at a low level, associated with the infection and necessary for the establishment of chronicity in cells? Could the expression of p53 lead to the transcription of other target genes that are not apoptotic during CHIKV infection? In order to investigate these questions, we decided to focus on C6/36 infection with CHIKV produced either in BHK-21 or C6/36 cell lines. The choice of using the C6/36 cell line initially was based on its permissiveness to CHIKV and that it allowed studying the antiviral cellular response by overcoming the RNAi innate response.

### 3.6 Time course of CHIKV infection in C6/36 cells and analysis of pro-apoptotic and antioxidant p53-target genes

The C6/36 cells were infected with CHIKV produced either in BHK21 or in C6/36, and the supernatants were collected at different times post-infection to quantify the production of infectious particles and the cells for RT-qPCR analysis (Figure 45 A, B, C and D). As before, we observed a difference in viral replication between the C6/36 infected with CHIKV-BHK21 and CHIKV-C6/36 (Figure 45 A.). From 2 hours, we detected more CHIKV-BHK21 viral RNA ( $\log_{10} 0.1$ ) than CHIKV-C6/36 ( $\log_{10} 0.01$ ), suggesting that CHIKV resulting from BHK21 cells infected the C6/36 cells more efficiently than CHIKV resulting from C6/36 cells. Then, from 6 to 12 hours, we observed an increase in the replication rate for the two viruses with a higher detectable quantity of CHIKV BHK-21.

In addition, the inoculum “Ino” (the viral inoculum is the start dilution with appropriate MOI used to infect cells) of the viral titer (Figure 45 B.) shows that C6/36 cells were infected with the same MOI (0.1) of CHIKV-BHK21 and CHIKV-C6/36. At 2 hours post-infection, no infectious particles were detectable, indicating that the residual particles were eliminated. We observed the same differences as qPCR analysis for virus production, with a higher quantity of viral particles from cells infected with CHIKV-BHK-21 ( $10^5$ ) than cells infected with CHIKV-C6/36 ( $4.10^3$ ). Then, from 6 to 12 hours, the tendency was the same and after 12 hours the quantity of viral particles was too high and the viral titration was saturated (data not shown).

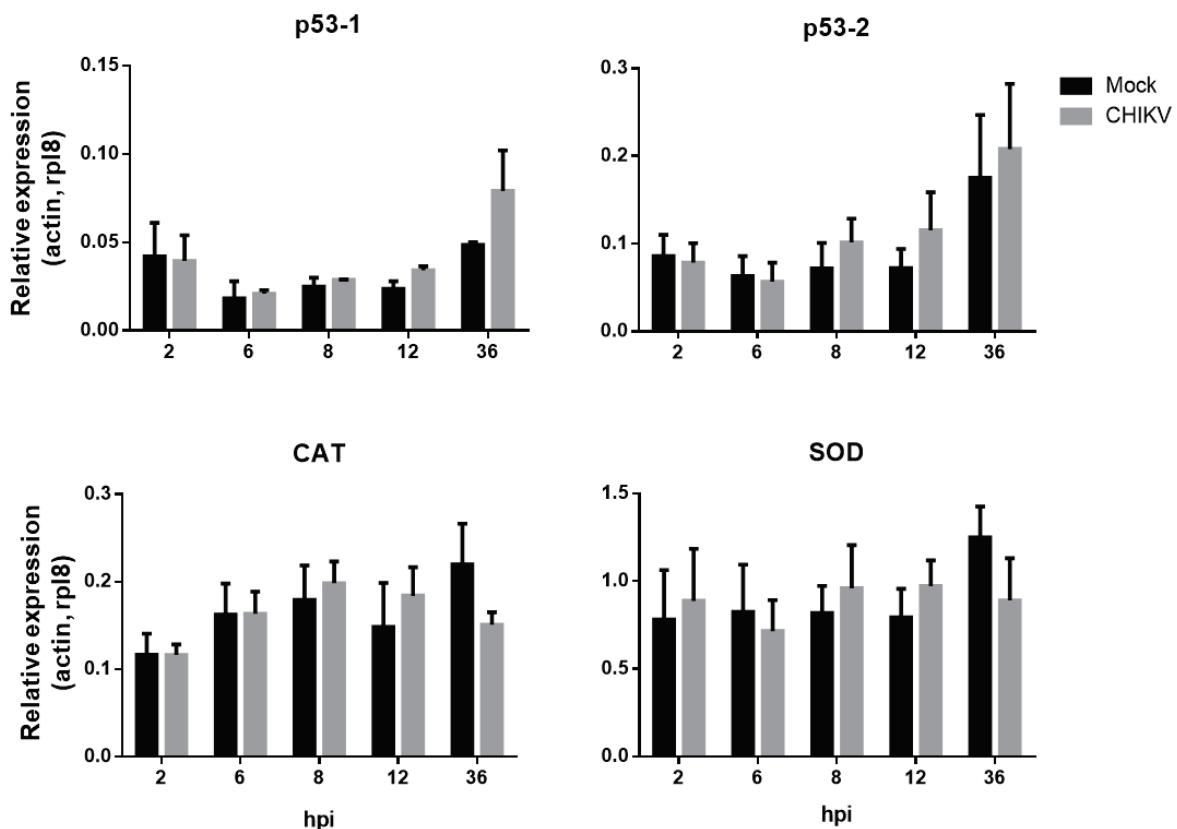
Taken together, we could hypothesize that more CHIKV-BHK21 infectious particles infected the C6/36 cells. Maybe the binding and entry of CHIKV-BHK21 was more efficient compared to the binding of CHIKV-C6/36. Moreover, new infectious particles were produced 2 hours post-infection, indicating that viral replication is faster than in mammalian cells. It seemed that the differences in viral replication and production were the cause of very early events in the CHIKV life cycle in the mosquito cell line.



**Figure 45: The viral infection and cellular response of C636 infected with CHIKV produced in mammal and mosquito cells.** The mosquito C6/36 cell line was infected with CHIKV produced in BHK-21 (CHIKV-BHK21) and in C6/36 (CHIKV-C6/36) cells at MOI 0.1 and collected for RT-qPCR analysis at 8, 24- and 48-hours post-infection and TCID50 at 2, 6, 8, and 12 hpi. The expression level of CHIKV nsp2 (vRNA=viral RNA), p53 and mx were measured by RT-qPCR and normalized on housekeeping genes actin and rpl8 (A, C and D.). The production of infectious particles in the supernatants of infected C6/36 was obtained by limiting dilution assays in VeroE6 and the results were reported as log<sub>10</sub>-TCID50/ml (B.). Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $<0.05$ .

Meanwhile, we wanted to know if this difference of infection had an impact on p53 and mx mRNA expression (Figure 45 C. and D.). The level of regulation of p53 mRNA did not change over time in infected cells compared to uninfected cells. At 8 and 12 hpi, we observed a non-significant increase in p53 mRNA in cells infected with CHIKV BHK-21 and in parallel, the increase of mx mRNA at 8 hpi (nonsignificant) and at 12 hpi (\*,  $p<0.05$ ). At 36 hpi, the level of mx mRNA expression in infected cells was comparable to its expression in uninfected cells. The infection of C6/36 with CHIKV produced in C6/36 did not induce regulations of p53 and mx mRNA over time.

Given that p53 mRNA was not regulated, regulation could be isoform specific. We quantified p53-1 and p53-2 mRNA plus two antioxidant enzymes, the catalase CAT and the superoxide dismutase SOD in C6/36 cells infected with CHIKV produced in BHK-21 cells (Figure 46). These results indicate that no regulation of the four transcripts could be detected during CHIKV infection.



**Figure 46: p53 isoforms mRNA and antioxidant enzyme CAT and SOD mRNA expression in the *Aedes albopictus* C6/36 cell line infected with CHIKV produced in the mammal BHK-21 cell line.** The mosquito C6/36 cell line was infected with CHIKV produced in BHK-21 cells at MOI 0.1 and collected for RT-qPCR analysis at 8, 24- and 48-hours post-infection. The expression levels of p53 isoforms (p53-1 and p53-2), mx, CAT and SOD were measured by RT-qPCR and normalized against housekeeping genes actin and rpl8. Data are the mean  $\pm$  SD of three independent experiments. Statistical analyses were performed with an unpaired t-test with a p-value  $>0.05$ , nonsignificant.

Taken together, these results suggest that infection with CHIKV produced in a mammalian cell would either infect more C6/36 cells or replicate more, compared to CHIKV produced in the same C6/36 mosquito cell. Moreover, when C6/36 cells were infected with the same quantity of viral particles, this difference in viral replication and production led to a significant increase

in *mx* transcript at 12 hours post-infection in the cells infected with virus resulting from BHK1 cells. The expression of this transcript was not accompanied with observable cytopathic effects (data not shown), suggesting that either a few portions of cells underwent cell death, or apoptosis was not induced or perhaps inhibited by CHIKV. Moreover, these results suggest that while viral replication was higher with CHIKV-BHK21 and induced higher expression of *mx* than CHIKV-C6/36, *mx* induction was not correlated to a better antiviral response in the C6/36 cell line. Thus, to go further it could be interesting to monitor the same experiment on U4.4 or Aag2 cells to compare the cellular response in a competent Dcr-2 cell line. Of course, another very interesting experiment would be the infection of *in vivo* mosquitos by blood feeding with CHIKV produced in mammalian or mosquito cells and analyze p53, *mx* transcripts and viral infections on sections of the epithelial cells of the midgut at an early stage of infection.

### 3.7 Conclusion

To sum up, we mainly focused on the p53-dependent apoptotic and antioxidant cellular response of the *Aedes albopictus* C6/36 cells infected with CHIKV, produced either in mammalian or mosquito cells. Our first objective was to explore the effect of CHIKV particles produced in mammal and mosquito cells on cellular response, knowing that C6/36 was not competent for the antiviral RNAi pathway. Thus, we observed a different cellular response from C6/36 infected with either mammal or mosquito infectious particles. Indeed, the infection of C6/36 with mammalian particles led to higher viral replication rate and higher production of infectious particles, as well as the upregulation of the pro-apoptotic p53-target gene *mx* in the late stage of infection. These results suggest that CHIKV of mammalian and mosquito origins influences the viral replication and the cellular response. However, the induction of the pro-apoptotic p53-target gene *mx* in the early stage of infection (with mammalian particles) did not influence cell death as no cytopathic effects were observable during the infection of C6/36 cells. We propose that following the detection of mammalian-CHIKV particles, CHIKV inhibits the induction of cell death downstream of *mx* expression, explaining the absence of cell death with the upregulation of *mx*.

We will further investigate the possible function of p53 and p53 isoforms in mosquito cell fate during CHIKV infection. To this end, we will knockdown the expression of p53-1 and p53-2 using silencing RNA and monitor the impact on viral infection, cell death and antioxidant response using C6/36 (deficient for RNAi pathway) and U4.4 or Aag2 (efficient for RNAi pathway) cell lines. In parallel, we will monitor the effect of *AeIAP* knockdown on viral infection. Indeed, if cell death is controlled by CHIKV downstream p53-traget gene *mx*, we could investigate the control of IAP during CHIKV infection.

## CHAPTER 2) Analysis of the effect of p53 isoforms on CHIKV infection in mammalian cell lines

### 1 Objectives

The role of p53 isoforms during CHIKV infection has never been studied and after showing an antiviral role of p53 we decided to go further by studying the possible isoform regulations and decipher their function during the infection of LHCN-M2 with CHIKV. To do this, we decided to study only the p53 N-terminal isoforms and more particularly  $\Delta 133p53\alpha$  and the  $\Delta 40p53\alpha$ . The former because it is one of the most characterized isoforms and it has been suggested that  $\Delta 133p53\alpha$  can act as a modulator of p53 in response to stress. Moreover, it has been demonstrated that during the infection of two different pathogens, the bacteria *Helicobacter pylori* and Influenza A virus, the  $\Delta 133p53$  isoform could negatively impact the activity of p53. The latter,  $\Delta 40p53\alpha$ , has been little studied in infectiology although it has been shown to have, among other functions, a dominant-negative effect on p53. For these reasons, it is interesting to investigate their potential effect on CHIKV infection. Thus, we examined the regulation of both isoforms during CHIKV infection of LHCN-M2 cells and then tried to decipher their function by overexpression.

### 2 Material and Methods

#### 2.1 Cell lines and viruses

HEK 293T, BHK-21, VeroE6 and U2OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco<sup>TM</sup> Thermo Scientific) supplemented with 10% Foetal Bovine Serum (FBS, HyClone).

LHCN-M2 (human skeletal myoblasts) immortalized cells were cultured in medium 4:1 Dulbecco's Modified Eagle Medium (DMEM, Gibco<sup>TM</sup> Thermo Scientific)/ Medium 199 (Gibco<sup>TM</sup> Thermo Scientific) supplemented with 15% FBS, 0.02% HEPES, 0.03 $\mu$ g/mL Zinc Sulfate, 1.4 $\mu$ g/mL Vitamin B12, 0.055 $\mu$ g/mL Dexamethasone, 2.5ng/mL recombinant human Hepatocyte Growth Factor and 10ng/mL recombinant human FGF-basic. LHCN-M2 cells were a kind gift of Drs Chun-Hong Zhu and Woodring E. Wright (Zhu et al. 2007). HEK 293T, BHK-21,

VeroE6, U2OS and LHCN-M2 cell lines were maintained at 37°C, 5% CO<sub>2</sub> in a humidified incubator.

Chikungunya virus (CHIKV) LR2006 strain from La Réunion was used to generate replicative viruses: from the plasmid clone, viral RNA (vRNA) was generated by *in vitro* transcription using mMessage mMachine kit, Ambion). Then, 5.10<sup>6</sup> BHK-21 cells were trypsinized, washed in PBS 1 X, resuspend in Opti-MEM medium (Gibco™ Thermo Scientific) and electroporated with 10µg of vRNA in a 0.4 cm cuvette using a Gene Pulser Xcell electroporation system (program used: 1 pulse, 270V, 950µF). Electroporated cells were seeded in T-75 flask cultured with DMEM, 10% FBS for 16h at 37°C, 5% CO<sub>2</sub> until medium change. The supernatant was harvested 24 hours later, filtered at 0.2 µm and then mixed with 0.5M sucrose (MP Biomedicals) and 50mM HEPES (Gibco™ Thermo Scientific) for conservation at -80°C. Viral stocks were titrated by TCID50 and plaque assay on VeroE6.

## 2.2 Generation of TP53 CRISPR-mediated knockout LHCN-M2 and U2OS cell line

The same protocols than in the first chapter have been used to generate knockout cell lines in this chapter. The LHCN-M2 sgRNA\_luc and sgRNA\_p53 were used and other CRISPR/Cas9 cell lines were generated using following primers “sgRNA\_10\_p53”:

sgRNA name	Sequence 5'-3'	
sgRNA_400_p53	Forward	CACCGCCCCGGACGATATTGAACAA
	Reverse	AAACTTGTTCAATATCGTCCGGGGC
sgRNA_402_p53	Forward	CACCGCCCCTGCCGTCCAAGCAA
	Reverse	AAACTTGCTTGGGACGGCAAGGGGC
sgRNA_luc	Forward	CACCGGGCATTTCGCAGCCTACCG
	Reverse	AAACCGGTAGGCTCGAAATGCC
sgRNA_10_p53	Forward	CACCGTCGACGCTAGGATCTGACTG
	Reverse	AAACCGAGTCAGATCCTAGCGTCGAC

**Table 8: List of human primers used for cloning of sgRNA in lentiCRISPRv2 vector**

### 2.2.1 Transduction of LHCN-M2 with doxycycline-inducible shRNA-Δ40p53

LHCN-M2 cells were transduced with lentivirus containing doxycycline-inducible pLKO-Tet-shRNAΔ40p53 (provided by CRCL, UMR INSERM 1052, Centre Leon Berard). Cells were treated with 5 or 20 µg/ml of doxycycline for 24 hours and then analyzed by Western blot using anti-p53 CM1 antibody.

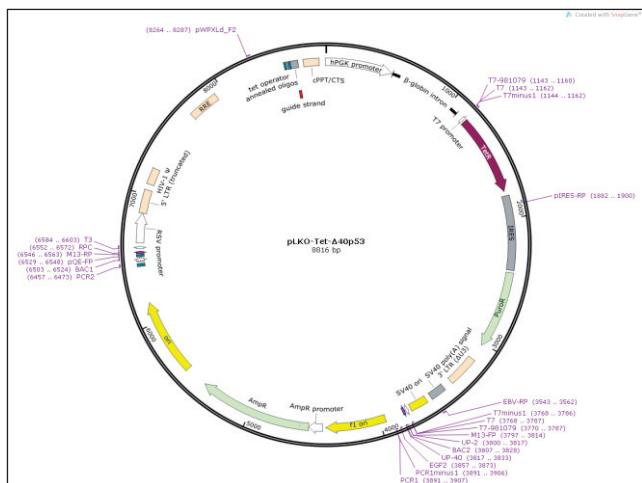


Figure 47: Map of plasmid pLKO-Tet-shRNA-Δ40p53.

## 2.2.2 Generation of Doxycycline-inducible system for overexpression of $\Delta 40p53\alpha$ and $\Delta 133p53\alpha$ isoforms

### 2.2.2.1 Production of VSVg pseudo particles

The generation of lentivirus allow us to generate stable inducible cell lines expressing Δ40p53α and Δ133p53α isoforms. Day first,  $2.5 \cdot 10^6$  HEK 293T cells were seeded in cell culture dish in 8 mL DMEM + 10% FBS. The next day, cells were co-transfected using calcium phosphate co-precipitation method by adding 8.3 µg of HIV packaging construct with a CMV promoter (psPAX2, AddGene 12260), 8µg of the pCW57.1-Tet-HA-Δ40p53α or Δ133p53α (AddGene) kindly provided by Dr. Andrea Paradisi and 2.5µg of VSV glycoprotein-expressing construct with CMV promoter (pVSVg, AddGene 8454). Media was changed 16 hours after transfection, and 24 hours later supernatant was harvested, filtered through 0.45 µm pore-sized membranes and stored at -80°C before transduction of target cells.

### 2.2.2.2 Verification of doxycycline-inducible system for overexpression of p53 isoforms in LHCN-M2 cells

The transduced LHCN-M2 cells were amplified during a week and then treated with 2 $\mu$ g/ml of doxycycline for 24 hours to induce the overexpression of tagged HA- $\Delta$ 40p53 $\alpha$  or HA- $\Delta$ 133p53 $\alpha$  (provided by CRCL, UMR INSERM 1052, Centre Leon Berard). To test the inducible overexpression of p53 isoforms we performed western blot (as described in the Material and method of Chapter 1) using anti-HA antibody (HA-Tag C29F4, Rabbit, Cell Signaling Technology 3724T) and anti-p53 CM1.

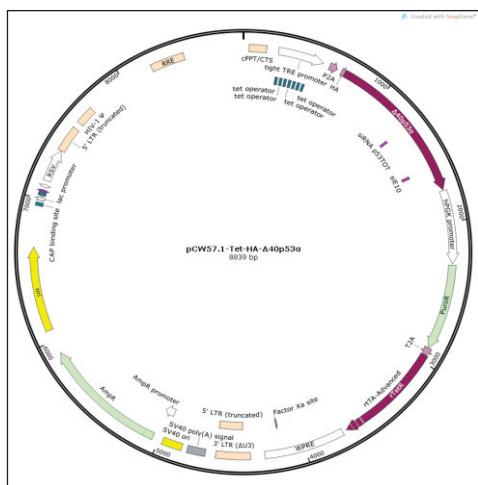


Figure 48: Map of plasmid pCW57.1-Tet-HA-Δ40p53α.

## 2.3 Cell viability

In order to monitor the effect of viral infection on LHCN-M2 cell lines viability, the ATP was quantified in cells and supernatants using CellTiter-Glo® luminescence cell viability assay kit (Promega). Cells were plated on 96-well plate day one at 10 000 cells/well in 100 $\mu$ l of LHCN-M2 medium. At several different time points 100 $\mu$ l of cell titer was added on cells and ATP quantification was measured by luminescence using a plate reader (Victor<sup>2</sup> plate reader, Perkin Elmer).

## 2.4 Immunostaining and flow cytometry analysis

After CHIKV infection in 48-well plate and at different times post-infection, cells were fixed in 2% final paraformaldehyde for 15minutes and then wash twice in PBS 1X and then incubated with anti-viral capsid primary antibody (1/800) in permeabilization solution A (0.1% saponin, 10% FBS, PBS 1X) for 1 hour at 4°C. After primary antibody incubation, cells were washed twice with solution A (0.1% saponin, 10% FBS, PBS 1X) before being incubated with FITC conjugated

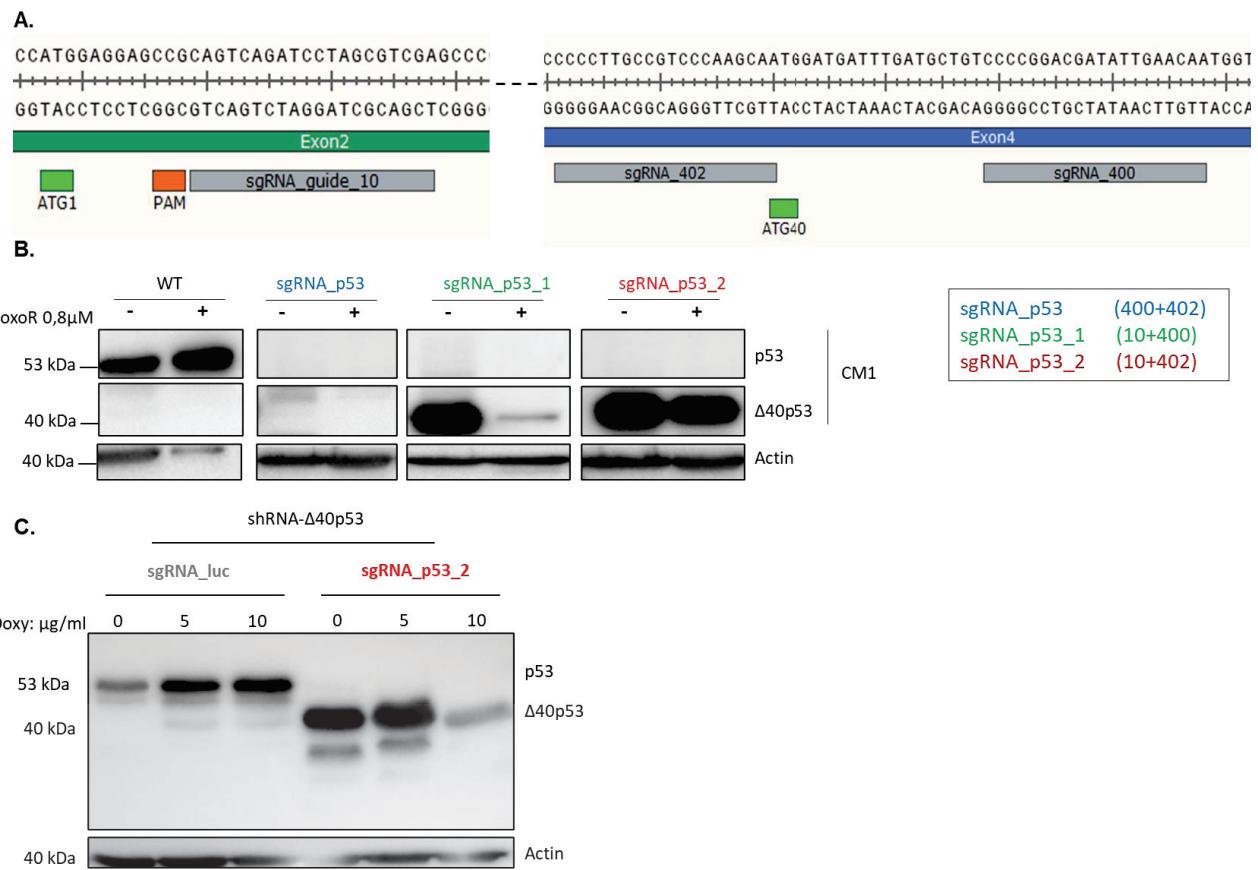
anti-mouse IgG secondary antibody (1/200) (F0257, Sigma Aldrich) in solution A (0.1% saponin, 10% FBS, PBS 1X) for 45 minutes at 4°C. After that, cells were washed with PBS 1X for twice and immunostained cells were analyzed using flow cytometer FACS Calibur, BD Biosciences.

## 3 Results

### 3.1 Generation of an endogenous Δ40p53 isoform overexpressing cell line using CRISPR/Cas9 technology

#### 3.1.1 Effect of Δ40p53 overexpression on CHIKV infection

Our first objective was to induce a complete knockout of *p53* gene using CRISPR/Cas9 technology, thus we designed a single strand RNA (sgRNA) targeting the 5' start of the gene to induce a reading frame shift. Thus, we designed an oligo targeting the exon 2 of *p53* gene not far from the first ATG1 codon, named sgRNA\_10\_p53. The same strategy as for part one was carried out, i.e. the transduction of two sgRNAs (sgRNA\_400\_p53 and sgRNA\_402\_p53) to improve the efficiency of the technology. Figure 49 A. presents the position of the different sgRNAs (10, 402 and 400) on the *p53* gene.



**Figure 49: Generation of  $\Delta 40p53$  endogenous overexpression cell line using CRISPR/Cas9 technology. A. Positions of Single guide RNA 10, 400 and 402 on p53 gene. B. Validation of full length p53 knockout LHCN-M2 and endogenous overexpressing  $\Delta 40p53$  isoform cell lines using CRISPR/Cas9 technology C. Confirmation on  $\Delta 40p53$  isoform expression using shRNA- $\Delta 40p53$  in the LHCN-M2 sgRNA\_p53\_2 cell line.**

After the generation of CRISPR/Cas9 cell lines, the profile of p53 was analyzed by Western blot after treatment of the different cell lines with 0.8  $\mu$ M of doxorubicin to detect the possible accumulation of p53. Unexpectedly, LHCN-M2 transduced with pseudo-particles containing the sgRNA\_10, presented a huge over-expression of a 40 kDa protein detected with anti-p53 CM1 antibody (Figure 49 B.) and the anti-p53 DO-11 antibody (data not shown).

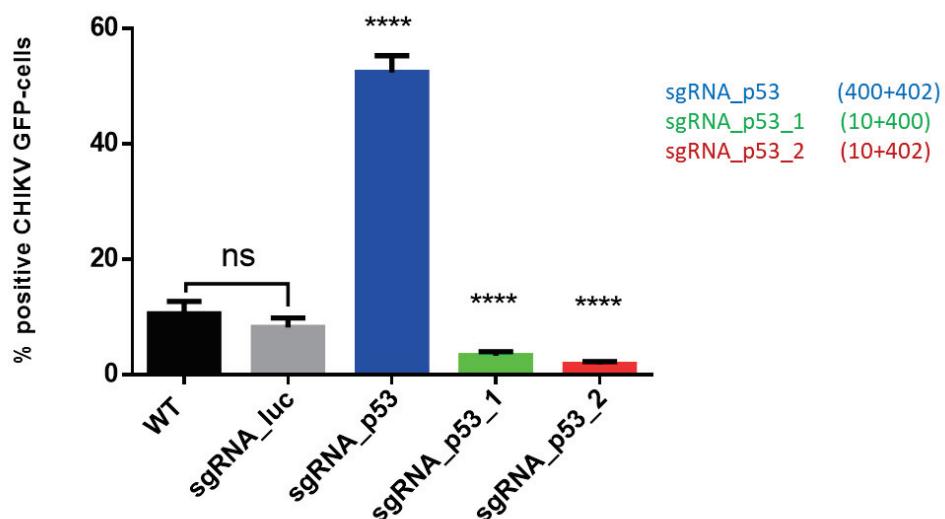
Moreover, following doxorubicin treatment of both the cell lines generated (sgRNA\_p53\_1 and sgRNA\_p53\_2) the profile of the 40 kDa protein was different, i.e. in the first one expression decreased drastically whereas in the second one expression was the same as in the untreated cells. We hypothesized that this 40 kDa protein could correspond to the  $\Delta 40p53$  isoform.

To test this hypothesis, we employed an RNA interference-related strategy using doxycycline-inducible shRNA system targeting  $\Delta 40p53$ . The shRNA  $\Delta 40p53$  were designed and kindly given by Dr. Andrea Paradisi (Cancer Research Center of Lyon – CRCL). The LHCN-M2 sgRNA\_luc and

sgRNA\_p53\_2 cell lines were transduced with shRNA-Δ40p53 plasmid containing the doxycycline-inducible system. Then, the cells were treated with 5 and 10 µg/ml of doxycycline for 24 hours and 50µg of protein in cell lysates was used for Western blot analysis using anti-p53 CM1 antibody (Figure 49 C.). We observed a reduction of the 40 kDa protein in LHCN-M2 sgRNA\_p53\_2 cell line after treatment with 10µg/ml of doxycycline. In parallel, we also transfected the cells with siRNA against Δ40p53 and observed a decrease in the expression of the 40 kDa protein (data not shown).

These results demonstrated that the 40 kDa protein over-expressed in the LHCN-M2 sgRNA\_p53\_2 cell line corresponded to the Δ40p53 isoform. In conclusion, we generated two different full-length p53 knockout LHCN-M2 cell lines which overexpressed the Δ40p53 isoform and responded differentially after DNA damage stress.

In the second step, we studied the impact of the endogenous overexpression of the Δ40p53 isoform on CHIKV infection. The LHCN-M2 sgRNA\_p53\_1 and sgRNA\_p53\_2 cell lines were infected with CHIKV-GFP at MOI 0.1 and then directly analyzed by flow cytometry detecting GFP (Figure 50). Interestingly, we observed a significant decrease of CHIKV-GFP infection in both cell lines compared to control sgRNA\_luc and WT cells.

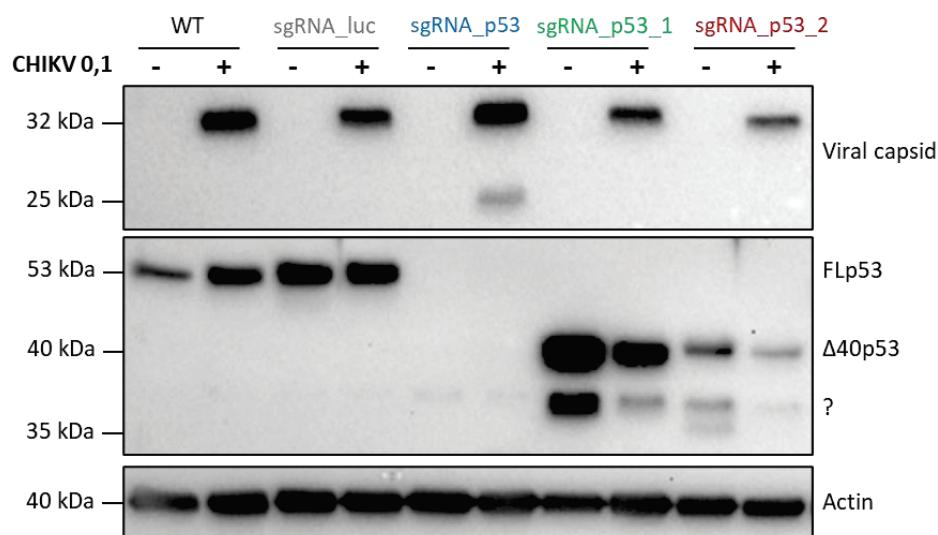


**Figure 50: Effect of endogenous overexpression of Δ40p53 isoform on CHIKV infection in LHCN-M2 cells.** Cells were infected with CHIKV-GFP at MOI 0.1 for 24 hours and then fixed in PFA 4% to detect the proportion of infected cells by flow cytometry analysis. Data are the mean ± SD of three independent experiments. Statistical analysis was performed with an unpaired Student t-test with a p-value <0.001.

Then, the expression of CHIKV capsid and p53 isoforms in LHCN-M2 wildtype, sgRNA\_luc, sgRNA\_p53\_1 and sgRNA\_p53\_2 cells lines was analyzed by Western blot 24 hours post-infection. The Western blot presented in figure 51 is representative of what we observed in three separate experiments.

We observed higher expression of viral capsid in LHCN-M2 WT cells than in sgRNA\_luc cells for the first experiment but in the two other experiments, the quantity of viral capsid was similar in both cell lines. The CHIKV-infected sgRNA\_p53 was used as a “positive control” of viral capsid detection. Then, in the three independent experiments, we observed the same quantity of viral capsid in the sgRNA\_p53\_1 cell line, and a strong reduction in the sgRNA\_p53\_2 cells, compared to the sgRNA\_luc cells. This result suggests that the endogenous expression of the Δ40p53 isoform negatively impacts the expression of viral capsid.

Interestingly, the detection of the Δ40p53 isoform showed that in the sgRNA\_p53\_1 and sgRNA\_p53\_2 cell lines the quantity of the isoform decreased in the infected cells compared to the mock cells. This result suggests that CHIKV infection induces a degradation of the Δ40p53 isoform.



**Figure 51: Viral capsid, p53 and Δ40p53 isoform protein expression in LHCN-M2 cell lysate after 24 hours of CHIKV infection.** Cells were infected with CHIKV at MOI 0.1 for 24 hours. The cells were collected, and 50 µg of proteins were loaded on SDS-PAGE for Western blot assay. Viral capsid SFV-C antibody was used to detect CHIKV and anti-p53 CM1 to detect the Δ40p53 isoform and other p53 isoforms. The antibody raised against housekeeping protein β-actin was used as loading control.

Finally, another protein, of about 37 kDa was detected in sgRNA\_p53 1 and 2 cell lines, and its expression decreased at 24 hpi compared to the mock cells. We hypothesized that this protein could be a  $\Delta$ 40p53 isoform, maybe a C-terminal isoform corresponding to  $\Delta$ 40p53 $\beta$  or  $\Delta$ 40p53 $\gamma$  whose expression also decreased during CHIKV infection.

In conclusion, we showed that in p53 knockout, overexpressing  $\Delta$ 40p53 isoform cells, the percentage of infected cells decreased compared to the sgRNA\_luc control cell line and the viral capsid decreased only in the sgRNA\_p53\_2 cell line. Moreover, the quantity of the  $\Delta$ 40p53 isoform decreased in infected sgRNA\_p53\_1 and sgRNA\_p53\_2. While the quantity of  $\Delta$ 40p53 was higher in the mock sgRNA\_p53\_1 cell line compared to the mock sgRNA\_p53\_2 cells, these results suggest that the decrease in viral protein and the percentage of infected cells were not dependent on  $\Delta$ 40p53 quantity. Finally, the reduction of viral capsid and  $\Delta$ 40p53 isoform in infected sgRNA\_p53\_2 suggests an anti-viral effect of the isoform and a degradation of  $\Delta$ 40p53.

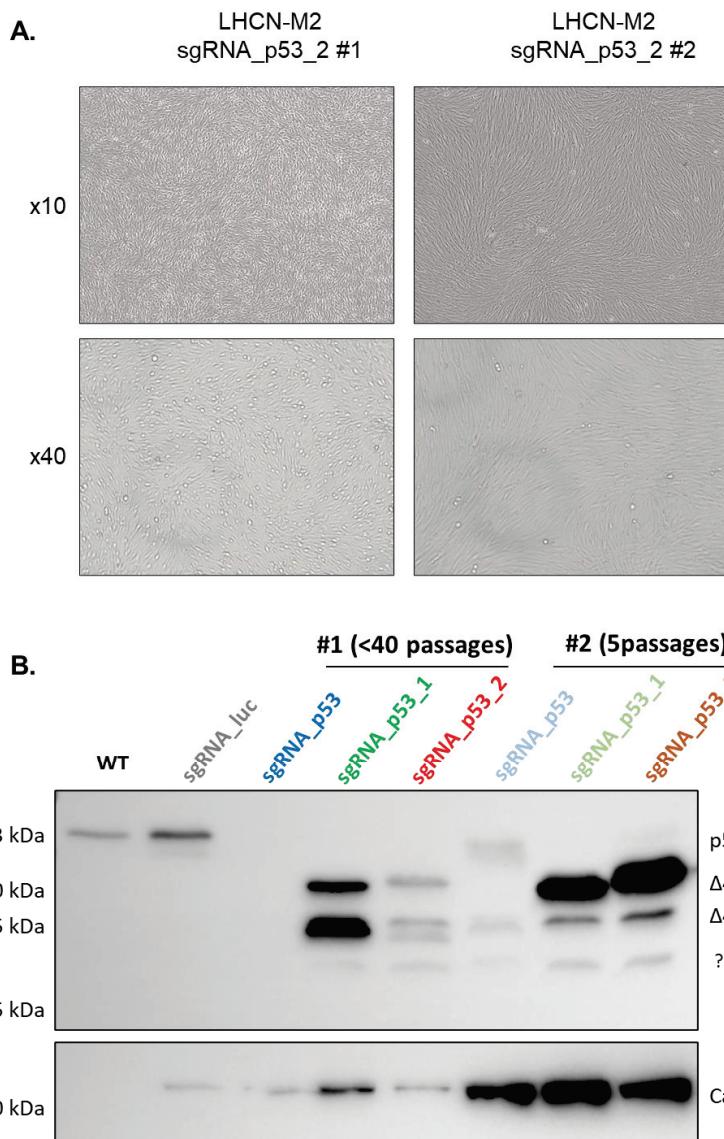
### 3.1.2 Change in cell morphology of the LHCN-M2 $\Delta$ 40p53 cell line

Next, we wanted to investigate the role of the  $\Delta$ 40p53 isoform using the sgRNA\_p53\_2 cell line as the expression of the viral capsid and the percentage of infected cells decreased compared to control cell line sgRNA\_luc. However, after dozens of passages in culture, the morphology of the sgRNA\_p53\_2 cell line changed with a more rounded shape (Figure 52 A. left panel), but not the sgRNA\_p53\_1 cell line. The sgRNA\_p53\_2 cell line seemed to have lost its skeletal muscle phenotype. We hypothesized that CRISPR/Cas9 had generated an off target. To test this hypothesis, we generated new cell lines sgRNA\_p53 1 and 2: the first cell lines were named #1, and the new cell lines #2 (Figure 52 A. right panel).

We again generated the LHCN-M2 sgRNA\_p53 and sgRNA\_p53\_1 cell lines and validated the p53 isoform expression by Western blot (Figure 52 B.). Moreover, we observed a decrease in the  $\Delta$ 40p53 isoform in both cell lines, first generation (#1), and in the newly generated cell lines (#2) we observed a higher quantity of the  $\Delta$ 40p53 isoform. In parallel, we detected a high protein detection of the Cas9 two weeks after the generation of cell lines #2 and it was still expressed after several months in the cell lines #1 (Figure 52 B.). The discrepancy in the

detection of Δ40p53 isoform could be due to the fact that the Cas9 nuclease participates in the evolution of the cell lines generated by continuing to cut double strand DNA.

As the CRISPR/Cas9 LHCN-M2 cell lines were not clonal, we hypothesized that the cells without overexpression of the Δ40p53 isoform would be selected against cells overexpressing the p53 isoform during successive passages in culture. Moreover, if we consider that the phenotype of the sgRNA\_p53\_2 (#1) cells is linked to off-target(s), then we could hypothesize that the cells presenting off-targets would be selected against the cells which over-expressed the Δ40p53 isoform, explaining why the expression decreased over time. Finally, off-target(s) could have been at the origin of the decrease of CHIKV infection and the viral capsid. Therefore, we first investigated the effect of the newly generated cell lines on cell viability and then on viral infection.



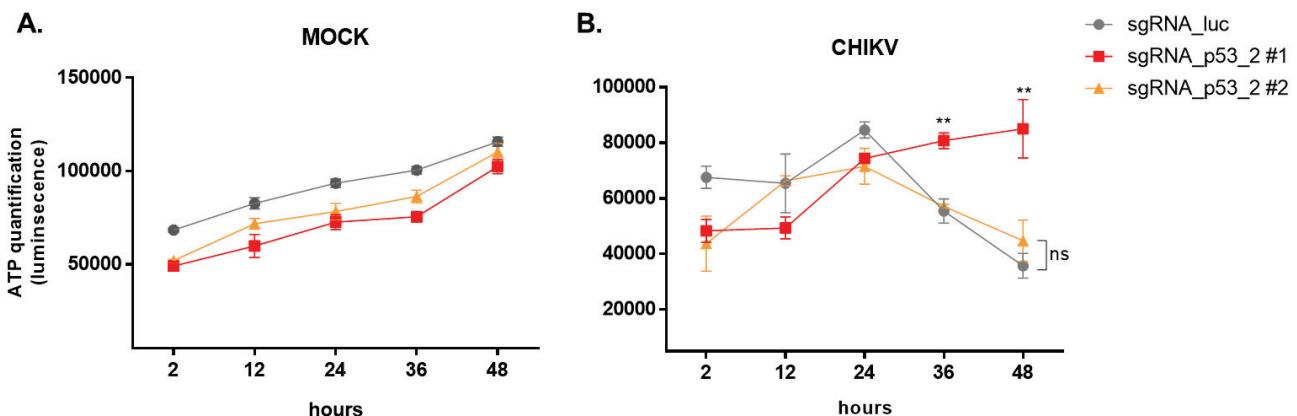
**Figure 52: Cell morphologies of LHCN-M2 sgRNA\_p53\_2 (#1 and #2) and p53 isoform protein expression in the new generated CRISPR/Cas9 cell lines.**

- A.** Photos of LHCN-M2 sgRNA\_p53\_2 #1 and #2 by light microscopy with a 10x and 40x objective. Cells were plated in a 48-well plate and 24 hours after taking the pictures.
- B.** Expression of p53 protein, p53 isoform proteins and Cas9 nuclease in old (#1) and new (#2) LHCN-M2 cells. The antibody anti-p53 CM1 was used to detect p53 and p53 isoforms proteins and the antibody anti-Flag to detect Cas9-Flagged by Western blot.

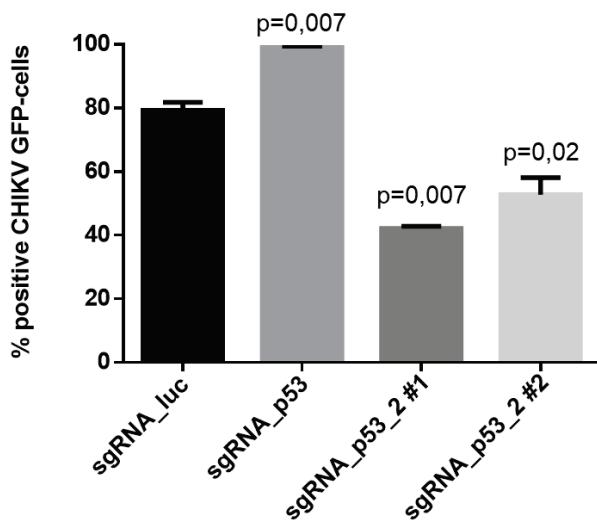
### 3.1.3 Effect of Δ40p53 isoform in the new generated CRISPR/Cas9 LHCN-M2 cell line on the cell viability of LHCN-M2 during CHIKV infection

We compared the effect of CHIKV infection on the viability of LHCN-M2 sgRNA\_p53\_2 #1 and #2 and then analyzed the percentage of infected cells by flow cytometry using a CHIKV-GFP. The cell viabilities of uninfected LHCN-M2 sgRNA\_luc and both p53 cell lines were similar from

2 to 48 hours (Figure 53 A.). Interestingly, after CHIKV infection, the cell viability of infected LHCN-M2 sgRNA\_p53\_2 #1 was significantly higher than the viability of control sgRNA\_luc and sgRNA\_p53\_2 #2 (Figure 53 B.). Moreover, a small proportion of infected sgRNA\_p53\_2 #1 cells were still alive after 48 hours of infection while almost the entire sgRNA\_luc and sgRNA\_p53\_2 #2 cell layer was destroyed by CHIKV (data not shown). However, we do not know if the surviving cells were still infected.



**Figure 53: Effect of CHIKV infection on viability of LHCN-M2 sgRNA\_luc, sgRNA\_p53\_2 #1 and #2.** Cell viability over time of uninfected (A.) and infected (B.) LHCN-M2 sgRNA\_luc and sgRNA\_p53\_2 (#1; #2). Cells were plated in a 96-well plate and infected with CHIKV at MOI 0.1. Then, the number of viable cells in culture was monitored at different time points by quantification of the ATP present in the well. Data are the mean  $\pm$  SD of three independent experiments. Statistical analysis was performed with an unpaired Student t-test with a p-value  $<0.05$ .



**Figure 54: CHIKV-GFP infection assay in sgRNA\_luc, sgRNA\_p53, sgRNA\_p53\_2 #1 and #2 LHCN-M2 cell lines.** Cells were infected with CHIKV-GFP at MOI 0.1 for 24 hours and then fixed in PFA 4% for flow cytometry analysis. Data represent the mean values of one experiment

obtained with two groups of three values. Statistical analysis was performed with a Student *t*-test with a *p*-value <0.05.

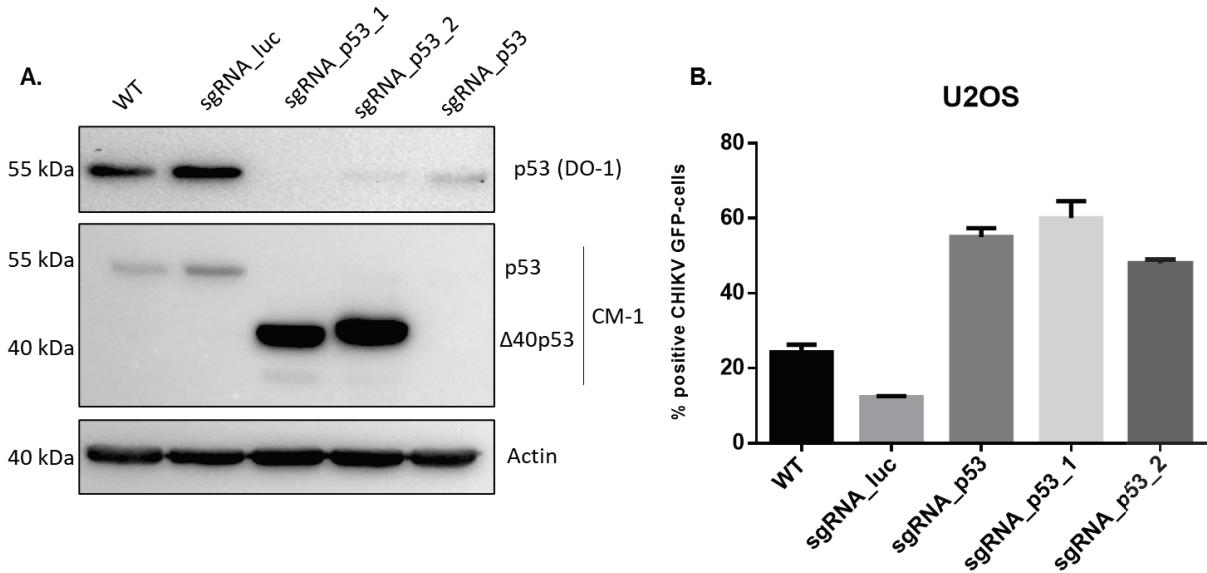
Subsequently, the three different cell lines were infected with CHIKV-GFP (MOI 0.1, for 24 hours) and the LHCN-M2 sgRNA\_p53 cell line was used as control. The percentage of positive CHIKV-GFP cells was higher in sgRNA\_p53 cells and decreased in sgRNA\_p53\_2 #1 and #2 cells compared to the sgRNA\_luc cell line (Figure 54).

Taken together, these results indicated that the sgRNA\_p53\_2 #1 cell line which presented a different morphology, was less infected by CHIKV and the infection did not affect its viability. Thus, the effect on infection and cell viability seemed to be specific to this cell line. To test our hypothesis, we generated CRISPR/Cas9 on another cell line, the human bone osteosarcoma U2OS tumoral cell.

### **3.1.4 Loss of effect of the Δ40p53 isoform on viral infection in the CRISPR/Cas9 U2OS cell line**

U2OS cells are less relevant for the tropism of CHIKV than the LHCN-M2 cells, but they do not present coding mutations in the *p53* gene. *p53* knockout U2OS cell lines were generated, and we could observe the knockout of *p53* in three U2OS cells and the overexpression of Δ40p53 in U2OS sgRNA\_p53 1 and 2, using anti-*p53* CM1 antibody (Figure 55 A.). Using the DO-1 antibody, we detected a low quantity of *p53* in sgRNA\_p53 and sgRNA\_p53\_1, suggesting the incomplete depletion of *p53*. Moreover, these results suggested that the over-expression of endogenous Δ40p53 was reproducible in another cell line, using the single guide RNA 10 (sgRNA\_10\_p53).

Then, *p53* knockout U2OS cell lines were infected with CHIKV-GFP at MOI 0.1 for 24 hours and the percentage of infected cells was investigated by flow cytometry (Figure 55 B.). The three U2OS cell lines were more infected compared to the U2OS sgRNA\_luc cells. Thus, the endogenous overexpression of the Δ40p53 isoform was not correlated with a decrease in infection of U2OS; indeed, the opposite effect was obtained. This could have been related to the cell type (U2OS versus LHCN-M2). To go further into this analysis, it would be necessary to repeat the infection of cells using a CHIKV-GFP strain and a wildtype CHIKV strain.



**Figure 55: Generation of p53 knockout U2OS cell lines and infection with CHIKV-GFP.**

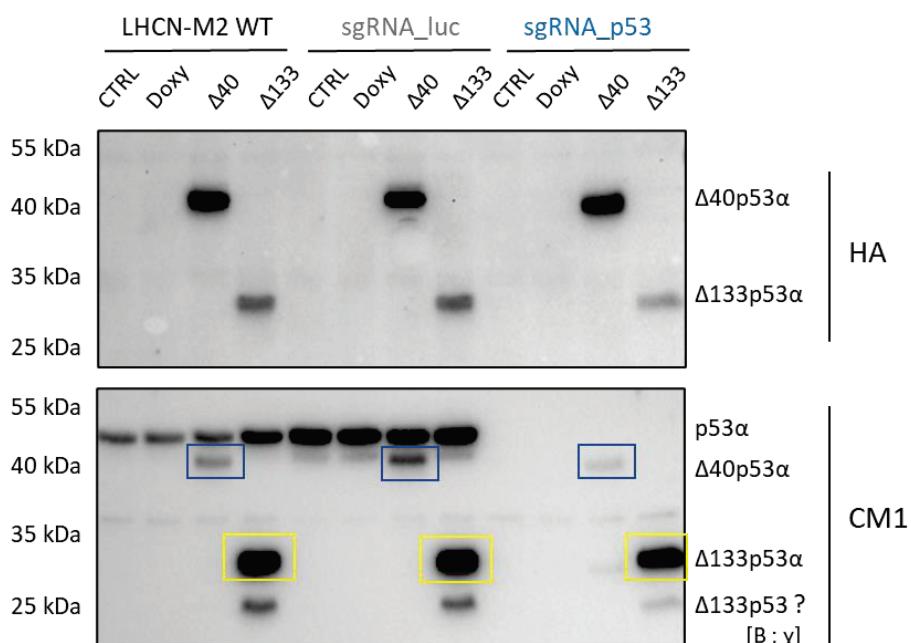
- A.** Expression of the p53 protein and Δ40p53 isoform protein in CRISPR/Cas9 U2OS cells. The antibody anti-p53 CM1 was used to detect p53 and p53 isoform proteins by Western blot.
- B.** CHIKV-GFP infection assay of U2OS WT, sgRNA\_luc, sgRNA\_p53, sgRNA\_p53\_1 and sgRNA\_p53\_2. Cells were infected with CHIKV-GFP at MOI 0.1 for 24 hours and then fixed in PFA 4% for flow cytometry analysis ( $n=1$ ).

In conclusion, it seems that only the LHCN-M2 sgRNA\_p53\_2 #1 induced an inhibition in CHIKV infection without impairing cell viability. Nevertheless, we confirmed a decrease in the percentage of infected cells in the new generated LHCN-M2 cell line (sgRNA\_p53\_2 #2), with the same guides 10 and 402. However, the involvement of the Δ40p53 isoform is not clear. Indeed, regarding the cellular morphology in the LHCN cell line and the contradictory results with the repetitive generated U2OS cell lines, it seemed that the effect of Δ40p53 isoform would be cell type specific.

### 3.2 Doxycycline-inducible system overexpressing $\Delta 40p53$ and $\Delta 133p53$ isoforms

#### 3.2.1 Generation of the LHCN-M2 doxycycline-inducible system for the over-expression of $\Delta 40p53$ and $\Delta 133p53$ isoforms

Another strategy was employed to investigate the possible effect of  $\Delta 40p53$  isoform on CHIKV infection by its overexpression in p53 knockout cells. Moreover, the potential effect of the  $\Delta 133p53$  isoform was also investigated. In this way we could investigate the effect of the isoform alone or in association with full length p53. The overexpression of  $\Delta 40p53\alpha$  and  $\Delta 133p53\alpha$  isoforms, generated as presented in materials and methods, were confirmed by Western blot analysis with antibodies against p53 CM1 and anti-HA, as the proteins are tagged in the N-terminal region (Figure 56). Using anti HA antibody, we observed that the three transduced LHCN-M2 cells overexpressed the  $\Delta 40p53\alpha$  isoform and  $\Delta 133p53\alpha$  isoform correctly and equivalently (Figure 56, top panel: HA).



**Figure 56: Doxycycline-inducible system for the over-expression of  $\Delta 40p53$  and  $\Delta 133p53$  in LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53.** Cells were transduced with VSV-g pseudo-particles containing pCW57.1-Tet-on-HA- $\Delta 40p53\alpha$  or  $\Delta 133p53\alpha$  isoform gene and selected with puromycin before induction of over-expression of p53 isoforms with Doxycycline 2 $\mu$ g/ml. The total cell lysates were collected, and 50  $\mu$ g of proteins was loaded on SDS-PAGE for Western blot assay using anti-HA or anti p53 CM1 antibodies. CTRL: no transduction, puromycin and doxycycline treatments; Doxy: cells treated with 2 $\mu$ g/ml of doxycycline;  $\Delta 40$ : cells transduced with pCW57.1-HA- $\Delta 40p53\alpha$ , selected with puromycin and one week later treated with 2 $\mu$ g/ml of doxycycline for 24 hours;  $\Delta 133$ : cells transduced with pCW57.1-HA- $\Delta 133p53\alpha$ , selected with puromycin and one week later treated with 2 $\mu$ g/ml of doxycycline for 24 hours.

In parallel, using anti p53 CM1 antibody, the detection of  $\Delta 40p53\alpha$  was much lower than that obtained before with anti-HA while it was the contrary for the  $\Delta 133p53\alpha$  isoform (Figure 56, bottom panel: CM1). Moreover, it seemed that the  $\Delta 133p53\beta$  or  $\Delta 133p53\gamma$  C-terminal isoform was recognized by the CM1 antibody, suggesting their expression followed the overexpression of the  $\Delta 133p53\alpha$  isoform using doxycycline-inducible system.

### 3.2.2 Effect of overexpression of p53 isoforms on CHIKV viral capsid

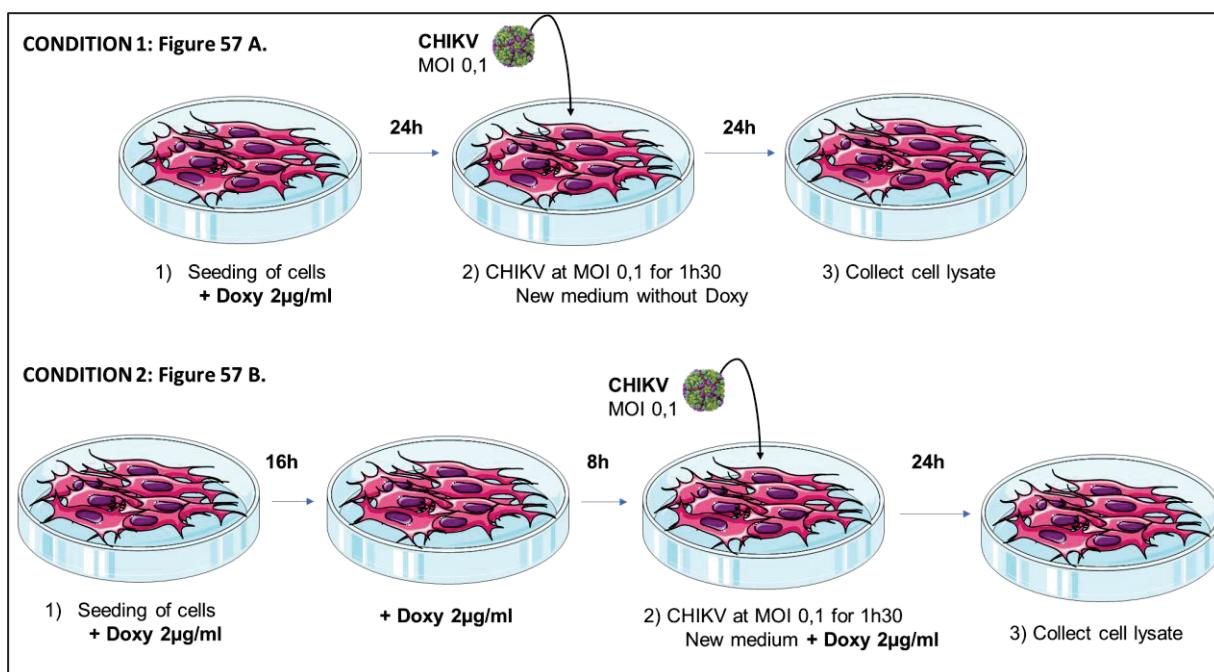
LHCN-M2 WT, sgRNA\_luc and sgRNA\_p53/ $\Delta 40p53\alpha$  cells were treated with 2 $\mu$ g/ml of doxycycline for 24 hours and then infected with CHIKV at MOI 0.1 for 24 hours (Figure 57 A.). The schematic workflow of this experiment (condition 1) is presented below. Regarding the expression of the  $\Delta 40p53\alpha$  isoform in uninfected cells, for the three cell lines, we observed significant induced expression of the isoform with the anti-HA antibody and less expression with the anti-p53 CM1 antibody. Curiously, at 24 hpi we were not able to detect the inducible expression of the  $\Delta 40p53\alpha$  isoform, and detected very few in sgRNA\_luc and sgRNA\_p53/ $\Delta 40$  using anti-HA and anti-p53 CM1 antibodies. Finally, regarding the expression of the viral capsid in both conditions (with and without doxycycline) we did not observe a difference of expression. We also investigated the percentage of infected cells by flow cytometry and did not observe a reduction in CHIKV infection (data not shown).

Given that the Old-World alphavirus nsP2 protein induces rapid degradation of the subunit of RNA polymerase II, Rpb1, and that the  $\Delta 40p53\alpha$  isoform is under the control of polymerase II, we hypothesized that CHIKV nsP2 induces the transcriptional shut-off of  $\Delta 40p53\alpha$ . In order to compensate the possible effect of nsP2 on cell transcription we treated cells with doxycycline several times: the schematic workflow of this experiment (condition 2) is presented below. We observed a detectable expression of  $\Delta 40p53\alpha$  isoform 24 hpi with the anti-HA antibody; however, it was still lower than the expression detected in mock cells (Figure 57 B.).

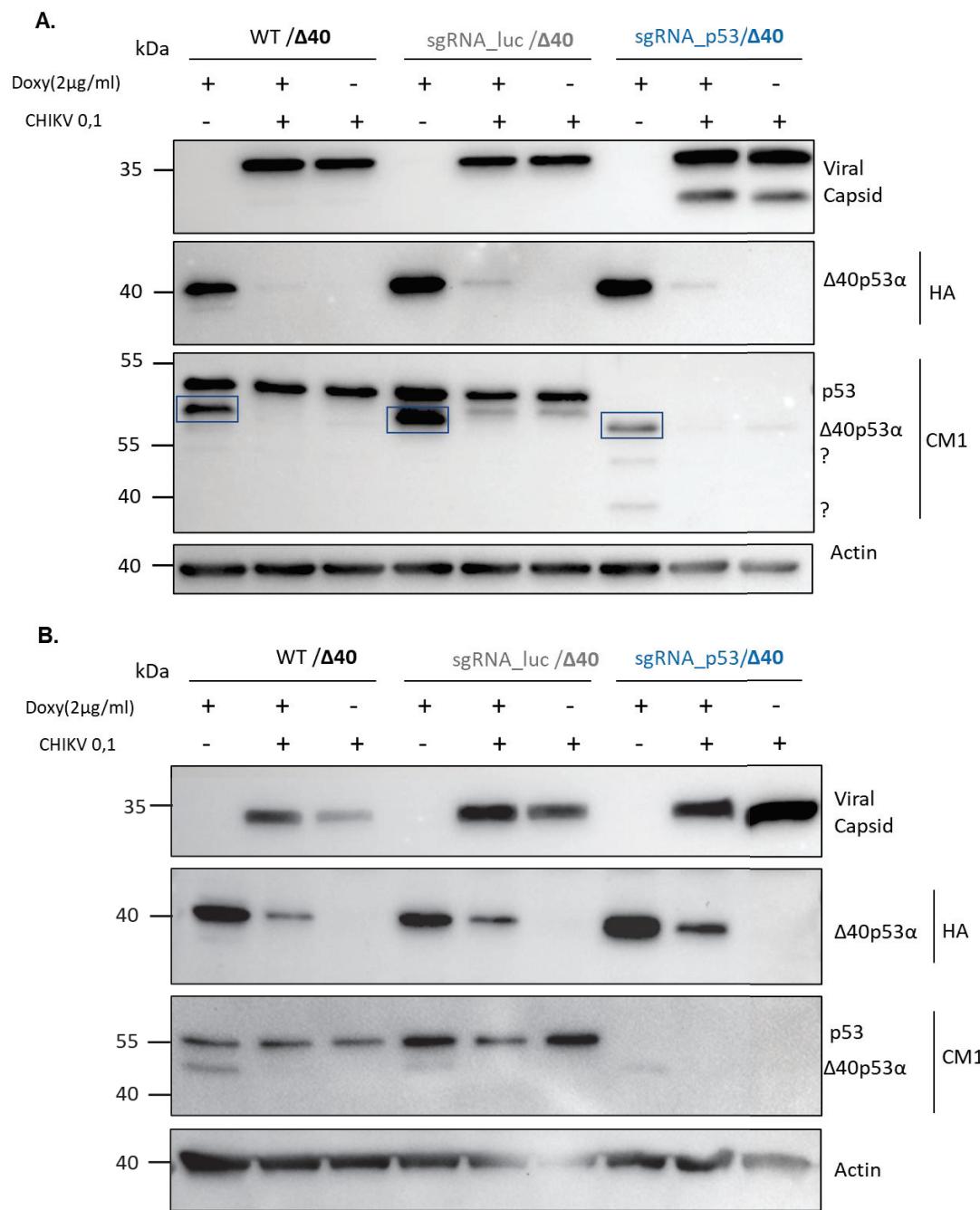
Interestingly, we detected a small reduction in viral capsid expression in infected sgRNA\_p53/ $\Delta 40$  with doxycycline compared to infected sgRNA\_p53/ $\Delta 40$  without doxycycline. On the contrary, we detected more viral capsid in WT/ $\Delta 40$  and sgRNA\_luc/ $\Delta 40$  infected cells with doxycycline, compared to WT/ $\Delta 40$  and sgRNA\_luc/ $\Delta 40$  infected cells without doxycycline.

This last result suggested that the expression of p53 and  $\Delta 40p53$  together could be correlated to an increase in viral capsid expression. The experiment should be repeated to confirm this result.

To conclude, the expression of p53 and  $\Delta 40p53\alpha$  isoform seemed to be correlated with an increase in the quantity of CHIKV viral capsid, while in the absence of p53, the overexpression of  $\Delta 40p53\alpha$  isoform was correlated to a decrease in the quantity of CHIKV viral capsid. The doxycycline-system must be improved to overexpress the  $\Delta 40p53$  isoform during viral infection and the percentage of infection and the production of infectious particles must be determined.



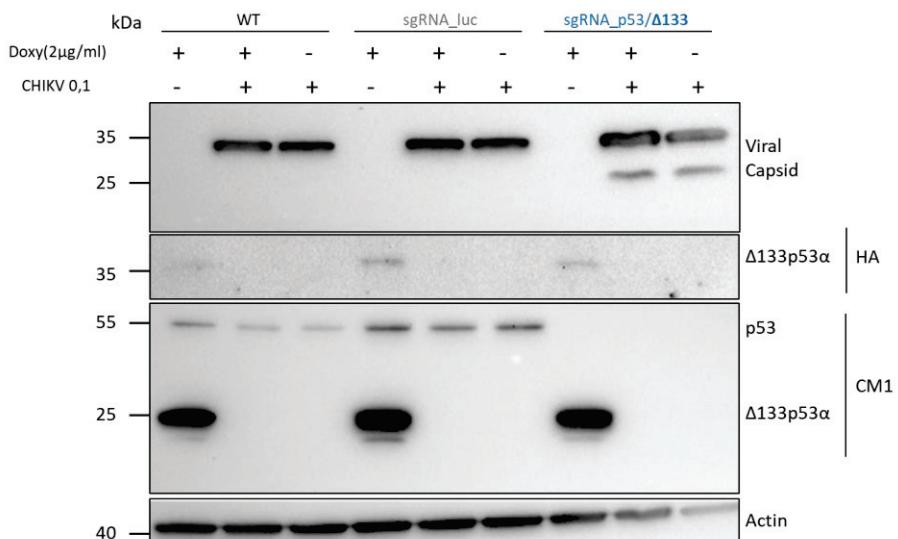
**Schematic workflow of the two conditions monitored for the overexpression of the  $\Delta 40p53$  isoform in LHCN-M2 cell lines (Figure 57).**



**Figure 57: Infection of LHCN-M2 sgRNA\_p53 overexpressing Δ40p53 $\alpha$  with CHIKV.**

- Cells were treated with 2 $\mu$ g/ml of doxycycline and 24 hours later infected with CHIKV at MOI 0.1 for 24 hours (n=2).
- Cells were treated with 2 $\mu$ g/ml of doxycycline and 24 hours later treated again before CHIKV infection. During the infection of cells with CHIKV for 1 hour and 30 minutes the medium was doxycycline free and after infection, the new medium was supplemented with doxycycline 2 $\mu$ g/ml (n=1).

Viral capsid SFV-C antibody was used to detect CHIKV and anti-HA or CM1 antibodies to detect HA-Δ40p53 $\alpha$  isoform. The antibody raised against housekeeping protein  $\beta$ -actin was used as loading control.



**Figure 58: Infection of LHCN-M2 sgRNA\_p53 overexpressing Δ133p53α protein with CHIKV.** Cells were treated with 2μg/ml of doxycycline and 24 hours later infected with CHIKV at MOI 0.1 for 24 hours. Viral capsid SFV-C antibody was used to detect CHIKV and anti-HA or CM1 antibodies to detect HA-Δ40p53α. The antibody raised against housekeeping protein β-actin was used as loading control (n=1).

The role of Δ133p53α isoform has been explored in the context of Influenza A virus and it has been shown that Δ133p53α and p53 β can act as regulators of viral production (Terrier *et al.* 2012). In the context of alphaviruses infections, the potential function of physiological p53 isoforms has never been explored. We investigated the effect of Δ133p53α isoform overexpression in p53 wildtype and p53 knockout LHCN-M2 cells on CHIKV infection.

As previously, we generated a doxycycline inducible-system overexpressing Δ133p53α isoform protein. We observed the overexpression of Δ133p53α isoform in non-infected and doxycycline induced cells but not in infected and doxycycline induced cells (Figure 58). In parallel, the quantity of the viral capsid was not different between these two conditions. The experiment was performed only once so it should be repeated, and the percentage of infected cells must be determined. To conclude, unfortunately, this assay did not identify a potential function of Δ133p53 isoform in the context of CHIKV infection and other approaches might be applied for the overexpression of Δ133p53 and Δ40p53 such as non-inducible cells.

### 3.3 Discussion

In the past few years, very few studies have investigated the role of p53 isoforms in the context of non-oncogenic viral infection. In addition, the direct effect of the p53 and p53 isoforms on the viral cycle has been demonstrated (Aloni-Grinstein *et al.* 2018; Austin *et al.* 2012; Terrier *et al.* 2012). These studies revealed the importance of p53 and p53 isoforms during viral infection, suggesting an effect for many viruses belonging to different families. Indeed, the modulation of host p53 pathways allows controlling various cellular antiviral responses such as apoptosis, immune response, or ROS production.

Our approach to studying a potential function of  $\Delta 40p53\alpha$  and  $\Delta 133p53\alpha$  isoforms in the human LHCN-M2 muscle cell line infected with CHIKV encountered technical difficulties and other difficulties which seem to be linked to the virus replication cycle.

We observed that in the CHIKV-infected CRISPR/Cas9 cell line sgRNA\_p53\_2 #1, the overexpression of  $\Delta 40p53$  isoform was correlated to a decrease in the percentage of infected cells with less viral capsid. In parallel, with the sgRNA\_p53\_1 cell line the same decrease in percentage of infected cells was detected, while the quantity of viral capsid was similar to that observed in control sgRNA\_luc cell line. The sgRNA\_p53\_1 cell line was generated using single guides 10 and 400 and the sgRNA\_p53\_2 cell line using single guides 10 and 402. The single guide RNA 10 seemed to be responsible of the endogenous overexpression of the  $\Delta 40p53$  isoform. The single guide 10 could have inserted a mutation in the region of the first ATG1 coding p53 without damage to the frameshift in the second ATG40 sequence coding  $\Delta 40p53$ . Consequently, the ribosome complex could not recognize ATG1, inducing a more significant ribosome entry in ATG40, and an exaggeration of the translation of  $\Delta 40p53$ .

In addition, as the  $\Delta 40p53$  did not contain N-terminal residues of the transcriptional activation domain TAD1 or the amino-acid motif that binds MDM2, this isoform escaped MDM2-mediated degradation and thus was more stable than full length p53. This might explain the amount of isoform expressed after the generation of the CRISPR/Cas9 cell lines. Nevertheless, during the experiment, this endogenous expression in LHCN-M2 sgRNA\_p53\_2 #1 cell line drastically decreased, and the cellular morphology changed. The percentage of infected cells was still lower than in control cell line. We wonder if it was the effect of  $\Delta 40p53$  overexpression in this

particular cell line that induced the change of cellular morphology and subsequently the level of expression of Δ40p53 protein, or was it the effect of the CRISPR/Cas9 method?

We showed that the Cas9 nuclease inducing the double strand DNA break was still expressed some weeks after the generation of the cell lines and even after months to a lesser extent. Moreover, as Cas9 nuclease and single guide RNA are integrated in the genome and constitutively active, this might affect cell fitness and specific sgRNA could target nonspecific gene generating off-targets. It is possible that the sgRNA\_p53\_2 #1 cell line suffered from off-target(s) which might explain the effect observed on cellular morphology and on CHIKV infection. As this cell line is not clonal, there are perhaps several populations and two of them could present the endogenous overexpression of Δ40p53 isoform on the one hand, and an unknown off target inducing the change of cell morphology on the other. We hypothesized that the second cellular population had been selected against the cellular population overexpressing the Δ40p53 isoform.

However, the infection of new generated cell line LHCN-M2 sgRNA\_p53\_2 #2 presented a similar decrease in the percentage of infected cells, while the infection of U2OS sgRNA\_p53\_2 led to an increase in the percentage of infected cells.

Even more strikingly, the viability of the LHCN-M2 sgRNA\_p53\_2 #1 cell line was not affected by infection and we detected infectious particles in the supernatants of the cells one week after infection (data not shown). This might signify that this particular cell line could be “persistently” infected with CHIKV. To test this hypothesis, infection with CHIKV could be challenged a second time to know if the survival cells can be infected without again impacting cell viability.

After challenges, the surviving cells could be isolated by serial dilution in a multi-well plate to ideally obtain one single clone in each well and infect them again to confirm the effect on the viral infection of every single clone. This experiment would allow sequencing and identifying mutations that confer the phenotype of the cells and/or the effect on CHIKV infection compared with the wildtype cell line. Moreover, the cellular transcriptome of survival cells could be analyzed by RNA sequencing.

To conclude, with this first approach, we do not know with any certainty if the Δ40p53 isoform impacted the infection of the human muscle cell line LHCN-M2 by chikungunya virus. Another approach was taken based on an inducible system overexpressing the p53 isoform.

After several repetitions we showed that the overexpression of  $\Delta 40p53\alpha$  disappeared 24 hours post-infection and one experiment carried out on  $\Delta 133p53\alpha$  gave the same result. We suggested that after the expression of CHIKV nsP2 protein, the degradation of subunit Rpb1 of RNA polymerase II induced the reduction in the expression of the  $\Delta 40p53\alpha$  and  $\Delta 133p53\alpha$  isoforms. Indeed, the doxycycline-inducible system of the plasmid is controlled by RNA polymerase II. We could generate the same plasmid under the control of RNA polymerase III. We showed that the 5S rRNA transcribed by RNA polymerase III presented the same cycle quantification value in LHCN-M2 cells infected with CHIKV overtime compared to mock cells, whereas gusb, rpl22 and rpl27 transcribed by RNA polymerase II, presented a difference of four cycles between 8 and 24 hours post-infection compared to mock cells.

However, the several treatments of LHCN-M2 cell lines with doxycycline allowed detecting a higher quantity of the  $\Delta 40p53\alpha$  isoform in three cell lines and it was correlated with a slight decrease in the detection of the viral capsid. We assume that the expression of  $\Delta 40p53\alpha$  had an effect on the viral capsid. It would be interesting to investigate the percentage of infection and the production of infectious particles to confirm the results obtained previously in the CRISPR-mediated endogenous overexpressing  $\Delta 40p53$  cell line.

Among the studies performed to assess the function of  $\Delta 40p53$ , it has been determined that in HCT116 and H1299 cells, which express exogenous  $\Delta 40p53$  but not p53,  $\Delta 40p53$  may inhibit starvation-induced autophagy. This inhibition arises from the degradation of dsRNA *via* the 3'-5' exonuclease activity of  $\Delta 40p53$  which could inhibit PKR/eIF2 $\alpha$ -induced autophagy (Zang *et al.* 2017). Thus, taken together we can hypothesise that the overexpression of  $\Delta 40p53$  isoform in the absence of p53 in LHCN-M2 cells inhibited pro-viral autophagy during CHIKV infection, leading to the impairment of viral capsid expression, and the decrease in the percentage of infected cells. To test this hypothesis, it could be interesting to analyze the autophagic flux engaged in LHCN-M2 cells overexpressing the  $\Delta 40p53$  isoform and compare it with that of the LHCN-M2 p53 wildtype cells expressing or not the  $\Delta 40p53$  isoform.

In LHCN-M2 expressing p53 and  $\Delta 40p53$ , we observed a higher expression of viral capsid compared to infected cells expressing only p53. This preliminary result shows that the expression of p53 and  $\Delta 40p53$  is correlated to an increase in capsid detection and maybe to the effect of  $\Delta 40p53$  on p53, controlling the antiviral activity of p53 through the Type-I interferon

signaling pathway. The experiment was conducted once, so it must be repeated and the percentage of infected cells analyzed.

In U2OS, the endogenous overexpression of  $\Delta 40p53$  did not lead to a decrease in CHIKV infection. It is possible that the function of the p53 isoform is different depending on the cell type. Further studies are needed to improve the molecular and cellular tools to decipher the potential function of  $\Delta 40p53$  in CHIKV infection. The generation of an inducible system dependent on the RNA polymerase III could be a solution, or a stable cell line that constantly overexpresses  $\Delta 40p53$ . We could also use another permissive cell line, less relevant for CHIKV tropism, but studied in-depth for p53 and p53 isoform expression, such as HCT116.

The investigation concerning the other isoform of interest,  $\Delta 133p53$  did not give results on CHIKV capsid expression. The cellular expression of the  $\Delta 133p53$  isoform protein was not detectable by Western blot using anti-p53 CM1 antibody which potentially recognizes the twelve human p53 isoforms. The overexpression of the  $\Delta 133p53$  isoform protein in LHCN-M2 wildtype and p53 knockout seems to be related to the same problem encountered with the  $\Delta 40p53$  isoform, *i.e.* a complete transcriptional shut-off of its expression.

In conclusion, we assume that following p53-dependent antiviral activity on CHIKV-infected LHCN-M2, the intertwining of p53 with p53 isoforms leads to a positive or negative regulator of p53 activity.



## GENERAL CONCLUSION

*Alphaviruses* are mainly transmitted to humans by mosquitoes from the *Aedes* genus, typically *Aedes albopictus* and *Aedes aegypti*. The chikungunya virus has recently spread from Asia and Africa to the continents of Europe and America, making it a worldwide threat and one of the most common arboviruses infecting humans. While in humans, chikungunya virus disease is characterized by fever, headache, and a typical acute infection sometimes followed by persistent arthralgia or myalgia, in mosquito the infection does not seem to cause significant pathology. At the cellular level, the infection of several mammalian cells with chikungunya virus inevitably leads to the death of the cell layer, whereas the infection of mosquito cells leads to the chronicity of cell layer without perceptible cytopathic effects. To date, no vaccine or specific treatments for humans have been developed and the control of mosquito populations requires more research to be confident about its effectiveness. This highlights the importance of continuing research on chikungunya virus biology and cellular response in mammals and mosquitoes, in order to develop in the future adapted antiviral treatments and vaccines for humans, and control the permissiveness of mosquitoes.

Thus, one objective of my project was to study the potential role of p53 on the cellular outcome and viral infection in mammals and insects infected with *Alphavirus*, mainly chikungunya virus and to a lesser extent Sindbis virus.

Firstly, we showed that the infection of a human immortalized skeletal muscle cell line with chikungunya virus led to the stabilization of p53. In order to investigate the potential function of p53 we generated a p53 depleted cell line and showed that in the absence of p53 expression, the cells were not able to produce Type-I interferon (IFN $\beta$ ) and could not engage the Type-I signaling pathway (*Mx1*, *IFIT1*). The p53 knockout led to an increase in the percentage of infected cells, highlighting the antiviral effect of p53. In parallel, given that p53 is also involved in antiviral apoptotic response for other arboviruses, we investigated the apoptotic p53 target genes such as *p21*, *puma* and *bax*. Surprisingly, no regulations of all genes were observed, suggesting an antiviral role of p53 involving innate immune response activity, independently of its antiviral apoptotic function during chikungunya virus infection. The antiviral activity of p53 could be correlated to the pathogenicity of infection and chronicity. Indeed, the activity level of p53 can be dependent on the patient and in the case of a “natural” p53 activation, the infection

can be rapidly eliminated. In the case of a inefficient p53 activation, the viral infection can persist and become chronic.

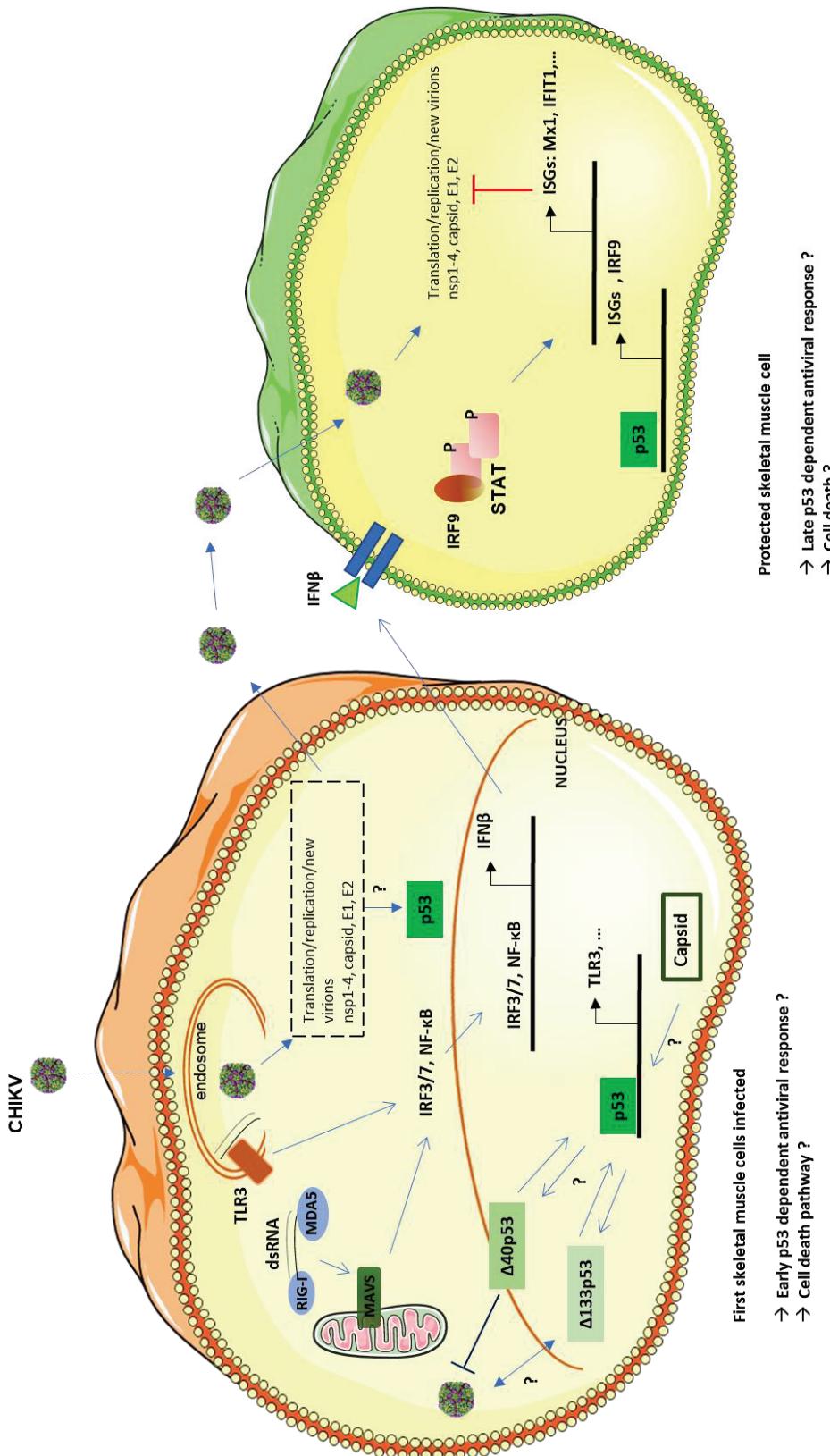
In addition to p53, twelve identified p53 isoforms were physiologically transcribed and we investigated the potential function of two of them:  $\Delta 40p53$  and  $\Delta 133p53$ . Our results showed that infection with chikungunya virus decreased in a genetically modified human skeletal muscle cell line overexpressing  $\Delta 40p53$ . Figure 59 shows schematically the antiviral function of p53 stabilization in a human skeletal muscle cell line infected with chikungunya virus.

The comparative effect of p53 on chikungunya virus infection in insects was investigated using the *in vivo* *Drosophila melanogaster* fly. Thanks to our collaboration with Pr. Mollereau, the *Drosophila melanogaster* p53-/- mutant strain was challenged with Sindbis and chikungunya virus. Interestingly, the effect we observed on viral infection was the opposite of that found in the human cell line, i.e. p53-/- flies were less infected than control w1118 strain. However, the survival of p53-/- mutants injected with chikungunya virus was also impacted but we hypothesized that in flies, p53 could present pro-viral controlling virus-induced oxidative stress. In order to support this hypothesis, it would be necessary to inject specific p53 depleted mutant strains (Dmp53 +/+ and  $\Delta Np53$ , and inversely) with chikungunya virus and Sindbis virus. Figure 60 represents schematically the impact of p53 isoforms on Sindbis virus and chikungunya virus.

Concerning the mosquito cell line, we observed that in the CHIKV-infected C6/36 cell line (non-competent for antiviral RNAi pathway), the apoptotic p53-target gene *mx* was upregulated without observable cytopathic effects. We hypothesized that CHIKV may control the induction of cell death downstream of Michelob\_mx induction in mosquito cells and further investigations are needed.

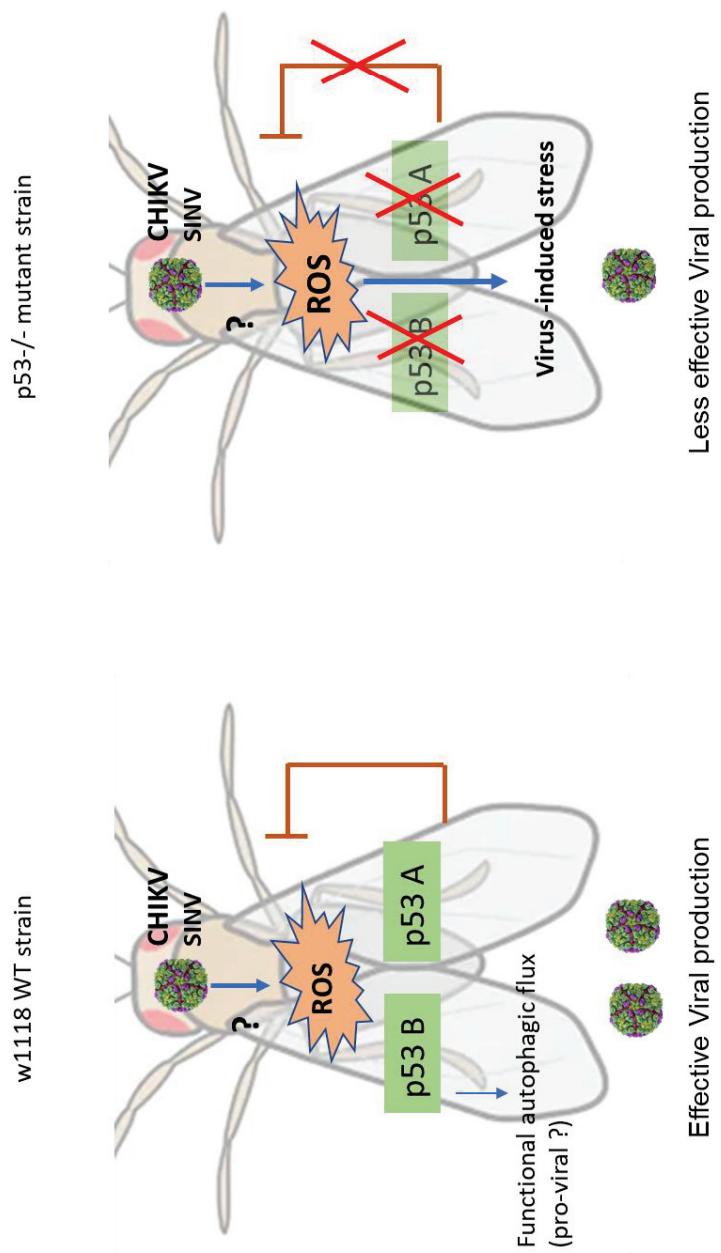
After the detection of viral RNA and/or viral proteins, it is likely that p53 in insects included innate immune signaling to respond to stress induced by infection. In permissive mosquitoes, the control of virus-induced stress through the p53 and p53 isoform signal transduction pathway could have been selected by arboviruses during evolution.

## GENERAL CONCLUSION – Role of p53 in mammals and insects infected with CHIKV



**Figure 59: Diagram illustrating the antiviral function of p53 in the CHIKV-infected LHCN-M2 human cell line based on our findings.**

- Early p53 dependent antiviral response ?
- Cell death pathway ?
- Late p53 dependent antiviral response ?
- Cell death ?



**Figure 60:** Diagram illustrating the hypothetical pro-viral function of *Drosophila* p53 A (Dmp53) and p53 isoform p53 B ( $\Delta$ Np53).

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