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Interaction between the influenza replication machineries and the cellular nuclear import system

Amélie Donchet

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THÈSE

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Présentée par

Amélie DONCHET

Thèse dirigée par le Docteur Thibaut CREPIN

préparée au sein de l'Institut de Biologie Structurale (IBS)
dans l'École Doctorale Chimie et Science du Vivant (EDCSV)

Interaction between the influenza replication machineries and the nuclear import system

Thèse soutenue publiquement le 19 janvier 2021,
devant le jury composé de :

Docteur Sandie MUNIER

MC, Université de Paris, Rapportrice

Docteur Roland MARQUET

DR, CNRS, Rapporteur

Professeur Martin SCHWEMMLE

PR, University of Freiburg, Examineur

Professeur Winfried WEISSEHORN

PR, Université Grenoble Alpes, Président du Jury

Docteur Thibaut CREPIN

DR, CNRS, Directeur de Thèse



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A mes étoiles,

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List of abbreviations

ARM repeats: Armadillo repeats
BLAST: Basic Local Alignment Search Tool
BRD: Bovine Respiratory Disease
CAS: Cellular Apoptosis Susceptibility
CCP4: Collaborative Computational Project Number 4
CCP4i: CCP4 Interface
cNLS: conventional/classical Nuclear Localization Signal
COOT: Crystallographic Object-Oriented Toolkit
Crm1: Chromosome region maintenance 1 protein
cRNA: complementary RNA
CTD: C-terminal domain
DNA: Deoxyribonucleic Acid
EM: Electron Microscopy
EMBL: European Molecular Biology Laboratory
ESCRT: Endosomal Sorting Complex Required for Transport
FA: Fluorescence Anisotropy
FAM: Fluorescein
FG-repeats: phenylalanine-glycine repeats
GDP: Guanosine DiPhosphate
GTP: Guanosine TriPhosphate
HA: Hemagglutinin
HEAT repeats: **H**untington, **E**longation factor 3, **P**R65/**A**, **T**OR
HEF: Hemagglutinin-Esterase-Fusion
HPAIs: Highly Pathogenic Avian Influenza viruses
HSQC: Heteronuclear Single Quantum Correlation
HTX: High Throughput Crystallization laboratory
IAV: Influenza A Viruses
IBB domain: Importin- β Binding domain
IBV: Influenza B Viruses
ICTV: International Committee on Taxonomy of Viruses
ICV: Influenza C Viruses
IDV: Influenza D Viruses
IPTG: isopropyl- β -D-thiogalactopyranoside
IRD: Influenza Research Database
Isavirus: Infection salmon anemia virus
ISBG: Integrated Structural Biology Grenoble
 K_d : Dissociation constant
kDa: kilo Daltons
LPAIs: Low-Pathogenic Avian Influenza viruses
M1: Matrix protein 1
M2: Matrix protein 2
MAD: Multi-wavelength Anomalous Dispersion
MDa: Mega Daltons
mRNA: messenger RNA
MW: Molecular Weight
MxA: Human myxovirus resistance protein 1
NA: Neuraminidase
NCBI: National Center for Biotechnology Information
ncNLS: non-conventional/non-classical Nuclear Localization Signal
NEP: Nuclear Export Protein
NES: Nuclear Export Signal
Neu5Ac: N-Acetylneuraminic acid
NLS: Nuclear Localization Signal

NMR: Nuclear Magnetic Resonance
NP: Nucleoprotein
NPC: Nuclear Pore Complex
NS1: Non-Structural protein 1
PA: Polymerase Acidic protein
PB1: Polymerase Basic protein 1
PB2: Polymerase Basic protein 2
PEG: PolyEthylene Glycol
pH: potential of Hydrogen
pHi: isoelectric point
PoII: DNA-dependent RNA polymerase II
PTM: Post-Translational Modification
PY NLS: Proline-Tyrosine rich Nuclear Localization Signal
RanBP1: Ran-Binding Protein 1
RanBP5: Ran-Binding Protein 5
RanGAP: Ran GTPase-Activating Protein
RCC1/RanGEF: Regulator of Chromosome Condensation 1 / Ran Guanine nucleotide Exchange Factor
RdRp: RNA-dependent RNA polymerase
RNA: Ribonucleic Acid
RNP: Ribonucleoprotein
SAXS: Small Angle X-rays Scattering
SEC: Size Exclusion Chromatography
SEC-MALLS: Size Exclusion Chromatography coupled with Multi Angle Laser Light Scattering
SNP: Single Nucleotide Polymorphism
SPR: Surface Plasmon Resonance
STEM: Scanning Transmission Electron Microscopy
SUMO: Small Ubiquitin-like Modifier
TEV: Tobacco Etch Virus
ToadV: Wuhan Asiatic Toad influenza Virus
TEM: Transmission electron microscopy
vRNA: viral RNA
WHO: World Health Organization
YFP: Yellow Fluorescent Protein

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Résumé détaillé

Les virus grippaux font partie de la famille des *Orthomyxoviridae*. Cette famille comporte sept genres de virus, dont les virus de la grippe de type A, B, C et D (IAV, IBV, ICV et IDV). Cette classification est destinée à évoluer, car la recherche n'a fait qu'effleurer la surface de la diversité virale : une étude de 2018 a identifié 3 nouveaux membres de la famille des *Orthomyxoviridae*, notamment un virus de crapaud à Wuhan. Ces trois virus présentent des identités de séquence plus importantes avec IBV qu'avec les autres virus grippaux. Le virus de crapaud, étudié durant ce travail de thèse, sera appelé ToadV. Les virus grippaux possèdent une solide base commune en terme de structure et de fonctionnement, mais ils ont également de claires spécificités.

Les virus grippaux sont des virus enveloppés, à génome ARN segmenté, simple brin et de polarité négative. L'unité répliquative du virus, appelée ribonucléoprotéine (RNP), est composée du segment génomique, encapsidé par de multiples copies de la nucléoprotéine (NP) et associé par ses extrémités 3' et 5' à une ARN polymérase ARN dépendante (RdRp) virale hétérotrimérique. NP est la protéine la plus abondante des RNPs et elle est nécessaire aux activités de transcription et de réplication virales. NP est également impliquée dans la protection du génome viral, dans l'interaction avec les partenaires cellulaires et dans le trafic cellulaire des RNPs. NP est une protéine virale centrale, dont les fonctions clés sont conservées entre les NPs de IAV, IBV, ICV et IDV (A/NP, B/NP, C/NP et D/NP) mais avec des spécificités structurales et fonctionnelles dépendantes du type viral.

Les ions et les petites molécules (< 30-60 kDa) diffusent librement dans le noyau des cellules. Cependant, les protéines plus grosses nécessitent d'être importées par transport actif. La voie la plus étudiée est le système impliquant la petite GTPase Ran et les récepteurs de transport nucléaire appelés importines- α et - β . Les importines- α , agissant comme des adaptateurs, interagissent avec un signal spécifique de la protéine à importer (cargo), appelé signal de localisation nucléaire (NLS), et avec l'importine- β , *via* son domaine d'interaction avec l'importine- β (domaine IBB). Ce domaine IBB, en absence de l'importine- β , agit comme auto-inhibiteur en se repliant dans les deux sillons de l'importine- α où les NLSs peuvent se fixer. Certaines importines- β n'ont pas besoin d'adaptateurs et peuvent directement interagir avec le NLS du cargo. Il existe deux types de NLS, les NLS classiques/conventionnels et les NLS non classiques/non conventionnels. Un NLS est généralement riche en Arginine

et en Lysine, et généralement composé d'un ou deux motifs basiques (NLS monopartite ou bipartite respectivement). Les importines sont très conservées au cours de l'évolution. Chez l'homme, il y a sept paralogues d'importines- α et au moins dix importines- β responsables de l'import nucléaire. Cette diversité s'accompagne d'une spécificité de cargos, de comportement et d'expression dans les cellules et tissus : les importines ne sont pas interchangeables.

Les virus grippaux font partie des rares virus à ARN à répliquer et transcrire leur génome dans le noyau de la cellule hôte. La machinerie répliquative ainsi que le génome ont donc besoin d'être importés dans le noyau, et pour cela détournent la machinerie des importines- α et - β . PB1, PB2 et NP possèdent des NLS pour interagir avec les importines : PB2 et NP avec les importines- α , PB1 avec une importine- β RanBP5, et PA est essentielle pour l'import efficace de PB1. L'import initial du génome dans le noyau sous forme de RNPs est supposé être médié par la NP, un autre des rôles essentiels de cette protéine virale dans le trafic viral.

Le NLS fonctionnel de A/NP et B/NP a été identifié sur l'extrémité intrinsèquement désordonnée N-terminale d'une longueur de 21 et 71 acides aminés respectivement (A/NP_{TAIL} et B/NP_{TAIL}). Le NLS de A/NP appartient aux NLS non conventionnels, interagit avec les importines- α 1, - α 3, - α 5 et - α 7, notamment avec une faible affinité pour l'importine- α 1 (constante de dissociation entre 1 et 5 μ M). De nombreux motifs basiques sont présents sur B/NP_{TAIL} et les résidus 30 à 71 ont été identifiés comme participant à l'interaction avec l'importine- α 7, qui est caractérisée par une constante de dissociation de 844 nM. C/NP et D/NP, de leur côté, présentent une longue extrémité intrinsèquement désordonnée de 62 et 52 acides aminés respectivement, mais en C-terminale. C/NP_{TAIL} et D/NP_{TAIL} possèdent des motifs basiques qui ressemblent clairement à des NLS conventionnels bipartite. Des travaux publiés durant ce travail de thèse par une équipe concurrente ont montré que C/NP_{TAIL} interagit avec les importines- α 1, - α 3, - α 4, - α 5 and - α 7, et son affinité pour l'importine- α 1 est de 50 nM.

Au début de ce travail de thèse, la caractérisation de la machinerie répliquative des virus de type D, et plus particulièrement de D/NP, était déjà initiée par l'équipe, en parallèle de ses travaux sur A/NP et B/NP. Ces dernières présentaient des affinités pour les importines- α trop faibles pour être considérées complètement fonctionnelles.

L'existence de ToadV et de sa NP n'était pas encore connue. Malgré le travail effectué sur les NPs grippales et leurs interactions avec les importines- α , de nombreuses questions restaient en suspens, d'autant plus que le mécanisme de fonctionnement d'une NP, ainsi que ses caractéristiques structurales et biochimiques, ne sont pas forcément transposables aux autres. Il a été décidé de caractériser dans un premier temps D/NP, structuralement ainsi que dans ses fonctions clés. Par la suite, dans l'optique de pouvoir comprendre comment les différents types grippaux accédaient au noyau et en quoi leurs stratégies convergeaient et/ou divergeaient, une stratégie expérimentale a été mise au point dans le but de pouvoir comparer aussi fidèlement que possible les différentes NP_{TAILS} et les différentes importines- α humaines. Enfin, avec la découverte inédite du virus grippal de crapaud, proche phylogénétiquement du virus de type B strictement humain, il a semblé judicieux d'initier sa caractérisation, aussi bien structurale que fonctionnelle, afin d'intégrer une vision évolutive aux fonctions cœur de la NP grippale et de mieux comprendre le fonctionnement de chaque NP individuellement.

Découvert en 2011 et classifié comme un nouveau genre grippal en 2013, le virus de la grippe de type D circule largement au niveau mondial chez les bovins. Peu de choses sont connues sur les protéines virales et son cycle.

A l'aide de la diffraction des rayons-X, de la microscopie électronique, du dichroïsme circulaire, de la chromatographie d'exclusion de taille couplée ou non à la diffusion de la lumière à plusieurs angles, de tests de stabilisation thermique, de l'anisotropie de fluorescence et de la résonance des plasmons de surface, la caractérisation de D/NP, à savoir ses activités d'interactions avec l'ARN et avec l'importine- $\alpha 7$, son comportement oligomérique et sa structure, a été réalisée.

D/NP forme principalement des tétramères en solution, et la concentration en sel n'a pas d'impact sur cet état oligomérique. La structure a été résolue à une résolution de 2.4 Å, montrant un corps replié très conservé. D/NP se localise dans le noyau de cellules infectées et/ou transfectées et, contrairement à A/NP et B/NP, D/NP possède une extrémité flexible intrinsèquement désordonnée en C-terminale, avec un évident NLS bipartite. L'interaction entre l'importine- $\alpha 7$ et D/NP_{TAIL} se caractérise par une affinité environ dix fois plus forte que celles déterminées pour A/NP_{TAIL} et B/NP_{TAIL}, de 100 nM, une affinité caractéristique des NLS fonctionnels. La délétion du motif basique

C-terminal du NLS conduit à une interaction défectueuse avec l'importine- $\alpha 7$ et une localisation nucléaire affectée, tandis que la délétion des deux motifs conduit à une abolition complète des deux mécanismes. Ces résultats ont fait l'objet d'une publication rattachée à ce travail de thèse.

Les fonctions clés de la nucléoprotéine grippale sont retrouvées avec D/NP : sa structure, son état d'oligomérisation, son NLS sur une extrémité désordonnée, sa localisation cellulaire. Cependant, chacun de ces mécanismes montrent aussi de claires spécificités, propre à D/NP.

Un large nombre d'études se sont intéressées à la caractérisation de l'interaction NP:importine- α , apportant de précieux renseignements au point de vue fonctionnel, moléculaire et atomique. Cependant, ces connaissances restaient lacunaires, autant du côté des importines- α que des NPs. Les seuls complexes étudiés dans le détail et impliquant A/NP et B/NP, A/NP_{TAIL}:importine- $\alpha 1$ et B/NP_{TAIL}:importine- $\alpha 7$, montraient une faible affinité, probablement au-delà de la limite de fonctionnalité d'un NLS. Au contraire, C/NP_{TAIL}:importine- $\alpha 1$ et D/NP_{TAIL}:importine- $\alpha 7$ présentent des affinités consistantes avec une activité fonctionnelle *in cellulo*, respectivement 50 et 100 nM. Cependant, alors que toutes les NPs grippales ont commencé à être investiguées, il y avait toujours un manque criant d'intégration des différentes importines- α , alors même qu'il est bien connu désormais qu'il s'agit d'entités uniques, spécifiques, avec leurs propres régulations et partenaires.

Grâce à la chromatographie d'exclusion de taille couplée (ou non) à la diffusion de la lumière, à la résonance des plasmons de surfaces et à l'anisotropie de fluorescence, la première stratégie comparative complète, impliquant toutes les NP_{TAILS} déjà décrites et toutes les importines- α , a pu être mise au point et a révélé des spécificités surprenantes. Les NP_{TAILS} ont été fusionnées à la YFP, de manière à conserver un contexte globulaire sans contribution de l'oligomérisation propre à la NP. Toujours capables d'interagir avec les importines, la caractérisation cinétique a pu être réalisée. Les vitesses d'interaction impliquant d'une part, les importines- $\alpha 3$, - $\alpha 5$ et - $\alpha 7$, et d'autre part, A/NP_{TAIL}, C/NP_{TAIL} and D/NP_{TAIL} se sont révélées extrêmement rapides, tant l'association que la dissociation. D/NP_{TAIL} est capable d'interagir avec une forte affinité avec toutes ces importines- α , sans spécificité identifiable. A/NP_{TAIL} et C/NP_{TAIL} présentent des affinités plus variables, chacune interagissant mieux avec une

importine- α particulière, respectivement l'importine- α 7 et l'importine- α 3. B/NP_{TAIL} s'est montrée extrêmement surprenante: les vitesses d'association et de dissociation se sont révélées encore plus rapides, et les affinités pour toutes les importines- α testées se sont avérées très faibles.

L'importine- α 1, présentant une propension à dimériser en solution, n'a pas pu être testée par la stratégie mise en place. A l'aide d'un test de compétition et d'anisotropie de fluorescence, des valeurs d'affinités ont pu être obtenues et ont révélées une forte affinité pour D/NP_{TAIL} et C/NP_{TAIL}, une affinité basse pour A/NP_{TAIL} et une affinité médiocre pour B/NP_{TAIL}.

Ainsi, alors que des différences étaient attendues dans les caractéristiques de ces complexes, elles se sont avérées encore plus importantes, avec une contribution égale de chacun des partenaires à l'interaction. Ces résultats ont fait l'objet d'une seconde publication au cours de travail de thèse. Ils établissent une base solide pour continuer à disséquer et détailler cette interaction hôte:pathogène extrêmement complexe et essentielle.

En 2018, une étude métagénomique a identifié plus de 200 nouveaux virus à génome à ARN, et notamment 3 nouveaux virus appartenant à la famille des *Orthomyxoviridae*. Un de ces nouveaux virus infecte un crapaud et, de façon assez surprenante, est plus proche des virus de type B que des autres virus grippaux. Sa NP (Toad/NP), de la même façon, est phylogénétiquement plus proche de B/NP que des autres NPs grippales. Une prédiction structurale a mis en évidence une architecture potentiellement similaire à celle de B/NP, avec une longue extrémité N-terminale désordonnée (Toad/NP_{TAIL}), un corps central replié de taille similaire (Toad/NP_{CORE}) au autres NPs et une courte extrémité C-terminale désordonnée. Cette prédiction a aussi identifié des traits très particuliers de Toad/NP_{TAIL}: elle pouvait potentiellement posséder un domaine replié. De plus, longue d'environ 130 acides aminés, cette extrémité N-terminale l'est près de deux fois plus que celle de B/NP et présente de nombreux motifs basiques. Il a donc été décidé de commencer à caractériser l'interaction de Toad/NP avec les importines- α via la stratégie SPR mise en place précédemment, ainsi que son interaction avec l'ARN et sa structure. La cristallographie, la microscopie électronique, la chromatographie d'exclusion de taille,

l'anisotropie de fluorescence et la résonance des plasmons de surface ont été utilisés à ces fins.

Toad/NP a tendance à former des tétramères en solution, et son état d'oligomérisation n'est pas impacté non plus par la concentration en NaCl, de façon similaire à D/NP. Toad/NP_{TAIL} interagit avec les importines- α d'une façon très similaire à B/NP_{TAIL}, avec des vitesses d'association et de dissociation très rapides, hors des limites de détection de l'appareil. Son affinité pour les importines- $\alpha 5$ et - $\alpha 7$ est très basse, hors des limites supposées d'un NLS fonctionnel, mais son affinité pour l'importine- $\alpha 3$ indique par contre qu'il pourrait s'agir d'une interaction fonctionnelle pour l'import nucléaire. Toad/NP_{CORE} interagit avec l'ARN de façon similaire à A/NP et B/NP, avec coopérativité, sans spécificité de séquence et avec une potentielle saturation du site de fixation de l'ARN avec un ARN 12-mer. Toad/NP possède en revanche une spécificité intéressante : son extrémité N-terminale semble réguler négativement l'interaction avec l'ARN, peut-être *via* une région riche en glutamate. La structure de Toad/NP_{CORE} n'a pas encore été résolue à ce jour mais le travail est encore en cours. Il est hautement probable que ce corps, comme le corps de toutes les autres NPs, soit structuralement très conservé. En revanche, des cristaux du domaine N-terminal ont permis de résoudre la structure d'un domaine replié en 3 longues hélices- α antiparallèles (acides aminés 4 à 68). Il s'agit du premier domaine replié identifié dans l'une des extrémités désordonnées des NPs des virus grippaux. Le rôle et la fonction de ces deux domaines (le domaine replié et le domaine désordonné) ne sont pas encore clairement identifiés et nécessite davantage de travaux.

La découverte et la caractérisation de Toad/NP a mis en évidence une forte conservation du fonctionnement de la NP malgré son tropisme vers un animal à sang froid et a également révélé des traits uniques de cette NP, comme ce domaine N-terminal très long, et partiellement replié.

En conclusion, ce travail de thèse a permis de disséquer les interactions entre les différents types de NP grippales et les importines- α de manière comparative, en évaluant la contribution de deux partenaires. Il s'agit d'une base importante et essentielle pour finalement comprendre cette interaction complexe et très régulée. Il a aussi permis de caractériser les NPs de deux nouveaux membres de la famille des virus de la grippe et mis en évidence à la fois la forte base conservée structurelle et

fonctionnelle des NPs grippales et des caractéristiques très spécifiques, propres à chaque type grippal et adaptés à son hôte.

Chapter 1: Introduction

I. Influenza viruses

The results detailed in this manuscript concern all influenza virus types. However, because influenza A is a major concern for the human health, the majority of the publications is based on influenza A viruses.

A. Features of influenza viruses

i. *Taxonomy*

Influenza viruses, as viruses with a segmented negative-sense single-stranded RNA genome, belong to the *Riboviria* realm, the *Negarnaviricota* phylum, the *Articulavirales* order and are part of the *Orthomyxoviridae* family (**Table 1**). According to the last release of the International Committee on Taxonomy of Viruses in July 2019¹³⁴, the *Orthomyxoviridae* family contains seven genera, all composed by one or two species. Amongst these seven genera, 55 *Orthomyxoviridae* viruses are currently unclassified.

Table 1: Full taxonomy of the Orthomyxoviridae family members.

<u>Taxonomy</u>	<u>Features</u>
<i>Riboviria</i> realm	RNA viruses
<i>Orthornavirae</i> kingdom	
<i>Negarnaviricota</i> phylum	Negative single-stranded RNA genome
<i>Polyploviricotina</i> sub-phylum	Cap-snatching RNA viruses
<i>Insthoviricetes</i> class	Contraction of influenza , isavirus and thogotovirus
<i>Articulavirales</i> order	Segmented genome
<i>Orthomyxoviridae</i> family	
<i>Alphainfluenzavirus</i> genus	
<i>Betainfluenzavirus</i> genus	
<i>Gammainfluenzavirus</i> genus	
<i>Deltainfluenzavirus</i> genus	
<i>Isavirus</i> genus	
<i>Quaranjavirus</i> genus	
<i>Thogotovirus</i> genus	

This classification is bound to evolve, as researchers have only scratched the surface of the viral diversity. The science enters the area of metagenomics and new studies are currently focusing on virus sampling in organisms that have been

overlooked before ^{151,188}. As an example, in 2018 a study identified 214 new RNA viruses found in reptiles, amphibians and fishes ³²⁹. In this study, three new *Orthomyxoviridae* family members were identified: the Wuhan Asiatic toad influenza virus, the Wuhan spiny eel influenza virus and the Wenling hagfish influenza virus. None of these viruses has been included in the ICTV taxonomy yet. Unexpectedly, these viruses present sequence identities closer to the narrow-host range influenza B viruses rather than to the wide-host range influenza A viruses. It has been liberally chosen to use Toad influenza virus (ToadV) to refer to the Wuhan Asiatic toad influenza virus studied in this manuscript, due to a clear lack of insights on the virus cycle and specificities.

In the *Orthomyxoviridae* family, only influenza A viruses (IAV) are further classified into subtypes, based on the antigenicity of their two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA). Up to now, 18 different hemagglutinins (H1 - H18) and 11 neuraminidases (N1 - N11) subtypes have been identified. Influenza B viruses (IBV) present only one HA and NA subtype while influenza C and D viruses (ICV and IDV) possess only one surface glycoprotein, the hemagglutinin-esterase-fusion (HEF), combining both HA and NA activities ^{117,120,335}.

The current nomenclature ³⁹³ for influenza virus includes the influenza type (influenza A, B, C or D), the host species from which it has been isolated (except for human strains), the geographical area, the strain number, the year of isolation and, for influenza A viruses, the HA and NA subtypes (HxNx).

-A/WSN/1933(H1N1),

-B/Memphis/13/03,

-C/Ann-Arbor/1/50/ and

-D/bovine/France/2986/2012 influenza strains

were used for this PhD thesis work along with the newly discovered Wuhan Asiatic toad influenza virus (ToadV). Proteins will be labelled as X/Y, with X the influenza type (A, B, C, D or Toad) and Y the name of the protein.

ii. Viral particle structure

Influenza viruses (**Fig. 1A and 1B**) are enveloped viruses with a lipid envelop deriving from the plasma membrane of the host cell, and so enriched in host lipids such

as cholesterol³¹⁷. During the virion release, several cytoplasmic and membrane host proteins are integrated into the envelope, such as lipid raft-associated proteins and cytoskeleton proteins³²⁷. By electron microscopy (EM), envelop-inserted HA and NA can be distinguished by their specific features, respectively a smaller globular head and stalk and a high flat head (Fig. 1A and 1B)^{31,229,308,402}. Approximately 350 trimeric HA and 50 tetrameric NA are inserted into a single viral particle envelop; this HA/NA ratio allows the functional receptor binding of HA¹¹⁵. Balance in HA and NA activities seems required for the fitness of the viral particles³⁷⁶. C/HEF and D/HEF are distributed hexagonally at the virion surface, with a distinct mushroom shape^{121,234}.

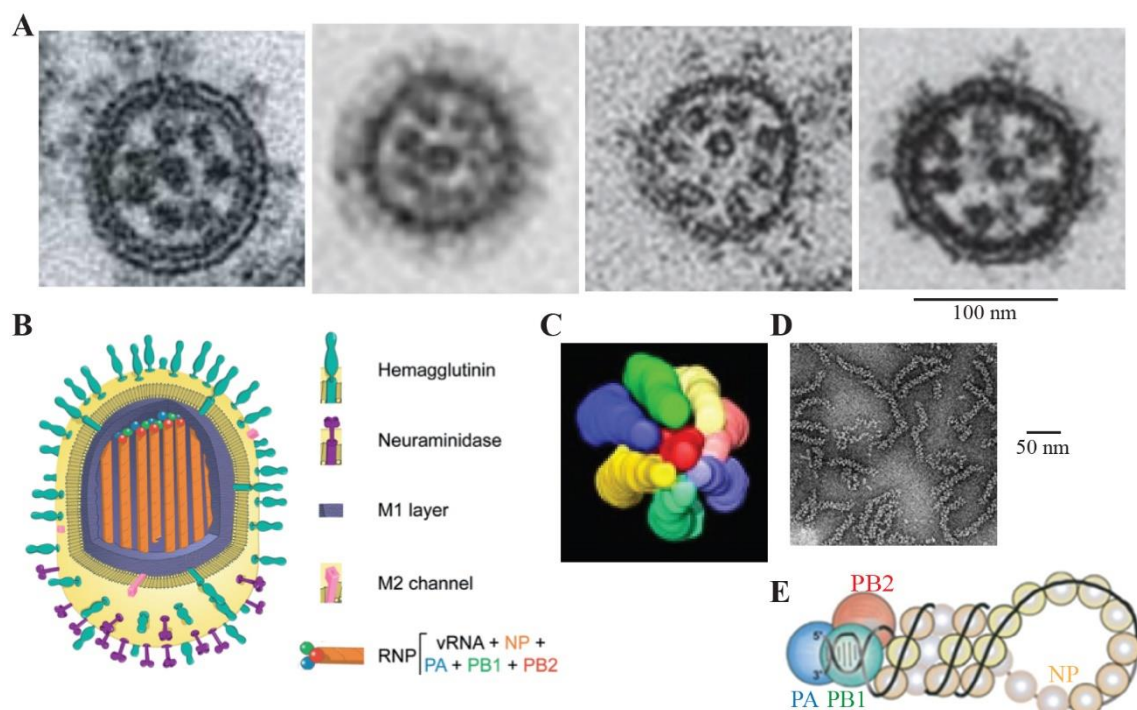


Figure 1: Viral particle structure and genome organization.

(A) Electron microscopy (EM) pictures of IAV, IBV, ICV and IDV particles, from left to right^{234,235,250}, (B) schematic representation of influenza A virus particle⁶², (C) modelization from STEM tomography of the packaging of the genome inside virions²³⁴, (D) EM picture of influenza ribonucleoproteins (RNPs) purified from virions³¹⁰, (E) schematic representation of a RNP, with the viral genomic segment in black, the nucleoprotein in light yellow and the three subunits of the polymerase in blue, green and red²³¹.

Influenza virions are pleomorphic^{43,115}. All influenza viral particles present similar sizes and structures, with both spherical and filamentous phenotypes. The size of the viral particles is usually around 100-120 nm in diameter (Table 2)^{26,117,155,254}. In addition, ICV filamentous particles can present an assembly in cord-like structures, up

to several micrometers in length ²⁴⁷. IAV, IBV and IDV particles occasionally form such structures, seemingly without packaged RNPs ^{42,200,234}. The morphology of the viral particle seems to be cell- and/or strain-dependent and is modified by serial passages in culture. Viral particles isolated from clinical samples tend to form more filamentous structures while cell culture-adapted or eggs-adapted virus shed virions far more homogeneous and spherical, as a result of host adaptation ^{42,159,300}.

Table 2: Summary of the influenza main features depending on the virus type.

Sph, Fil and Cord respectively stand for Spherical, Filamentous and Cord-like. * While reassortment can occur for IBV due to the two circulating lineages, this does not give rise to an antigenically distinct virus, only to a genetically distinct virus.

<u>Virus type</u>	IAV	IBV	ICV	IDV	ToadV
<u>Viral particle phenotypes</u>	Sph: 120 nm Fil: 120 nm Cord: several µm	Sph: 120 nm Fil: 150 nm Cord: several µm	Sph: 100 nm Fil: 100 nm Cord: several µm	Sph: 100 nm Fil: 100 nm Cord: several µm	?
<u>Genomic segments and main proteins</u>	Eight segments S1: PB2 S2: PB1 S3: PA S4: HA S5: NP S6: NA S7: M1/M2 S8: NS1/NEP	Eight segments S1: PB2 S2: PB1 S3: PA S4: HA S5: NP S6: NA S7: M1/M2 S8: NS1/NEP	Seven segments S1: PB2 S2: PB1 S3: P3 S4: HEF S5: NP S6: M1/M2 S7: NS1/NEP	Seven segments S1: PB2 S2: PB1 S3: P3 S4: HEF S5: NP S6: M1/M2 S7: NS1/NEP	Eight segments?
<u>Viable reassortment</u>	Yes, except some strain combinations	No*	No	No	?
<u>Reservoir</u>	Aquatic birds	Humans	Humans	Cattle	Toad?
<u>Tropism</u>	Wide spectrum Mammals and birds	Narrow spectrum Mainly humans	Narrow spectrum Humans and swines	Medium spectrum Mammals	?

Using electron tomography and EM, each segment of the viral genome, can be visualized as a dense rod-like structure (**Fig. 1A and 1D**). Eight of these structures, called ribonucleoproteins (RNPs), are localized at the distal end of influenza virions (**Fig. 1A, 1B and 1C**) ^{234,250}.

iii. The ribonucleoproteins, the replicative units of the virus

The functional replicative unit is composed by the viral RNA genome (vRNA) with viral proteins necessary and sufficient for an efficient replication and transcription. In influenza viruses, such a macromolecular complex is called an RNP (**Fig. 1**). One RNP is composed by a single genomic segment, encapsidated by multiple copies of the nucleoprotein (NP) and with one heterotrimeric RdRp (PA:PB1:PB2) associated to both 3' and 5' extremities (**Fig. 1E**)^{7,230}. This structure is helicoidal, supercoiled and highly flexible (**Fig. 1D and 1E**)^{46,162,277}. The dimensions of an RNP are respectively approximatively for 30-120 nm x 10-15 nm, depending on the encapsidated segment^{46,162,277,309}. The oligomerization of RNA-bound NP gives RNPs their rod-like structure^{280,353}. Influenza viruses possess 7 or 8 genomic segments and consequently 7 or 8 unique, distinct RNPs. Interestingly, even if ICV and IDV possess only seven viral genomic segments, it has been shown that all influenza viruses present a 7 + 1 packaging pattern of RNPs in virions (**Fig. 1C**)^{115,234}. Whether ICV and IDV package an extra vRNA segment or one of the host RNA is still unclear²⁴⁹.

iv. Genome organization, protein content and host tropism

IAV and IBV possess eight viral RNA genomic segments whereas ICV and IDV genomes are composed by seven segments^{117,265,298}. Each segment encodes for one or several proteins. Some segments are known to contain distinct open reading frames, encoding distinct proteins. Transcribed mRNAs can also be processed by the cellular splicing machinery, to generate other proteins (**Fig. 2**)^{67,90,233,408}. The segments are numbered and named depending on their length and the main protein they encode (**Table 2, Fig. 2**). The difference in segment number is due to the difference in the viral glycoprotein composition: IAV and IBV possess HA and NA while ICV and IDV only possess HEF. While the full genome is not yet available for ToadV, two glycoproteins (HA and NA) are encoded by the genome, meaning that it seems to be made of eight segments, like both IAV and IBV. The details of the proteins is shown with the **Figure 2**.

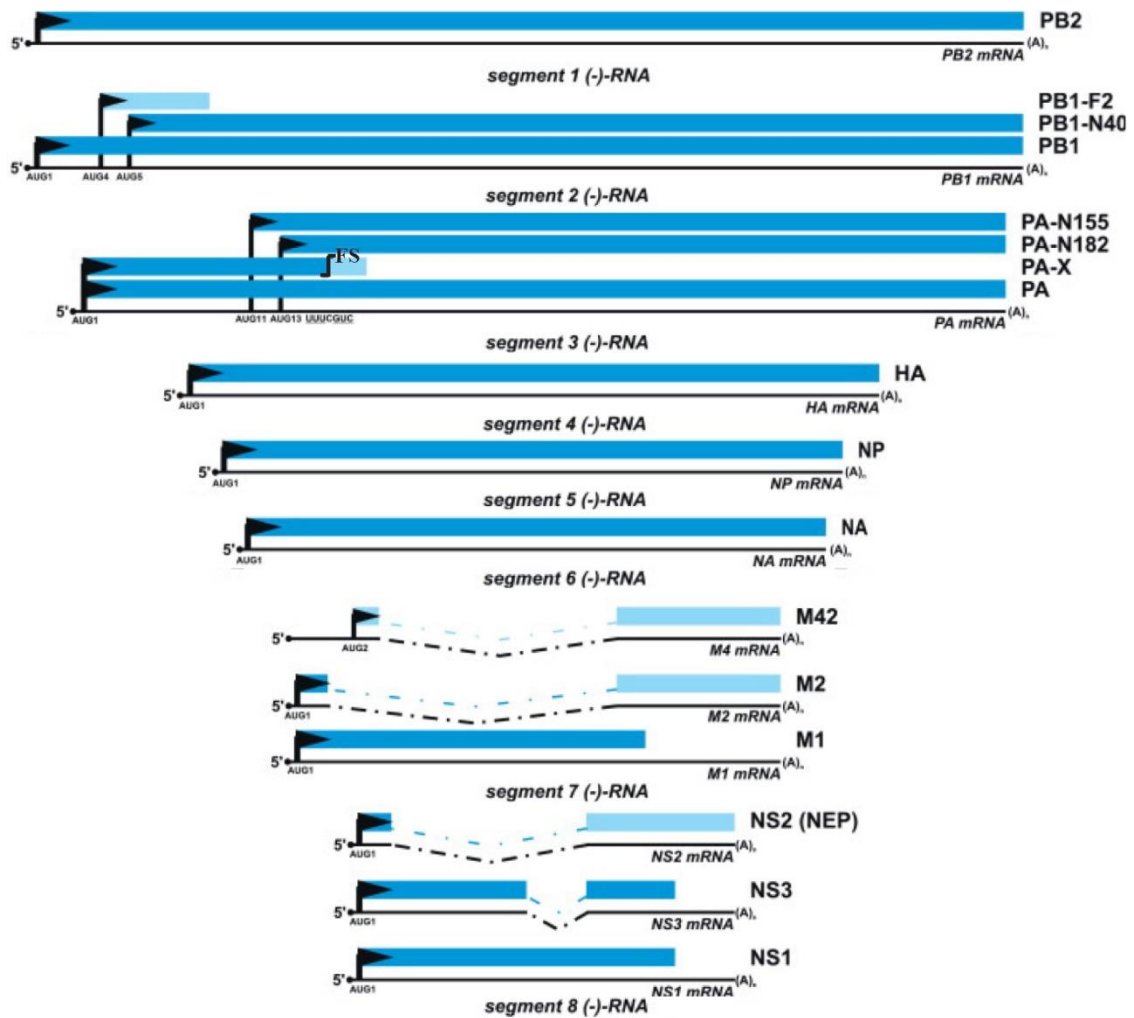


Figure 2: Main and accessory proteins encoded by influenza A viral mRNAs.

Adapted from ³⁷⁰. Black arrow and dotted lines represent respectively the mRNA translation start and spliced parts. Each blue shade corresponds to one of the 3 open reading frames. For segment 3 PA-X mRNA generation, one frameshift occurs (FS).

Beside the main proteins, several accessory type-dependent proteins have been identified. Up to now, seven non-essential proteins have been described for IAV, which seem to play many roles in the regulation, the virulence and/or the host adaptation processes (**Fig. 2**) ^{370,409}.

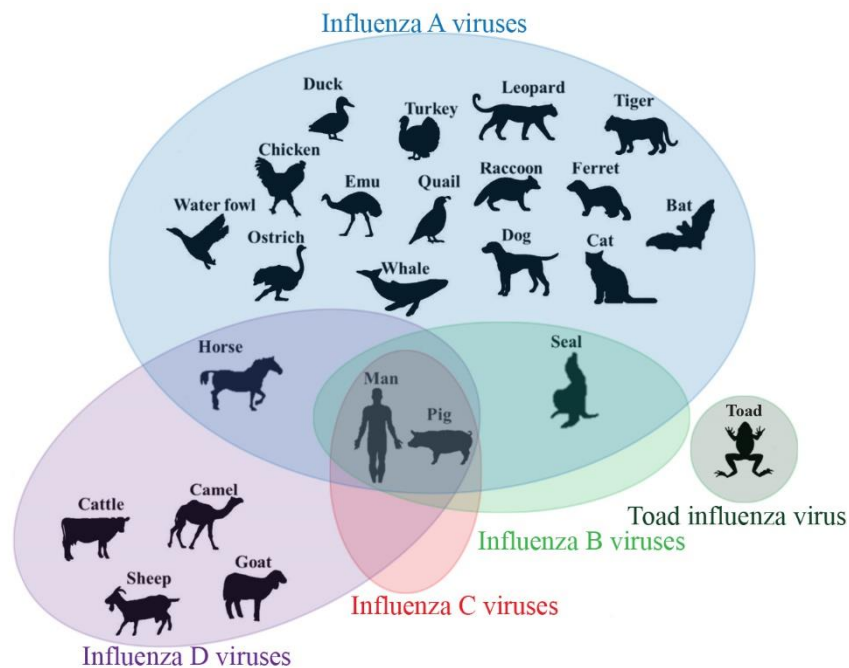


Figure 3: Host tropism of influenza viruses.

The known host range of each influenza virus is labelled as a coloured background, with blue for IAV, light green for IBV, red for ICV, purple for IDV and dark green for ToadV. Adapted from ¹⁷¹.

Each influenza virus possesses its own specific host tropism, which relies partly on the behaviour and specificities of its proteins (**Fig. 3, Table 2**). Aquatic birds are the reservoir for IAV but they are able to infect a wide spectrum of animals. Some serological reports show evidence of infection in reptiles and amphibians or at least some sort of cross-reactivity, in addition to mammals, bats and birds ^{36,369,387,415}. IBV and ICV present a restricted host spectrum: IBV mainly infect humans, but some cases have been reported in seals, and ICV can infect swine in addition to humans ^{255,260,417}. IDV have been discovered in 2011, initially identified as an ICV subtype, but officially considered as a new influenza genus in 2013. IDV show a large spectrum of host organisms in mammals, from camelids to pigs and goats, but their reservoir is suspected to be the cattle ^{68,116,117,285}. They can replicate in ferrets, the animal model for human infection ¹¹⁷. Humans, and more particularly people working closely to the cattle, have been shown to seroconvert by producing anti-influenza D antibodies ^{117,363,391}. Because of its recent discovery, ToadV has not been studied so far, without any further information in terms of reservoir and tropism.

Several factors restrict the host and the cell tropisms. The growth temperature is one of them. IDV have been found to efficiently replicate at both 33°C and 37°C, likely

helping in its broad host range ^{117,124,391}. IAV and IBV present an optimal growth temperature of 37-39°C and 35°C respectively, in agreement with the temperature of their respective reservoir hosts and cell environments ²⁰⁰. ICV tend to grow better at lower temperatures (*i.e.* 33°C rather than 37°C) ^{117,254}.

In term of cell tropism, the main and most studied factor is the HA/HEF glycoprotein, which binds receptors modified with sialic acids (**Fig. 4A**). The A/HA activation seems to be done through a restricted number of proteases and its optimal temperature ranges from 33 to 39°C depending on the HA type and the targeted host. In contrast, B/HA demonstrates a large panel of activating proteases with an optimal temperature of 33°C, well adapted to human airways ¹⁸². D/HEF and C/HEF are structurally well conserved and are both able to bind to a large spectrum of host tissues, but with a better D/HEF attachment to the cell surface than C/HEF. D/HEF presents a more open binding cavity, likely allowing more diverse receptors and/or partners interactions ³³⁵. C/HEF is rather sensitive to the temperature, with an optimal close to 33°C ³⁴⁸. Interestingly, IDV is the most resistant of all influenza viruses to an acidic or hot environment and its stability was shown to be mainly due to D/HEF ⁴¹⁶.

Several other viral proteins are involved at host:pathogen interfaces and are responsible for these differences in host and cell tropisms such as NS1, the polymerase subunits and NP.

The subunits of the RdRp, and in particular PB2, are considered as determinants of the host tropism ^{23,351}. It is now well-known that the A/PB2 E627K substitution is an important marker of the mammal adaptation, for the replication at lower temperatures ^{343,352}. When residue 627 is in its avian form, the D701N substitution can compensate, allowing a successful replication, probably in its interaction with the cellular nuclear import machinery ^{24,341}. Other discreet substitutions can also compensate for a fully avian phenotype at these two positions, as seen in the case of the pandemic 2009 H1N1 strain ²¹⁴.

NP can be found at several host:pathogen interfaces. Several mutations (N319K, G102R, M105K or D375N) have been identified as positions involved in nuclear import ^{93,94,291,292}. Other mutations (G16D, L283P, F313Y or Q357K) allow the viral escape from the MxA restriction ²⁰².

Outside of a few specific mutations, the diversity of adaptive mutations suggests that adaptation to a new host is a largely synergic mechanism, with several distinct mutations likely required to overcome this barrier ^{203,319}.

NS1 is strongly involved in the host defences regulation, and more particularly against interferon signalling ¹¹⁰. In H5N1 viruses, the residue 92 tends to be a glutamate or an aspartate, and could lead to a stronger resistance to antiviral responses and to higher virulence ^{271,324}. A/NS1, B/NS1, C/NS1 and D/NS1, while sharing the same antiviral activities, are not completely interchangeable for the replication and the host response inhibition purposes ²⁵¹.

While not directly involved in the host tropism, M1 and M2 seem to possess a very specific behaviour depending on their viral type.

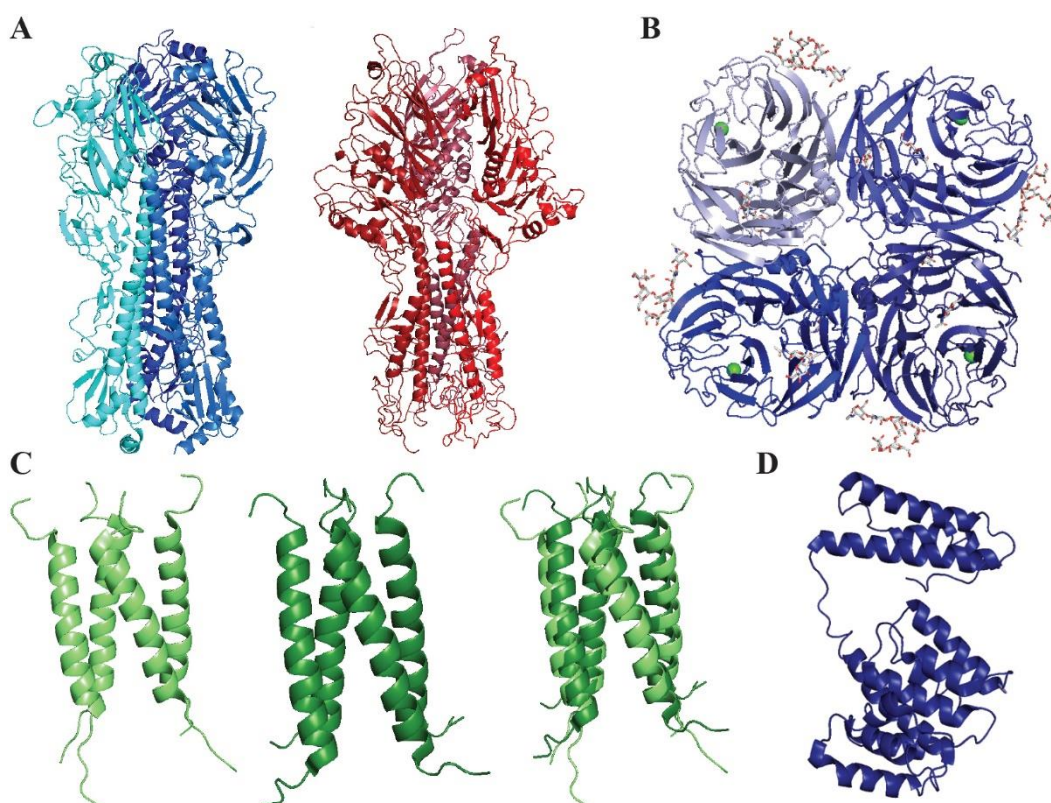


Figure 4: Structure of influenza HA, HEF, NA, M2 and M1 proteins.

(A) Crystal structures of trimeric A/HA (blue, PDB 3LZG) and C/HEF (red, PDB 1FLC). (B) Crystal structure of A/NA (PDB 5HUK). (C) Crystal structures of the open channel of B/M2 (left), the close conformation (middle) and the superimposition (right) (PDB 6PVR and 6PVT). (D) Cryo-EM structure of the A/M1 matrix protein (PDB 7JM3).

The influenza M2 protein is an ion channel essential during the viral cycle and more particularly for the cytoplasmic release. A/M2 and B/M2 (**Fig. 4C**) share some features, such as a mostly proton channel role, histidines playing the part of proton sensor and activator and a similar transmembrane domain length. They also seem to present specificities, with a different pH of activation and a different direction of the proton flux^{179,198,420}. In contrast, C/M2 and D/M2 are primarily chloride ion channel with a slight proton permeability, suggesting a different mechanism in the viral cycle^{21,125,158,227}.

The highly flexible influenza M1 protein (**Fig. 4D**) is heavily involved in the structure of the viral particle and the budding process³⁰³. M1 is often found at the interface between viral and host partners, and it is known to interact with viral RNPs, several viral and cellular factors, and lipids.

v. Evolution: antigenic drift and antigenic shift

Influenza viruses are known for their fast continuous evolution, depending on two mechanisms: the antigenic drift and/or the antigenic shift.

Like the majority of RNA virus RdRp, the influenza RdRp does not possess a proofreading mechanism. Therefore, the influenza RdRp integrates about one error every genome replication, at a rate of about 10^{-5} - 10^{-6} substitution/nucleotide site/cycle^{23,66,269,340}. It has been shown previously that IAV present a higher mutation rate than IBV, with rates of about $2 \cdot 10^{-3}$ and $0.6 \cdot 10^{-3}$ substitutions/nucleotide site/year respectively^{248,390}. Influenza viruses are under a strong selective pressure from both the host immune system and the host machineries they hijack. NA and HA (**Fig. 4A and 4B**) are in the front line, recognized swiftly by the immune system and the main target for host antibodies^{141,276}. This requires a fine balance between escape mutations and/or mutations increasing the virus fitness and non-deleterious mutations to preserve the protein functions. This process is called the antigenic drift and occurs also in host adaptation in cell culture³³⁴.

The antigenic shift is a brutal event that is due to the direct jump from one host species to another and/or the reassortment. The latter is specific to the segmented nature of the viral genome. It occurs when two viruses co-infect the same cell and exchange one or several genomic segments during the packaging step. However, it is

required for the two viruses to be of the same type and compatible¹⁸⁷. Genomic segment exchange often leads to pandemic strains of IAV^{319,384}. As an example, the pandemic 2009 H1N1 genome came from four distinct IAV parental strains^{225,253}.

vi. Disease and epidemiology

Influenza viruses are mostly known due to the seasonal epidemic and sporadic pandemic they cause in humans. Seasonal influenza is an acute and contagious disease affecting the human respiratory tract with several non-specific symptoms, such as high fever, cough, asthenia, headaches and muscular aches. In most cases, no complication arises. However, severe or deadly outcomes can befall to high-risk people, including pregnant women, immunocompromised or people affected by a chronic disease, the elderly and new-borns. Upon influenza virus infection, they could develop severe respiratory tract infection, associated or not with coinfection, and extreme inflammatory reaction. The severity is intrinsically linked to the virus and the host immune response¹⁴⁹. IAV and IBV are responsible for these yearly events, infecting around 10% of the world population, resulting in 3 to 5 million severe cases and 290 000 to 650 000 respiratory-related deaths each year¹³⁸. The average annual economic burden of a seasonal outbreak has been recently estimated at \$11.2 billion in the USA or €78.3 million in Germany^{284,318}.

Seasonal epidemics occur during fall and winter in temperate climates⁴⁰⁰. In the tropical and equatorial parts of the globe, influenza viruses circulate all around the year, with unpredictable epidemics, assuring virus constant exchange between the Northern and the Southern hemispheres^{312,374}. The virus is mainly transmitted through infectious droplets emitted by infected patients, but it can also be spread through the contact with an infected surface or person^{213,252}.

IAV and only IAV can cause pandemics, corresponding to worldwide sporadic occurrences: only IAV possess several antigenically distinct HA and NA proteins, leading to large-scale outbreaks for a lack of previous immunity.

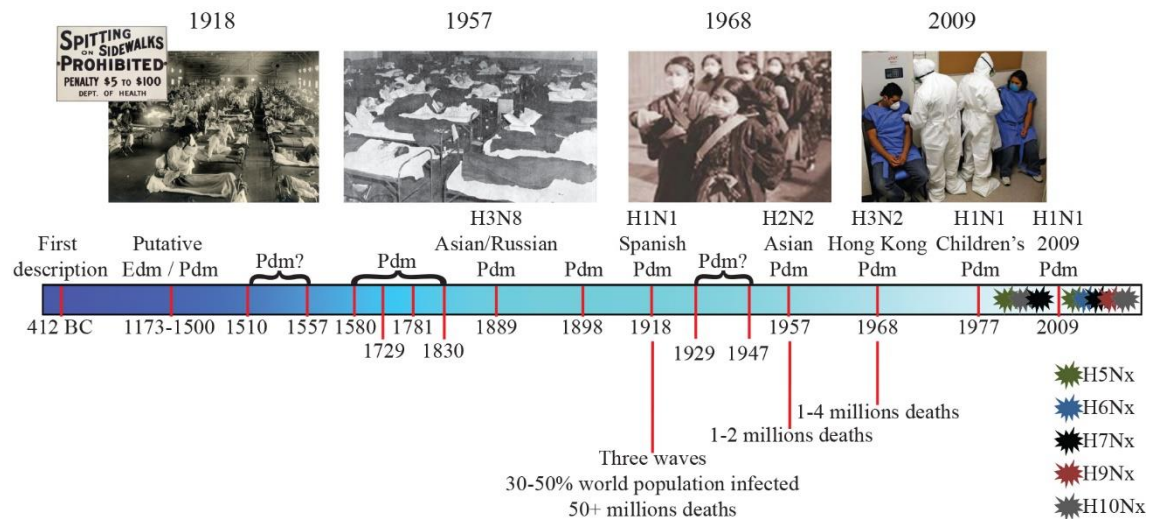


Figure 5: Major historical outbreaks in the shared human and influenza history.

Putative and confirmed pandemics (Pdm) and epidemics (Edm) are illustrated as red bars. Sporadic outbreaks of deadly highly pathogenic avian influenza viruses are shown as spiky forms of distinct colours for distinct HA types. This schematic representation is not at scale.

The human history is tightly intertwined with influenza viruses (**Fig. 5**): in 412 B.C. in Ancient Greece, Hippocrates described a disease strikingly similar to influenza and no less than 13 pandemics have been identified these last 400 years^{174,279,315}. The worst pandemic in modern history was caused by a H1N1 influenza virus, submerging the world in three waves in 1918-1920. As World War I was raging across the world, involved belligerents did not report the pandemic. Spanish censorship-free newspapers did, and thus the pandemic was wrongly named “the Spanish flu”. The mortality rate was estimated at least 2.5-5%, far higher than any other influenza outbreak. The virus infected almost half of the world population, killing at least 50 million people, mainly young adults^{145,330,400}. Beyond the new antigenic variant and the virus intrinsic virulence, other reasons can explain this catastrophe¹³⁵. In 1918, the responsible agent was unknown and therapeutic, prophylactic and sanitary measures were lacking^{144,208,336}. Antibiotics to treat severe secondary bacterial infections were not discovered yet, becoming widely available from 1939 onwards.

Since the 1920’s, four pandemics occurred, with a high death toll but far from the level of 1918-1919 (**Fig. 5**). Nowadays, experts are closely monitoring several influenza viruses, and more particularly zoonotic viruses of the H5Nx, H6Nx, H7Nx, H9Nx and H10Nx subtypes^{222,400}. Sporadic transmission of these viruses to humans occurs regularly, without sustainable human-to-human transmission. However, the

mortality rate in these infections can be up to 50%, suggesting that a pandemic could result in a disaster ^{147,323,394}.

In addition to human infections, influenza viruses widely circulate in animals, more particularly in birds for IAV ^{169,262}. While wild aquatic birds tend to be asymptomatic or present little symptoms, this is not the case in poultry ²²⁶. Low-pathogenic avian influenza viruses (LPAIs) and highly pathogenic avian influenza viruses (HPAIs) tend to spread quickly in bird populations. LPAIs tend to induce in worse cases mild symptoms, such as a decrease in egg production and slight respiratory issues; HPAIs, in contrast, usually result in a severe disease, killing domesticated birds in a few days, with a mortality rate that can be close to 100% ^{262,347,392,401}. The severity of the IAV infection depends on the strain and the host and ranges from little to no symptom to systemic infections and high mortality rates ³³⁸.

Even if its epidemic and circulation patterns remain largely unknown, ICV infect humans and more particularly children, leading to a mild respiratory disease ³²¹. Swine are also susceptible but with no evident signs of the disease.

IDV reservoir seems to be the cattle. The clinical symptoms for IDV alone are typically weak but IDV coinfection with other pathogens seems to be involved in a more severe disease, called the bovine respiratory disease (BRD) ^{80,216}. Swine and various farm animals such as goat but also camel present an anti-IDV serology but the pathology has not been extensively studied. Swine and animal models like ferret do not present influenza-like symptoms and lesions ^{79,117}.

Regarding the newly identified influenza viruses infecting cold-blooded animals, it is not known if they can induce symptoms, infect other animals or cause any diseases.

B. Influenza virus cycle

The first step of the viral cycle is the attachment to the host cell through HA/HEF (**Fig. 6 and 4A**). Both recognize sialic acid molecules, highly expressed and displayed on cell surfaces.

A/HA and B/HA recognize the N-Acetylneuraminic acid (Neu5Ac) sialic acid, found on glycolipids or glycoproteins ³⁴⁴. Human A/HA bind preferentially to α 2,6-linked

Neu5Ac, abundant on human epithelial cells in the upper respiratory tract ^{47,375}. In contrast, avian A/HA interact with α 2,3-linked Neu5Ac, predominantly found in the digestive tract of aquatic bird and the human lower respiratory tract ¹³⁷. Both α 2,3- and α 2,6-linked sialic acids are highly expressed in the pig trachea ^{136,375}. ICV and IDV HEF bind to the N-Acetyl-9-O-acetylneuraminic acid ³⁰¹. The high HA/HEF density could compensate the weak binding affinity by a high avidity ^{316,349}.

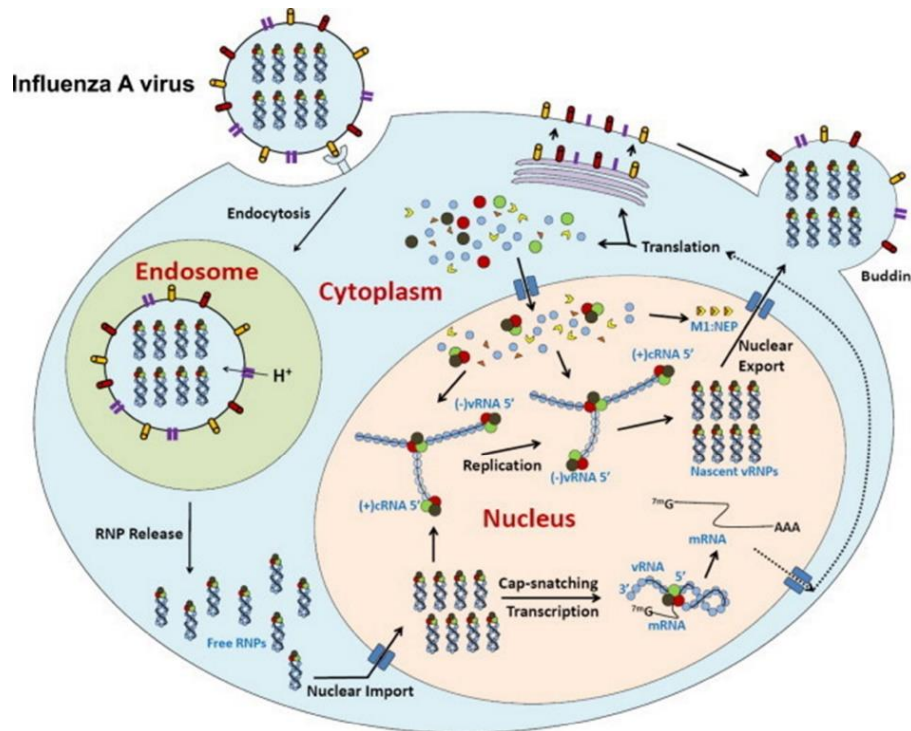


Figure 6: Influenza viral cycle.

The viral proteins are represented with different forms and colours: NP, PA, PB1 and PB2 are represented as round forms in blue, brown, green and red respectively. M1 and NEP are triangular forms, in orange and yellow respectively. HA, NA and M2 are cylindrical forms, red, yellow and purple respectively. Adapted from ⁴²³.

The viral entry in cells mainly occurs through the clathrin-dependent mechanism but clathrin-independent endocytosis and macropinocytosis have also been reported ^{210,270,331,377}. Influenza virions then go through the endosomal/lysosomal pathway. From early endosomes to lysosomes, the internal pH of the endosomal particles drops from 6.5 to 4.5. The ion channel M2, inserted into the virion envelop, leads to the internal acidification of the viral particle, resulting in the M1 dissociation from the RNPs (**Fig. 4C**) ²⁰⁴. HA/HEF goes through a conformational change, triggering the viral

particle envelop fusion with the endosomal membrane and the RNPs release in the cytosol^{96,210,424}.

The RNPs then require access to the nucleus to initiate the transcription/replication steps. Influenza viruses hijack the host nuclear import machinery, and in particular the importin- α/β pathway (cf. part. II)^{28,54}. RNPs are rapidly translocated through the nuclear pore complex, and transcription is initiated by the viral polymerase²⁰⁵. Viral messenger RNAs (mRNAs) are directly transcribed from the viral genome. They are exported in the cytoplasm, where they will be translated by the host machinery⁵⁴. Newly synthesized subunits of the RdRp, NP, M1, NS1 and NEP will be imported or will diffuse in the nucleus to contribute to the synthesis of viral RNAs^{28,57,93,381}. Replication is the other important process in the nucleus. For this, negative-sense genomic RNAs are firstly used to generate positive-sense RNA (cRNA) which is used as a template for genomic negative-sense RNA synthesis. As the viral RNAs are never naked, the encapsidation by both NP and the RdRp occurs simultaneously to their synthesis. Once reconstituted, RNPs are exported in the cytosol by using the cellular nuclear export machinery, M1 and NEP. NEP interacts with the cellular Chromosome Region Maintenance 1 (Crm1) export protein and M1, which in turn interacts with RNPs^{2,37,72,126,240,267,385}. RNPs are then addressed to the lipid rafts at the plasma membrane, where HA/HEF, NA and M2 are already localized to be packaged into virions¹³².

A functional viral particle requires the seven/eight individual genomic segments packaged in a single particle. While the molecular basis behind this step is still unclear, selective packaging of RNPs is the favoured hypothesis^{25,97,98,235}. A viral RNA segment seems to present at its extremities packaging signals, which interact directly with other distinct RNP packaging signals^{88,89,197,228}. In addition, parts of coding regions are suspected to enhance a correct packaging^{99,105}. They bring together distinct segments, resulting in a close parallel alignment of distinct RNPs. The budding of infectious virions is a mechanism poorly understood. However, the budding seems to be initiated at lipid rafts by the presence of the RNP complexes, HA/HEF, M1 and NA, in an ESCRT-independent manner^{238,313,317,388}. M2, localized at the border of lipid rafts, is responsible for the membrane scission^{204,302}. NA then cleaves sialic acids from receptors associated with HA, making free the new virion.

C. Viral transcription and replication machineryi. *Nucleoprotein*

The nucleoprotein is the most abundant protein of the RNPs and also in the infected cells. NP is required for both viral transcription and replication activities and could act as a switch between the two processes ^{15,325}. NP is also involved in the viral RNA genome protection, in the interaction with cellular partners and in the cellular trafficking.

Table 3: Sequence identities between influenza types.

Sequences were recovered from the Influenza Research Database ⁴¹⁹. The strains used for alignment are those used in this work.

	A/NP	B/NP	C/NP	D/NP	Toad/NP
A/NP	100	37.2	21.8	21.1	22.5
B/NP		100	23.1	23.8	28.7
C/NP			100	37.5	22.1
D/NP				100	20.1
Toad/NP					100

Globally, sequence identities between all NP types range from 20 % to 38 %, with biochemical properties and structure predictions rather well conserved (**Fig. 7, Table 3**). A/NP, B/NP, C/NP, D/NP and Toad/NP molecular mass and protein chain length vary between 56 and 67 kDa, ranging from 498 to 607 amino acids.

NP is a highly basic protein with a basic groove, a feature to be linked to its RNA binding activity (**Fig. 8B**) ^{242,243}. NP does not seem to bind to RNA bases but rather to the phosphate skeleton ¹³. It is still unclear how NP and RNA interact, as recent studies showed that NP was not distributed homogeneously on viral genomic segments with a preferential binding mode ^{183,184,396}. The monomeric form of NP is probably the active form in the interaction with RNAs, with the NP oligomerization triggering the formation of the RNP architecture; the high affinity binding of the trimeric A/NP to RNA could impair the oligomerization process required for the RNP formation ¹⁷⁸. A/NP and B/NP bind RNAs with nanomolar affinity, with cooperativity, but behave differently depending on the salt concentration and the RNA length ^{13,70,71,178,407}. The hypothetical length of vRNA bound by one monomer of NP in the context of the RNP is thought to be around

24 nucleotides^{46,207,259}. Interestingly, NP-bound RNAs are still susceptible to RNases, consistently with the suspected binding pattern of RNAs, wrapped around NP^{143,207}.

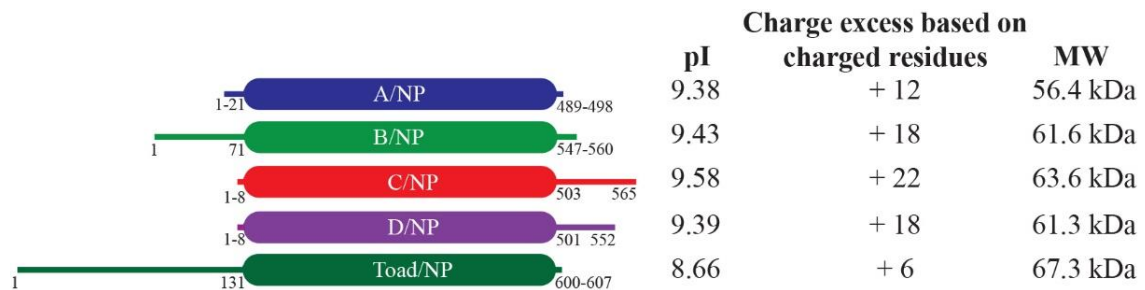


Figure 7: Biochemical features of influenza nucleoproteins.

Intrinsically disordered tails and structured domains are represented as thin lines and boxes respectively.

Influenza NPs are globular, tightly folded proteins. To date, several influenza NP structures have been solved, but none of them with RNA (**Fig. 8**). More particularly, two A/NP and one B/NP have been crystallized and solved as trimers and tetramers respectively, highlighting the NP tendency to form oligomers in solution and in absence of RNA. NP oligomerization is affected by the RNA, the salt concentration, its concentration, its phosphorylation state and the temperature^{39,178,353,365}.

While A/NP and B/NP share only 37% of sequence identity, they are structurally well conserved (**Fig. 8C**). They are folded mainly in α -helices. The two major differences come from **i**) the oligomerization loop orientation in the crystal structure, due to the oligomeric state (trimer *v.s.* tetramer) and **ii**) a longer N-terminal (N-ter) domain not visible in the structure and intrinsically disordered (i.e. 21 residues for A/NP against 71 for B/NP). The structure of the monomeric R416A mutant gives precious insights into the oligomerization mechanism (**Fig. 8D**). In the native structure, the Arg-416 is localized in a protruding loop (from residue 402 to 428) making the interaction interface with a second protomer. The Arg-416 makes an essential salt bridge with the side chain of the Glu-339. The R416A mutant loop, in contrast, was not extended and was folded into its own NP-binding groove (**Fig. 8A, 8C and 8D**). Putatively due to the oligomerization loop conformation, the monomeric mutant is drastically impaired in its interaction with RNA (micromolar affinity)^{27,40,353}. The B/NP oligomerization

mechanism seems similar to the A/NP one, forming a conserved salt bridge between residues R472 and E395.

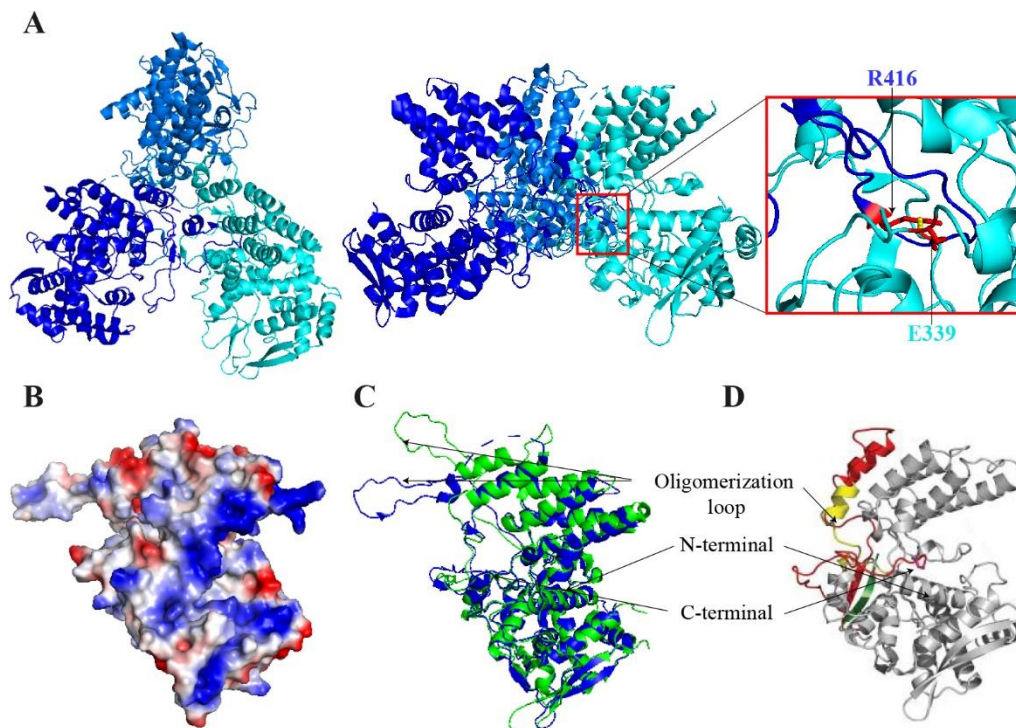


Figure 8: Influenza nucleoprotein structures and structural features.

(A) Crystal structure of trimeric influenza A/NP with the essential salt bond responsible for the oligomerization process, (B) Electrostatic potential of a protomer of the trimeric A/NP, with positive charges in blue and negative charges in red (C) Superposition of A/NP (PDB 2IQH), coloured in blue; with B/NP (PDB 3TJ0), in green (D) Monomeric R416A mutant of A/NP, adapted from ⁴⁰.

In the *Orthomyxoviridae* family, another virus, the infectious salmon anemia virus (Isavirus) and its NP (ISA/NP) have been studied. ISA/NP has been crystallized as a dimer. It is structurally well conserved with influenza NPs, but it possesses a supplementary partly structured N-ter domain (**Fig. 9**) ⁴²². ISA/NP seems to be mainly dimeric in solution. Each monomer binds to 12 nucleotides long RNA molecules and 24 nucleotides long RNAs seem wrapped around ISA/NP, as it has also been suggested for influenza NPs. The activity or the functionality of the unique N-terminal domain of ISA/NP has not been elucidated yet. It is interesting to note that ISA/NP N-ter extremity (NP_{TAIL}) is longer than those of A/NP and B/NP (respectively 111, 21 and 71 amino acids). Furthermore, A/NP_{TAIL} and B/NP_{TAIL} have a well-defined function, playing an essential role in the nuclear translocation (cf. part III.). Although, due its

size, B/NP_{TAIL} could probably have a yet unravelled function.

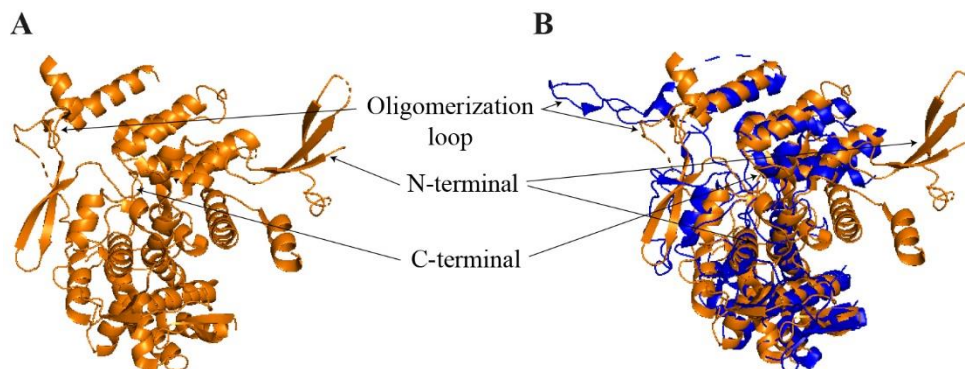


Figure 9: Structure of Isavirus NP.

(A) Crystal structure of ISA virus NP (PDB 47EWC) (B) Surimposition of A/NP (PDB 2IQH), coloured in blue, with ISA/NP (PDB 47EWC), in orange.

While no structure for C/NP or D/NP was solved at the beginning of this PhD thesis work (cf. Chapter 2), it was possible to make assumptions on their potential folding. The disorder prediction indicates that in contrast to A/NP and B/NP, C/NP and D/NP present only a few unstructured N-terminal residues and a long intrinsically disordered C-terminal (C-ter) region spanning about 50-60 residues (**Fig. 7**). This change from an N-ter NP_{TAIL} to a C-ter one is puzzling. Identity between C/NP and D/NP sequences is 38%, similar to the one between A/NP and B/NP, suggesting that their folding could very well be alike.

ii. *Heterotrimeric RNA-dependent RNA-polymerase*

The influenza RdRp is an intricate heterotrimeric complex of PA, PB1 and PB2, each of them being composed of 700 to 800 residues with a total molecular mass close to 270 kDa.

The influenza polymerase structure has eluded researchers for a long time, due to issues in producing it in a soluble amount. The issue was tackled by firstly solving individual domains of the complex, like the PB2-627 domain^{311,352}. Subunit interfaces were initially roughly determined: PA_{C-ter} interacting with PB1_{N-ter}, which in turn interacts with PB2_{N-ter} through its C-ter part. EM analysis showed that the polymerase is a tightly folded complex, suggesting supplementary inter-subunit interaction regions. These last

few years, major breakthroughs have been achieved using X-rays crystallography and cryo-EM allowing to solve structures of IAV, IBV, ICV and IDV polymerases (**Fig. 10**).

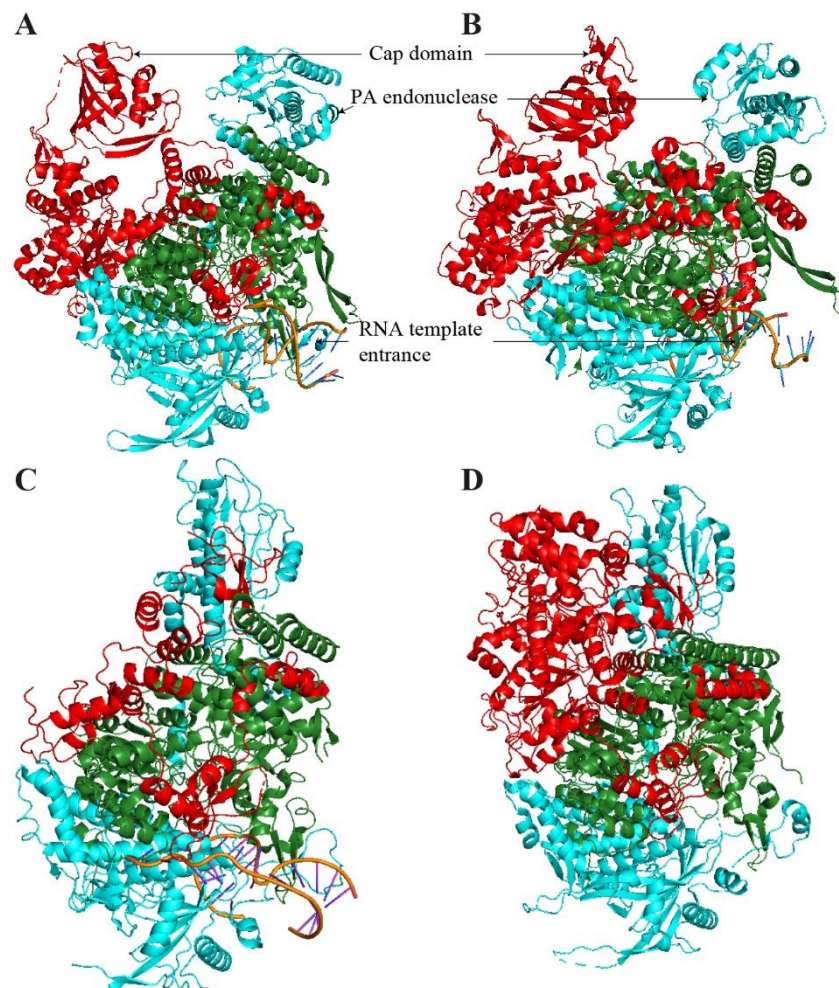


Figure 10: Bat IAV, IBV, ICV and IDV RdRp structures.

(A) Crystal structure of bat IAV RdRp (PDB 4WSB) and (B) crystal structure of IBV RdRp (PDB 4WSA). (C) Crystal structure of ICV RdRp (PDB 5D9A) with bound RNA (D) Cryo-EM structure of IDV Apo RdRp (PDB 6KUT). PB1, PB2 and PA are respectively in green, red and cyan. Well-defined domains such as PB2 cap domain and PA endonuclease domain have been identified when fully crystallized. RNA promoter is coloured in orange.

The first two influenza RdRp structures solved were a bat IAV RdRp and an IBV RdRp (**Fig. 10A and 10B**)^{273,289}. These structures reveal further subunit interfaces, with each subunit tightly entangled with each other. They nevertheless possess an important intrinsic flexibility, as other structures were released with domains caught in alternative positions^{119,359}. These two first macromolecular complexes are structurally well conserved, bare for the intrinsic flexibility of the cap-binding domain of PB2, which

presents itself in a different orientation. Both structures were solved with a bound promoter RNA. Structural insights have also been snatched from transcribing and replicating IAV, IBV, ICV and IDV polymerases, giving precious step-by-step descriptions of the polymerase activity (**Fig. 10C, 10D and 11**)^{75,119,168,272,380}.

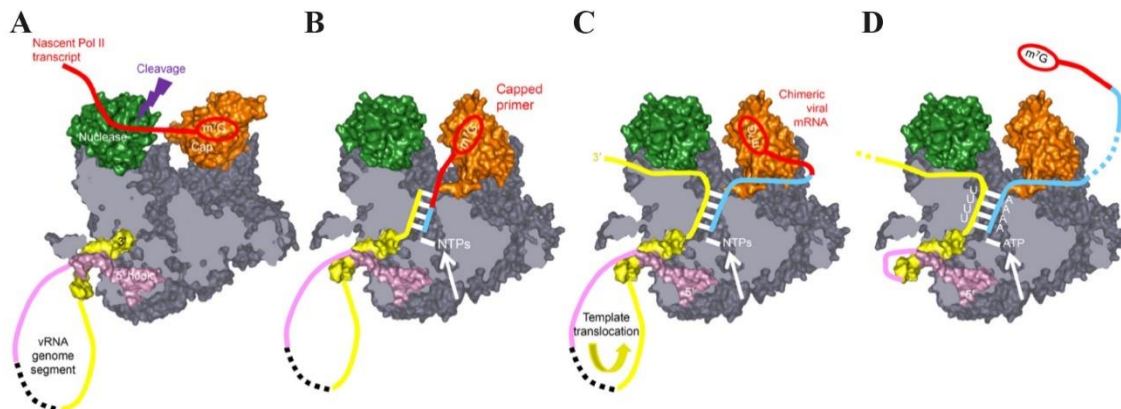


Figure 11: Reconstituted model of influenza RdRp transcription.

(A) PB2 performs its cap-snatching activity on a cellular mRNA and PA cleaves the mRNA before (B) PB2 rotates to present the cleaved capped primer to PB1 (C) that performs the elongation up to (D) the stuttered polyadenylation. Adapted from²⁷⁴.

Each subunit presents essential specific features and activities. PB1 is the core protein for transcription and replication, possessing the RNA polymerase activity. PB2 and PA present activities related to transcription and more particularly its initiation: as the influenza polymerase does not possess a guanylyl-methyltransferase activity for the mRNA capping and as viral mRNAs use the cap-requiring cellular machineries for transport and translation, a mechanism called ‘cap-snatching’ is used (**Fig. 11**)^{29,275}. Briefly, PA is able to bind to the C-terminal domain (CTD) of the cellular DNA-dependent RNA polymerase II (Pol II)^{195,379}. PB2 binds to the cap of host mRNAs, by snatching it from the transcribing cellular Pol II¹⁰⁸. PB2 presents this capped RNA through a rotational change to the PA subunit. With its endonuclease activity, PA cleaves the host capped mRNA 10 to 13 nucleotides downstream⁵⁸. These few nucleotides left are then used as primers by PB1 to synthesize the viral mRNA. The polyA tail is synthesized in a particular way by the RdRp, by stuttering over a patch of 5 to 7 uridines at the 5’ part of the vRNA^{84,278}. The influenza RdRp has been shown to adopt two major distinct conformational states, *i.e.* transcriptionally active or inactive. It has been shown that the interaction between the influenza RdRp and the cellular

RNA polIII is required for the RdRp to switch from an inactive state to an active state³⁷⁹. The dissociation timing from the cellular polIII remains obscure.

In contrast to transcription, due to the negative sense of the viral genome, influenza polymerase realizes the replication in two steps (**Fig. 12**). Firstly, viral segments of positive sense are synthesized to serve as a template. This template then goes through another round of synthesis to perform the actual viral genome replication and generate negative sense genomic segments¹¹⁸. PB1 seems to be able to initiate and terminate the replication on its own, in a primer-independent manner, but making replication 300 times slower than transcription²⁸⁸. Interestingly, during the replication, several RdRp complexes can be found on the viral RNA, a feature absent during the transcription^{148,218}.

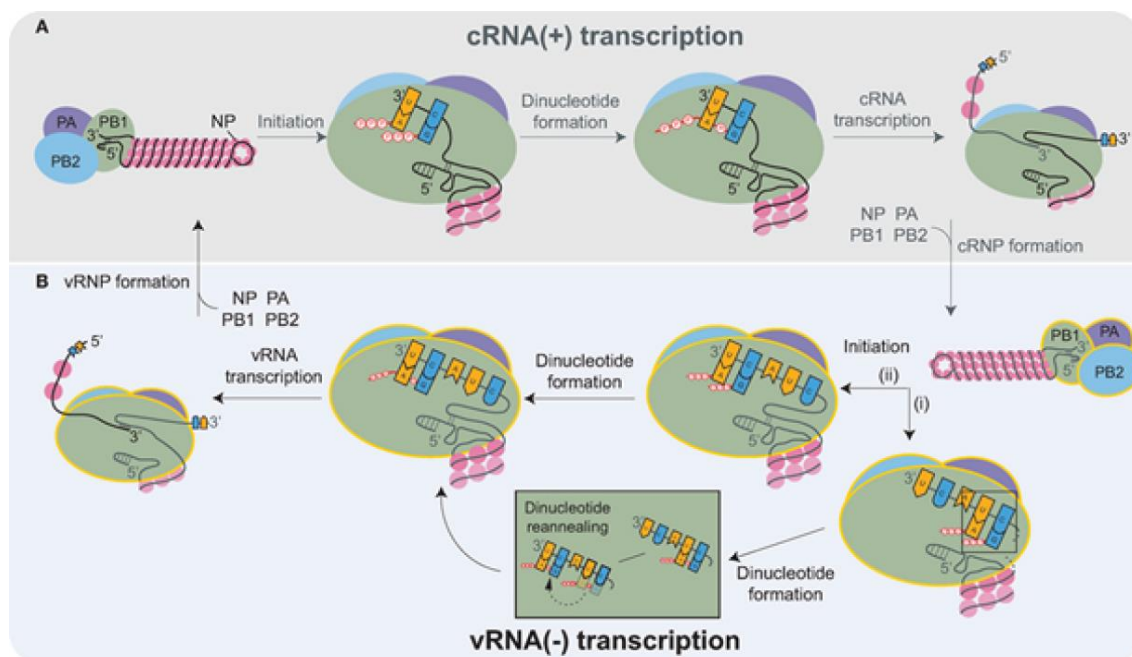


Figure 12: Replication of the influenza genome.

The replication occurs in two steps: **(A)** the viral replication machinery transcribes cRNAs and promotes cRNPs assembly followed by **(B)** the transcription of vRNAs. Adapted from⁶⁵.

The balance between transcription and replication is not understood. However, the current model suggests that the switch from the former to the latter is not clearly cut but rather a progressive transition. The polymerase dimerization is thought to play an important role in the replication, and more particularly, in the synthesis of negative-sense RNAs from positive sense RNAs^{75,378}. The NP:polymerase interaction is also a viral factor putatively playing a part in this process. Investigations of the role of several

cellular factors such as the cellular PolIII and cellular capped-mRNA have been carried out ³⁷¹. A second model emerged during the last decade, based on coexisting replication *in trans* and transcription *in cis*, where the newly synthesized RdRps would perform the replication while the RdRp imported with the viral genome as RNPs would do the transcription ^{83,372}. In spite of huge breakthroughs this decade, further work is required to dissect completely this particular transition.

II. Nucleocytoplasmic shuttling of proteins

In cells, a huge number of different chemical and biological processes occur. Compartmentalization is a key feature to separate them, leading to a higher efficiency and allowing distinct specific functions to be carried out at the same time ³⁰⁷.

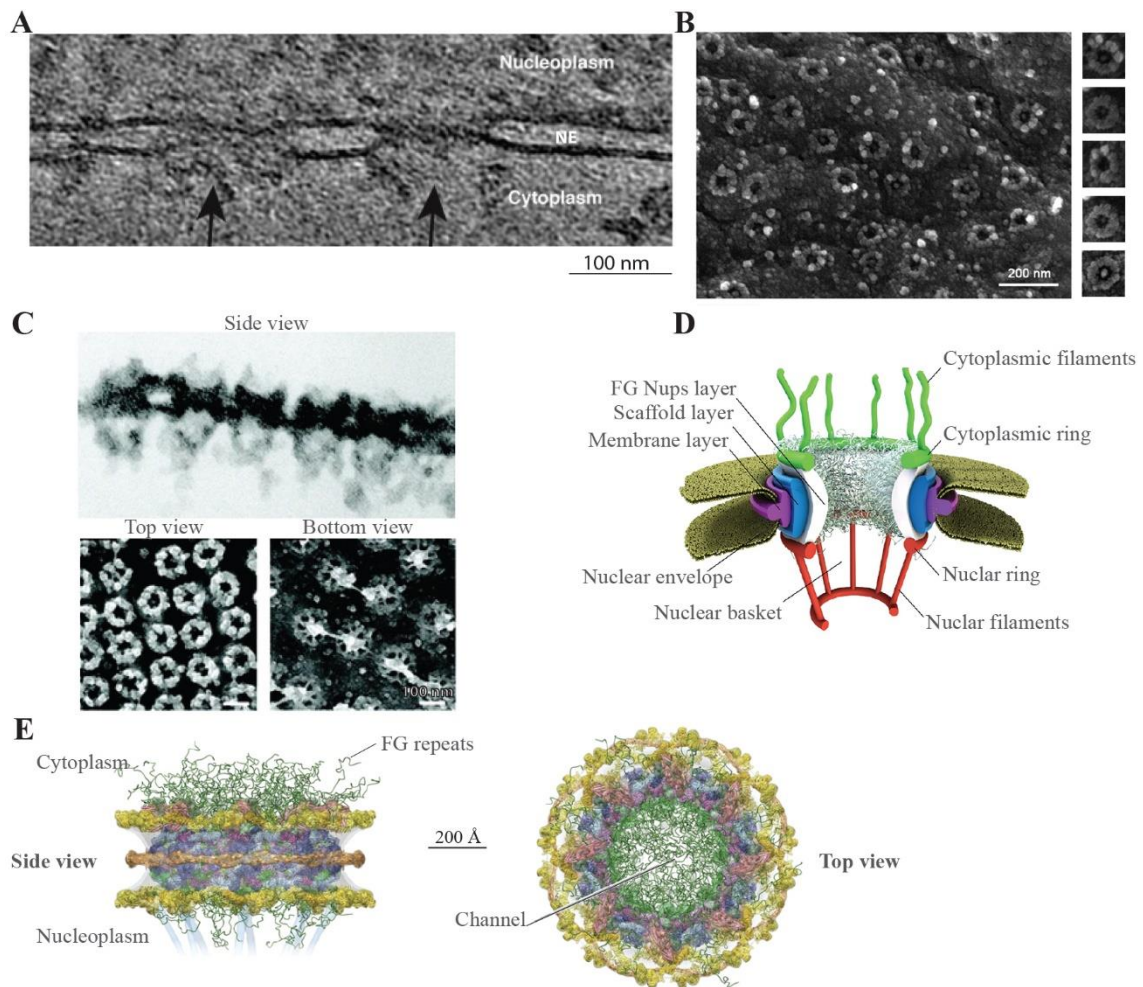


Figure 13: Nuclear pore complex (NPC).

(A) Picture of tomographic slices of HeLa cell nuclear envelope with NPCs identified with black arrows. NE stands for nuclear envelope. Adapted from ³⁰. (B) Field emission scanning electron microscopy pictures of COS-1 cell nuclei. Adapted from ³²⁶. (C) Transmission electron microscopy (TEM) pictures of the nuclear envelope with NPCs. Cytoplasmic filaments and nuclear baskets can be seen respectively in the top and bottom view (cellular/nuclear TEM pictures) as well as in the side view picture. Adapted from ⁷⁴. (D) Schematic view of the NPC. Adapted from ¹⁰. (E) Structure of the NPC with modelled disordered FG-repeats. Adapted from ¹⁶⁰.

The physical separation, either membrane-based or protein-based, is commonly found in eukaryotes and to an increasing extent in prokaryotes^{52,59,92}. One example is the nucleus, a compartment containing the largest part of the genetic information, housing the gene regulation, the RNA processing and the transcription. While the driving force behind the emergence of this particular organelle is still unclear, several hypotheses are currently debated, most of them agreeing to an efficiency and integrity advantage^{142,191,206,397}. In contrast with other membrane-based organelles, in eukaryotes, the nucleus is separated from the cytoplasm by two membranes (**Fig. 13A**). A way to cross this physical barrier is through a large macromolecular complex acting as a channel, called the nuclear pore complex (NPC) (**Fig. 13**)^{56,78,81}.

This complex, conserved across all eukaryotes, is made of several hundred copies of around thirty distinct nucleoporins and reaches a molecular weight of about 60 MDa in yeast, up to about 120 MDa in vertebrates (**Fig. 13E**)^{4,201,290}. Nucleoporins localized in the NPC channel are enriched in disordered phenylalanine-glycine repeats (FG repeats), represent almost half of the molecular weight of the NPC and are essential for the restriction of the NPC permeability and for the active transport (**Fig. 13D and 13E**)³⁰⁶. The NPC shows an eight-fold symmetry, with a diameter and a height estimated at 100-150 nm and 30-80 nm respectively, from yeast to vertebrates (**Fig 13**)^{4,306,410}.

It is of note that a single cell possesses between several hundred to thousands NPCs and the composition of these NPCs varies depending on the cell type or maybe even within one single cell^{95,194,212,258,287,320}.

A. Nuclear transport pathways

The NPC allows ions, small molecules and proteins (usually <30-60 kDa) to diffuse freely through the 40 nm wide channel. Some features of this passive diffusion are still debated, such as the existence of a clearly cut size threshold of 40 kDa or a progressive threshold^{30,156,263,264,360}. However, for bigger proteins, active transport by nuclear transport receptors is often required, although some proteins could directly interact with the FG-repeats localized in the channel, allowing a transporter-independent nuclear translocation^{50,129,172,173,266}. The most studied transporter-dependent pathway is the system involving the small GTPase Ran and the importins-

α/β nuclear transport receptors^{1,219,241}. The nuclear transport can also be mediated by the cytoskeleton, which allows a faster nuclear delivery³². Some molecules to be translocated (called cargo molecules) can be imported by alternative transport receptors, like lectins⁶⁹. The cargo translocation can also be calcium-dependent, modulated by the calmodulin¹¹³.

Several pathways exist for the import of cargo molecules that seem to be able to use more than one system, likely in order to prevent the complete blockade of any essential metabolic and/or regulatory pathways if one transport system is impaired.

B. The nuclear transport pathway based on Ran and karyopherins

i. The Ras-like small GTPase Ran

The keystone of the Ras-like small GTPase Ran system is based on the RanGDP/RanGTP gradient existing between the cytosol and the nucleus^{103,139}.

Two nucleoside-bound forms of Ran coexist: the GDP-bound Ran form, mostly found in the cytosol and the GTP-bound form abundant in the nucleus. Like the majority of small GTPases, the GTP hydrolysis mediated by Ran is very slow. Two proteins, RanGAP and RanBP1 are found in the cytosol to accelerate the GTP hydrolysis^{22,211}. Following the gradient, the GDP-bound Ran ends up in the nucleus. Another partner of Ran, the chromatin-associated RCC1 (also called RanGEF), is localized in the nucleus and induces nucleoside exchange from GDP to GTP²². Once more in the GTP-bound form, Ran is redirected towards the cytosol by the gradient.

These differential concentrations of GTP and GDP due to Ran partners lead to the gradient, the shuttling of both Ran forms and drive the bidirectionality of the transport.

ii. The nuclear import and export: importins- α , importins- β , and exportins

The Ran import system relies on nuclear transport receptors (**Fig. 14**). Import and export receptors, usually members of the karyopherin superfamily, use distinct nucleoside-bound forms of Ran, to make this system efficient⁴⁹.

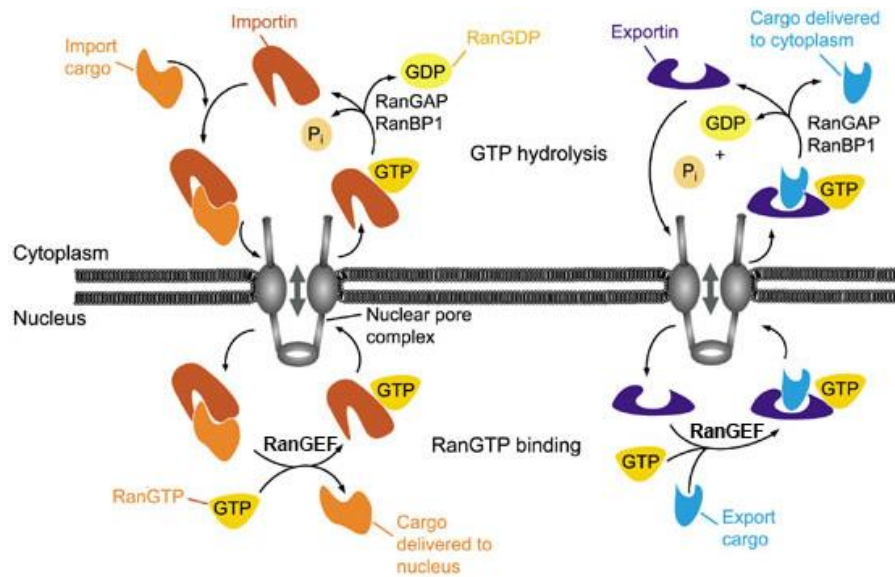


Figure 14: The nuclear transport pathway based on Ran.

The small GTPase Ran mediates two nuclear transport processes: the nuclear import via the importins- α/β and the nuclear export relying on the exportins. Adapted from ³⁴².

The nuclear export is mediated by proteins called exportins that interact with the GTP-bound form of Ran. These exportins recognize specifically their cargo through a specific signal called Nuclear Export Signal (NES), usually made of hydrophobic residues ^{109,339}. The most known and studied is the leucine-rich NES ¹⁷⁶.

The nuclear import is mediated by importins, and more particularly importins- α and importins- β . Importins- β require the GDP-bound form of Ran for the efficient import of its cargo, as RanGTP induces the cargo dissociation, and can directly bind the cargo for its transport ^{102,295}. In some cases, adapter proteins, namely importins- α , are required. They are able to interact with the cargo through a specific signal called a Nuclear Localization Signal (NLS) and with the importin- β through an autoinhibitory importin- β binding (IBB) domain that is otherwise folded into the importin- α NLS binding pockets ¹⁰¹. Upon the hydrolysis of GTP and the availability of importins- α/β and the cargo, a likely cooperative, synergic mechanism allows the formation of a complex between the importin- β , the importin- α and the cargo (or more directly between an importin- β and a cargo with a specific NLS) ^{193,373}. As the NLS binding pockets of the importin- α are free from the IBB domain, bound by the importin- β , the cargo can be loaded onto the dimeric transport complex and translocated into the nucleus. The transport through the NPC channel is mediated by transient interactions between the

importin- β and the FG-repeats of nucleoporins, although the precise mechanism is still debated ^{18,104}. The release of the cargo in the nucleus relies on the conformational change of the importin- β induced by a RanGDP-to-GTP exchange, along with the IBB domain autoinhibition and the nucleoporins contribution ^{100,114,185,186}.

iii. The nuclear localization and export signals

NLSs can be split into two families: the conventional/classical (cNLS) and the non-conventional/classical NLS (ncNLS) ^{123,167,181,406}.

Table 4: Reference NLS sequences and consensus sequences for 6 classes of conventional and nonconventional basic NLSs interacting with importins- α .

Basic residues are represented in blue. The red X can be replaced by any residue. Z stands for any amino acids but Asp and Glu. * Most optimal sequences of bipartite NLSs show a central linker region enriched with acidic residues and terminal regions enriched with Pro and basic residues. Adapted from ¹⁶⁷.

Reference NLS	Sequences
Monopartite SV40 Large T Antigen NLS	PKKKRKV
Bipartite nucleoplasmin NLS	KRPAATKKAGQAKKKK
NLS class	Consensus sequences
Class 1	1/ KR(K/R)R 2/ K(K/R)RK
Class 2	(P/R)XXKR(Z)(K/R)
Class 3	KRX(W/F/Y)XXAF
Class 4	(R/P)XXKR(K/R)(Z)
Class 5	LGKR(K/R)(W/F/Y)
Bipartite	1/ KRX ₁₀₋₁₂ K(K/R)(K/R)* 2/ KRX ₁₀₋₁₂ K(K/R)X(K/R)*

The most detailed NLSs belong to the classical NLS group and are usually made of small patches of basic residues, rich in arginine and lysine. This group presents two types of basic patches: monopartite and bipartite NLSs. The first NLSs identified in the monopartite and bipartite categories are respectively the Simian Virus (SV) 40 Large T antigen and the nucleoplasmin NLSs (**Table 4**) ^{61,152,299,332}. The discovery of these NLSs was the starting step to determine the consensus sequences, albeit loose ones (**Table 4**). Monopartite NLSs present a single basic patch separated in diverse classes and consensus sequences ¹⁶⁷. Bipartite NLSs present two highly basic patches separated by a linker of 10 to 12 residues with a consensus sequence usually KRX₁₀₋

$_{12}K(K/R)X(K/R)$ or $KRX_{10-12}K(K/R)(K/R)$. Longer linker sequences have also been described^{86,180}.

In addition, some NLSs do not show this basic feature but rather a hydrophobic pattern interacting with the importin- α using the same binding pockets as the basic NLS^{38,399}. The classical NLSs require the importin- β -bound importin- α adapter protein to be translocated, while non-classical NLSs tend to bypass these proteins and to interact directly with importins- β . Such atypical cargos can present a proline-tyrosine rich sequence (PY NLS)^{366,406}. PY NLSs are not as studied as basic NLSs but work is done towards their characterization. They tend to be longer, disordered and more variable. Identifying NLSs is a tricky task, also due to the fact that some NLSs are not linear but conformational^{6,161}.

The dissections of nuclear localization signals have been greatly improved these last decades but the full story is not completely clear, with few other mechanisms and NLSs still investigated³³⁷. With several thousand cargos requiring nuclear translocation in a timely manner and new putative motifs identified recently, it is to be expected that some discreet patterns still elude discovery¹⁶¹.

C. Similarities and specificities of importins- α

Importins are highly conserved through evolution (cf. **Annexe 1**)²⁰⁹. Whereas the yeast possesses only one importin- α and 14 importins- β , the human genome encodes as much as seven forms of importins- α and at least ten importins- β involved in nuclear import^{41,256,342}.

The seven human importins- α are split in three groups (**Fig. 15A**)²⁸². The $\alpha 1$ clade includes the importin- $\alpha 1$ and - $\alpha 8$, which are the more divergent, with a sequence identity of 53 %. The $\alpha 2$ clade includes the importin- $\alpha 3$ and the importin- $\alpha 4$ that present a strong sequence identity (86 %). The last clade ($\alpha 3$) includes the importin- $\alpha 5$, - $\alpha 6$ and - $\alpha 7$. These importins- α are well conserved, with sequence identities between 79 % and 84 %.

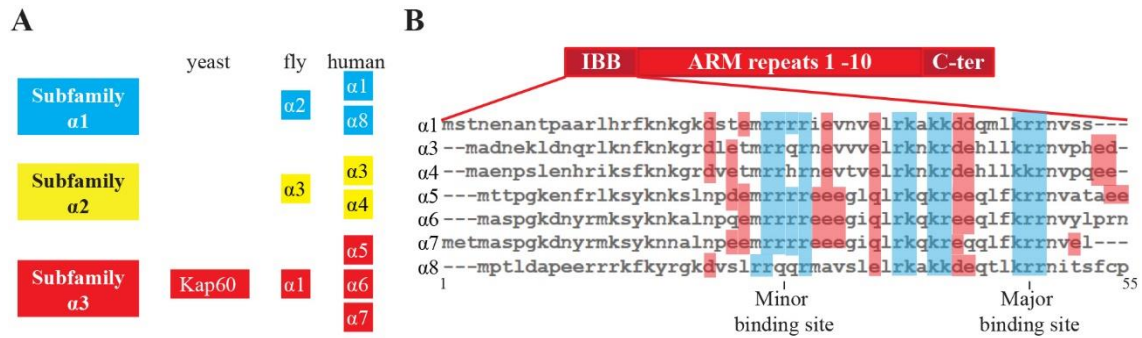


Figure 15: Evolution, diversification and global organization of importins- α .

(A) Diversity and subfamilies of importins- α in yeast, fly and human. Blue, yellow and red correspond respectively to the $\alpha 1$, $\alpha 2$ and $\alpha 3$ subfamilies. Inspired from ²⁸². (B) Schematic organization of importins- α and importin- β binding (IBB) domain alignment. Basic and acidic charged residues of the NLS-like sequence are respectively in blue and red. Basic patches binding to NLS binding pockets are identified as 'minor binding site' and 'major binding site', accordingly to the auto-inhibited state. Sequences and accession numbers were recovered from NCBI/Uniprot databases for human (*Homo sapiens*) importins- α (cf. **Annexe 3**).

All importins- α present a common, conserved architecture (**Fig. 15B**) ¹⁶⁴. They are crescent-shaped, rather flat proteins, composed by one N-terminal IBB domain (with a NLS-like sequence), responsible for the auto-inhibition, the binding to the importin- β and the cargo release in the nucleus ^{44,114,193}. The IBB domain is made of two basic patches separated by an acidic linker sequence of around 15 residues and flanked by acidic residues. Following this flexible domain, importins- α are composed by 10 armadillo (ARM) repeats, each one composed of repeats of hydrophobic residues forming three α -helices. The C-terminal part is an acidic patch, responsible for the nuclear export of the cargo-free importin- α by the Cellular Apoptosis Susceptibility protein (CAS) ¹⁷⁵. The best-characterized importin- β , the importin- $\beta 1$, on the other hand, is structured by 19 HEAT repeats, with each repeat composed by two α -helices. However, other importins- β present a variable number of HEAT repeats: for example, RanBP5 is composed of 24 HEAT repeats ³⁴⁵. While ARM and HEAT repeats are ubiquitous, closely related structures, the latter type of repeats confers a higher degree of flexibility, while ARM repeats result in a more rigid structure ^{5,154,283}.

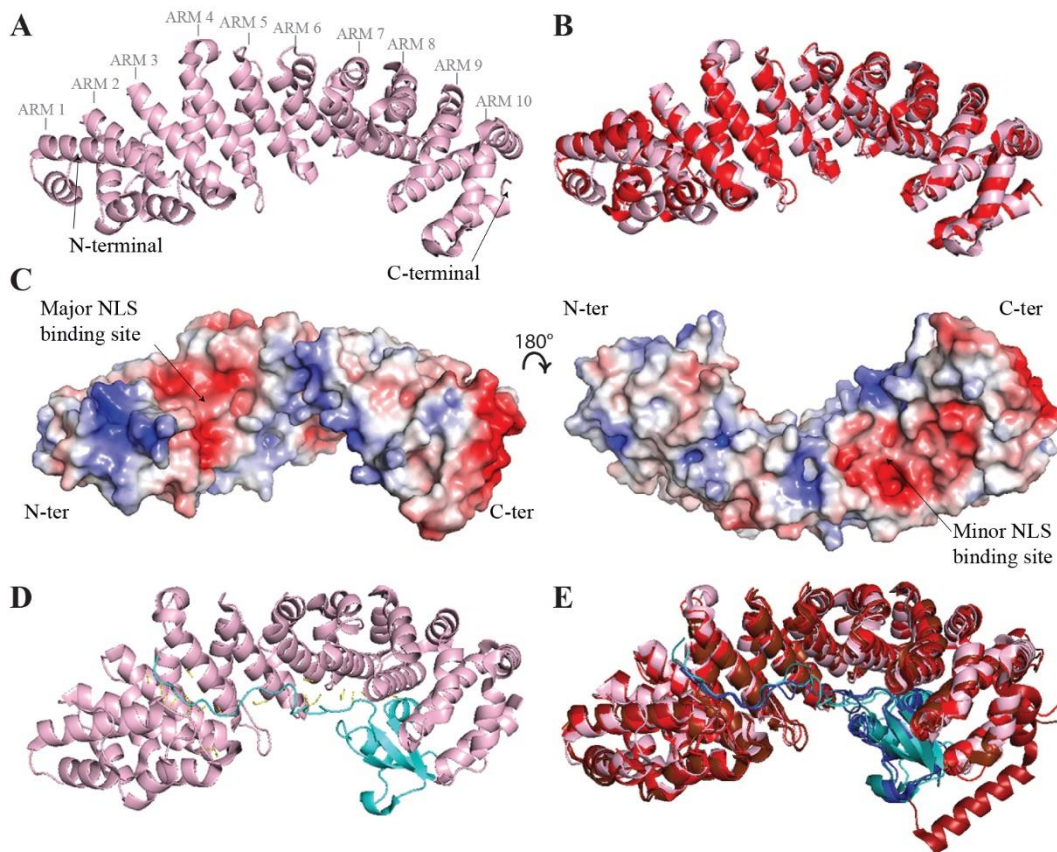


Figure 16: Structure of importins- α .

(A) Crystal structure of Δ IBB importin- α 1 in the free state (PDB 3WPT). (B) Structural comparison of Δ IBB importin- α 1 (light pink) and Δ IBB importin- α 3 (PDB 6BVZ) (red) in their cargo-free state. (C) Δ IBB importin- α 1 represented as surface with its electrostatic potential and NLS binding pockets. (D) Crystal structure of Δ IBB importin- α 1 in complex with influenza PB2 NLS (PDB 4UAF), bound to both NLS binding sites. (E) Structural comparison of complexes involving PB2 NLS with Δ IBB importin- α 1 (cyan with light pink), Δ IBB importin- α 3 (PDB 4UAE) (deep teal with red), Δ IBB importin- α 5 (PDB 2JDQ) (blue with dark red) and Δ IBB importin- α 7 (PDB 4UAD) (dark blue with chocolate).

Importins- α present two NLS-binding pockets. The major binding pocket, localized over the ARM repeats 2 to 4, is the interaction site for most of the monopartite NLSs with sites to accommodate five NLS residues. The minor binding pocket possesses four binding positions and is part of the ARM repeats 6 to 8. Bipartite NLSs tend to associate with both binding sites (**Fig. 16**)⁴⁸. Interestingly, some NLSs tend to interact so strongly with importins- α that they can directly compete with the IBB domain and take its place, without any contribution of the importin- β . Crystallographic structures of importins- α show few differences between paralogs and while they possess some sort of flexibility, it is not to the extent of the one of importins- β , which goes through major structural changes (**Fig. 16B and 16E**)²⁸³.

Despite these shared features, importins- α are not interchangeable proteins. While it has been shown that cargoes are able to interact with several paralogs of importins- α , they possess a preferential adapter protein^{91,286}. Similarly, importins- α also show preferential binding to specific cargoes^{166,389}.

An interesting specificity of importins- α comes from their biochemical properties. It has been shown that the importin- $\alpha 3$ is unusually flexible for an importin- α , but far from the flexibility of importins- β ²⁸³. It has been proposed that this peculiar importin- α was conserved through evolution for fast nuclear translocation, and/or for interacting with specific cargoes, with less common NLS or possessing a globular domain close by, requiring the adapter to curve and bend to bind it efficiently³¹⁴. The importin- $\alpha 3$ seems less well adapted to interact with classical monopartite NLSs. In contrast, the importin- $\alpha 1$ appears rigid and tends to better interact with monopartite NLSs. The importin- $\alpha 7$ seems to be more flexible than the importin- $\alpha 1$ but not as much as the importin- $\alpha 3$ ²⁸³. For other importins- α , such analysis has not been carried out yet.

Another specificity is the strength of the importin- α auto-inhibition. It has been found that the importin- $\alpha 1$ presents the strongest auto-inhibition while the importin- $\alpha 3$ auto-inhibition is the weakest; the importin- $\alpha 7$ auto-inhibition seems in between^{217,283}. This is partly because subtle differences exist between paralogs, more particularly in the NLS sequence of the IBB domain, and in the flanking regions that are more or less acidic (**Fig. 15B**).

Importins- α are expressed in all cell types and tissues to some extent, the only exceptions being the importins- $\alpha 6$ and - $\alpha 8$, which are more restricted respectively to the testis and to the ovaries. However, some tissues and cell types are enriched in one or several importin- α paralogs, and importins- α themselves are not homogeneously expressed. For example, based on the Human Protein Atlas, the importins- $\alpha 7$ is highly expressed in a large number of tissues while the importin- $\alpha 1$ is moderately expressed in most tissues, sometimes highly, lowly or not expressed at all^{357,367}. This is particularly interesting when put in a more cellular context, with importins- α specificities towards cargoes. For example, the importin- $\alpha 3$ seems to be the most expressed importin- α in the mammalian respiratory tract, followed by the importin- $\alpha 7$, while importin- $\alpha 1$ is lowly expressed in most respiratory tissues^{246,358}. In contrast, the importin- $\alpha 1$ is highly expressed in most types of cancers and as most of the research

is based on immortalized and/or cancer cell lines, caution is required for the design and the interpretation of studies involving such an importin and such cells ¹¹².

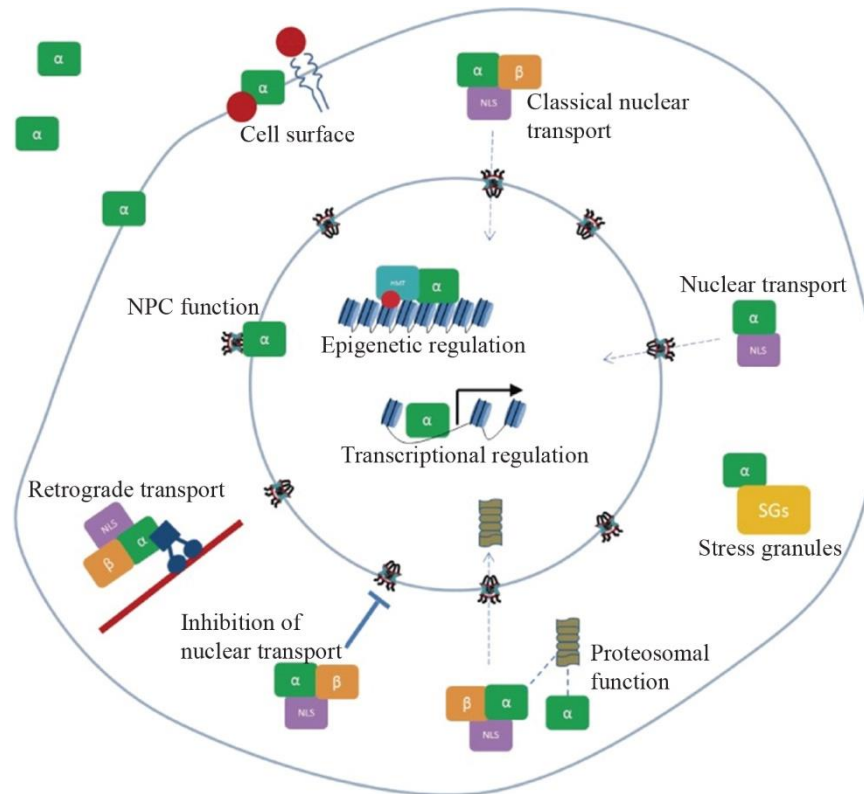


Figure 17: Diversity of the roles played by importins-α in cells.

Besides their well-known role in nuclear transport, importins-α are involved in numerous other processes in cells, as regulatory proteins or in trafficking. Adapted from ²⁵⁶.

In addition to their role in the nuclear import, importins play other, more discreet parts in the cell life (**Fig. 17**). The importin-α has been reported for sequestering a cargo in the cytoplasm, adding another layer for the regulation of nuclear processes ^{87,322}. Importins-α are expressed in a development-dependent, cell type-dependent manner: the regulation of the nuclear import through the importins-α differential expression is one of the switches used during the cell development, the differentiation and the aging ^{153,165,354,364,413}. It is of note that knockout or knockdown of specific importins-α often lead to issues in reproduction and development. Such a defect can be lethal ^{220,305}. Importins-α can also be found in an active form on the cell surface of a few cancer lines, where they putatively can bind to growth factors and transport them to the nucleus ²⁵⁶. Importins play essential roles during the mitosis, for the regulation

of NLS-bearing proteins and for the spindle assembly^{107,395}. They can also control the gene expression, by transporting transcription factors or by binding DNA during various stresses or in cancer cell lines^{19,412}. Their role as cytoplasmic chaperones has been investigated, to prevent the aggregation of highly basic proteins, although only a few importins- β have been identified yet¹⁴⁰.

D. *In vitro* and *in cellulo* interaction parameters of the complex between cargo and importins: affinity and rates

A number of studies focused on the molecular basis for the interaction between importins and cargo proteins. While the *in cellulo* affinity, thermodynamics and rates of interactions eluded researchers for a long time, dissecting these parameters was more doable using *in vitro* characterization with purified partners. Most of the current knowledge comes from a large number of biochemical, biophysical and structural methods, which bring precious information about these interactions. *In vitro* characterizations highlighted that all partners in this complex nuclear import pathway (*i.e.* cargo proteins, importins, NPC proteins) have a surprisingly high affinity for each other. Interactions between cargo proteins and IBB-truncated importins, between importin- α and importin- β or between importins and nucleoporins typically yield a dissociation constant in the nanomolar range^{18,35,76,192,356}. Δ IBB importins- α often show a nanomolar affinity for NLSs, with sometimes a low nanomolar affinity to bipartite NLSs or a low micromolar affinity. Some cargo proteins are even able to compete with the IBB domain. It has been shown previously that bipartite NLSs can displace the NLS-like sequence of the IBB domain in the absence of the importin- β ^{190,283}. The same bipartite NLSs tend to interact strongly (K_d around 10 nM) with several importins- α *in vitro*, regardless of their cargo specificities. In mouse, the affinity between the importin- β and the importin- α 2 was measured at around 15 nM while the affinity for the sole IBB domain was 7 nM. Pre-associated importin- β :importin- α 2 complex binds to the SV40 Large T-Antigen and nucleoplasmin NLSs with a dissociation constant of 40 nM³⁵. A functional NLS is expected to possess an affinity *in vitro* in the nanomolar range. Outside of this *in vitro* range, the cargo would be too tightly or too loosely bound to its transport receptor.

As it has been shown that the affinity correlates well with the nuclear localization, the majority of studies realized on importin: cargo complexes rely on the dissociation constant obtained *in vitro* to assess the efficiency and functionality of the nuclear import *in cellulo*¹²². However, while this relationship is still true, it simplifies to an extreme the relationship between the transporter and its cargo. As in all interactions, the strength is an insightful parameter but the rates are equally important^{20,51}. The rates of association and dissociation are the parameters determining the affinity, and they give precious insights on the half-life of a complex. This is what makes the difference between an efficient and an unproductive interaction: too slow or too fast an interaction leads either way to an issue in addressing the right partner to the right location on time, to carry out its fundamental function.

The major issue with dissociation constants determined *in vitro* for this kind of interaction comes from the fact that a nanomolar affinity correlates with a complex half-life of several minutes³⁵⁶. However, with the nucleocytoplasmic shuttling of importins being around 30 milliseconds, this raises questions about the results obtained *in vitro* in light of what can be observed *in cellulo*^{170,411}. These last few years, studies have been tackling this issue. Several recent studies showed that the cell environment considerably affects the nuclear transport^{33,34,356,361}. Looking at importins: nucleoporins or importins: cargos interactions, it has been shown *in vitro* that non-specific binding, even in the form of extremely diluted bacterial lysate, drastically reduced the affinity between partners. It has been estimated that the environment contribution to nuclear transport interactions is similar to a specific interaction in the micromolar range with a non-specific competition in the millimolar range^{33,361}. These estimations are this time much more in agreement with the nuclear import rates, as the half-life of such a complex is in the millisecond range.

E. Nuclear import regulation through cellular machinery

i. Importin- α :importin- β 1 sub-complex versus importins- β as transport receptors

Importin- β transport receptors are perfectly able to bind and translocate cargo proteins by themselves, without importins- α . The direct interaction of importins- β with

cargo leads to faster rates and a more efficient nuclear localization than when the importin- α is required ²⁹⁷. Furthermore, the importin- α -mediated nuclear import is energy-consuming: while one nucleocytoplasmic cycle relying on importins- β requires the hydrolysis of one GTP to GDP, if importin- α is required to play its part as an adapter, the shuttling cost increases to 2 GTP-to-GDP hydrolyses, for importin- α recycling. This raises the question of the conservation and the generation of new importins- α through evolution.

It has been shown that, despite these drawbacks of using importins- α in nuclear import, they have their own advantages: it adds a supplementary, larger layer of regulation and control and offers a broader, preferential choice for cargo loading and delivery depending on the cellular requirements and conditions ^{296,297}.

ii. Post-translational modifications

As mentioned previously, the nuclear import is a multi-layered, highly regulated process. Another obvious and easy way to achieve this level of control is through post-translational modifications (PTMs) on transport receptors, cargo proteins or even NPC proteins.

Phosphorylation is the most described PTM and is heavily involved in transport regulation. It is well documented that the phosphorylation of cargos can improve or worsen the affinity for an importin and consequently the nuclear delivery ^{236,355}. In extreme cases, it can completely abolish the interaction or unmask a NLS. Methylation, acetylation, ubiquitination and SUMOylation have also been reported to regulate cargos:transporters assembly, similarly to phosphorylation ^{17,281,355,382}. For instance, acetylation of an importin- α seems to enhance its binding to importin- β and Ran is regulated in almost all its functions by this PTM ^{12,163}. All of these PTMs are not simply dichotomic; they can be inhibiting or activating modifications, with the most diverse mechanisms. PTMs of the NPC proteins are equally diverse and extensive: phosphorylation, acetylation, ubiquitination, SUMOylation, O- and N-glycosylation have been reported ¹⁶. They are involved in NPC permeability, assembly and disassembly during mitosis.

It is to be expected that other, less-studied PTMs are likely regulating the nuclear import, and further work is required to continue to shed light on this complex mechanism.

iii. Other regulatory mechanisms

Cytoskeleton networks and more particularly microtubules and associated transporters are able to regulate this specific nuclear trafficking pathway and are involved in viral nuclear trafficking^{221,304}. Interestingly, unusual regulatory pathways are also involved in nucleocytoplasmic transport, such as lipids signalling or NLS/NES proteolysis^{14,77}.

III. Nuclear import of the influenza replication machinery

While influenza viruses are amongst the only RNA viruses performing replication and transcription in the nucleus, several RNA viruses require some sort of nuclear targeting, for replication and/or immune regulation purposes ⁹. Therefore, an efficient interaction with the cellular nuclear import machinery is one of the keystones for the viral cycle and is determinant for virulence and fitness ²⁹². The influenza replication machinery requires nuclear import at two major points of the viral cycle: firstly in the RNPs context and secondly for the newly-synthesized replication machinery proteins. PB1, PB2 and NP interact with either importins- α or importins- β , and PA is required for successful nuclear import of PB1. They present basic patches, a sign of potential NLSs (**Table 5**). Studies have long try to dissect this particular host:pathogen interaction, but many questions are still unanswered ^{205,239,257}.

Table 5: NLS sequences of the influenza A replication machinery.

Basic residues are represented in blue.

Protein	NLS type	Sequences
A/NP	ncNLS	NLS1: ³ T KGT KRSYEQM ¹³
	Bipartite NLS	NLS2: ¹⁹⁸ KRGIND RNFWR GENGR RTR ²¹⁶
A/PB1	Bipartite NLS?	¹⁸⁷ RKRR VRDNMT KKM V TQ R TIG KRK Q R ²¹¹
A/PB2	Bipartite NLS	NLS1: ⁴⁵⁰ GIESIDNVMGMIGILPDMTPSTEMSM R GV RISK MGVDEYSSAE
		KIV ⁴⁹⁵ NLS2: ⁷³⁶ KRKR NSSILTDSQTAT KRIR MAIN ⁷⁵⁹

A. Nucleoprotein

The nuclear import of NP and its interaction with importins- α (**Fig. 18**) are likely one of the most extensively studied of all influenza replication machinery proteins.

Two basic clusters have been identified in A/NP as putative NLSs. The first one (NLS1) is localized on the flexible N-ter NP_{TAIL}, within the 20 first residues, is considered to be part of the category of non-conventional NLS and binds to the minor binding pocket of importins- α ^{215,239,257,381}. The second (NLS2), considered as a bipartite NLS, is localized in a loop, spanning residues 198 to 216 ^{261,386}. However its role is unclear:

NLS2 basic patches are structurally too close to constitute a platform for importin binding, NLS2 is not easily accessible and mutagenesis did not prevent nuclear import, only nucleolar localization^{53,243,414}. A/NP interacts with several paralogs of importins- α , namely importin- α 1, - α 3, - α 5 and - α 7^{93,215,257,294,381}. The crystal structure and characterization of the A/NP_{TAIL} in complex with the mouse Δ IBB importin- α 1 shows that the A/NP_{TAIL} binds to the minor NLS binding pocket of importins- α with a weak affinity for the importin- α 1, from 1 to 5 μ M^{232,403}. A/NP NLS2 showed an even lower affinity, with a dissociation constant of 72 μ M. The synergic behaviour of both NLSs has been suggested to explain the functionality of the nuclear import at such low affinities⁴⁰³. This last hypothesis is still debated. The sole NP_{TAIL} is also suggested as the unique A/NP NLS: while the NLS on the A/NP_{TAIL} presents a weak affinity for importin- α 1, it seems more plausible for it to be the A/NP NLS than the internal putative NLS sequence.

B/NP, C/NP and D/NP do not seem to present an internal basic NLS sequence. However, the N-terminal flexible tail of B/NP, while longer (71 amino acids), presents several basic residues across its sequence (**Fig. 7**). No classical NLS pattern could be predicted from the sequence alone, but works realized during this PhD thesis validated the B/NP_{TAIL} NLS activity. B/NP interacts slightly more strongly with importin- α 7, with an affinity of 844 nM. Interestingly, a long stretch of residues is involved in the interaction, from residue 30 to 71¹⁷⁷.

In the meantime, it has been shown that the C-terminal flexible tail of C/NP and D/NP (C/NP_{TAIL} and D/NP_{TAIL}) are also highly basic, possess what seems to be clearly bipartite NLSs, and are able to bind to importins- α (**Fig. 7**). The C/NP_{TAIL} is able to interact with importin- α 1, - α 3, - α 4, - α 5 and - α 7 and its affinity for importin- α 1 is around 50 nM, a dissociation constant in complete agreement with previously characterized functional NLSs³⁵⁰. Point mutations of the second patch of basic residues (residues 546-549) impaired dramatically the nuclear import and the affinity for importin- α . Structural and nuclear import insights have been gained on D/NP during this PhD and will be addressed in the Chapter 2.

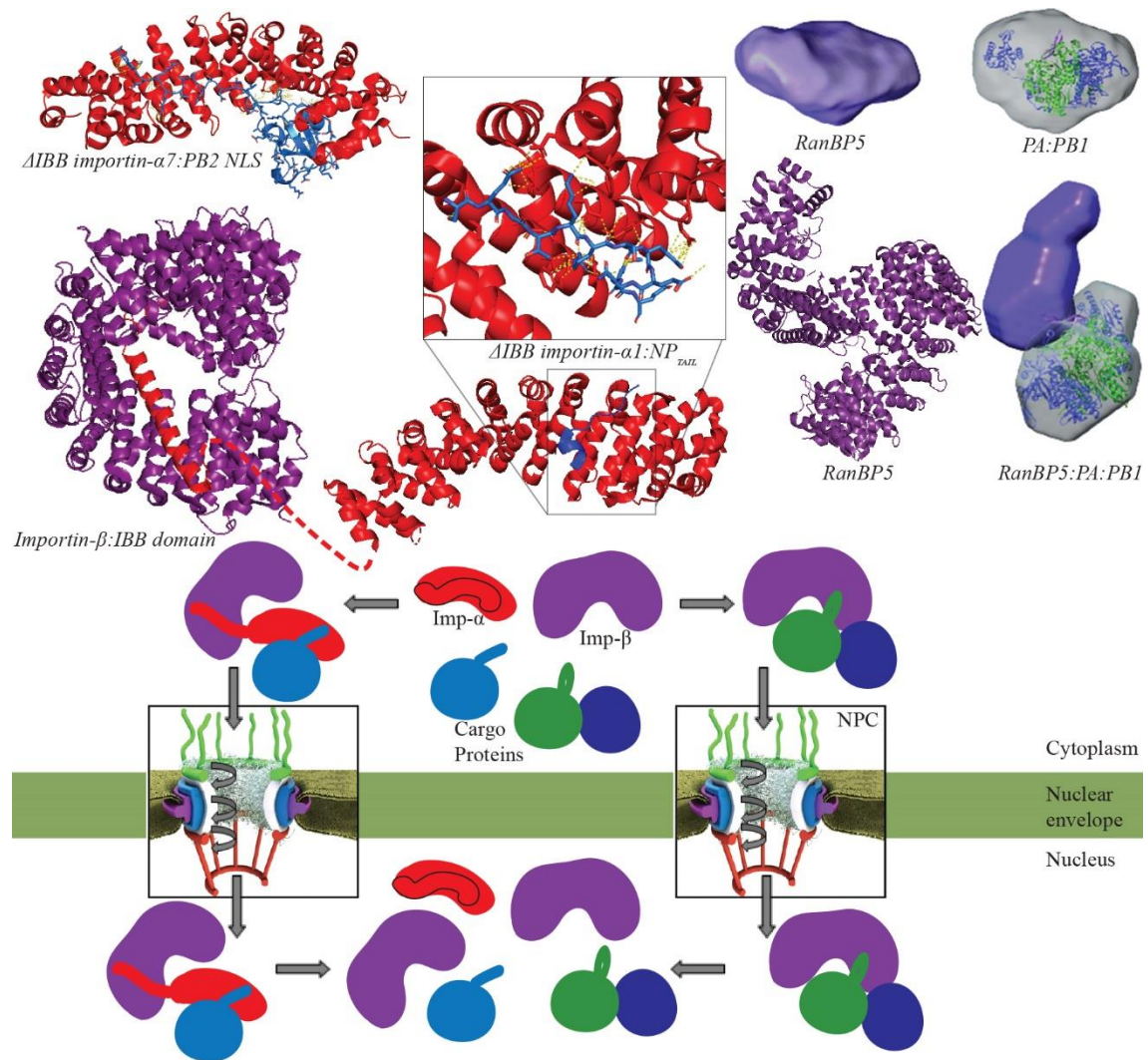


Figure 18: Summary of the nuclear import of the influenza replication machinery.

Schematic representation of nuclear import of influenza NP and RdRp subunits along with crystal structures of RanBP5 (PDB 6XTE), Δ IBB importin- α 7:PB2 NLS (PDB 4UAD), Δ IBB importin- α 1:NP NLS (PDB 4ZDU) and of importin- β :IBB (PDB 1QGK) complexes. SAXS envelopes were adapted from ³⁴⁶. Schematic representation of NPC was adapted from ¹⁰.

The affinities determined so far of A/NP and B/NP for importins- α are too low to lead to an efficient nuclear transport. Another mechanism and/or partner is clearly still eluding discovery to explain the nuclear import functionality observed *in cellulo* during the infection course.

It is of note that most of the work to determine *in vitro* affinities between importins- α and NPs is realized using only peptides -whether the NP_{TAIL} or the putative internal A/NP NLS peptide-, which is both an essential step and a bias. It is now known that for some cargos, both the NLS sequence and the 3-dimensional context are important.

NP is not different: other A/NP residues such as amino acids 102, 105 and 375 are able to mutate upon adaptation to Δ importin- α 7 mice, to improve the binding to other importin paralogs, such as importin- α 3 in the case of residues 102 and 375 ²⁹¹. The interaction modelling showed that at least another non-NLS interface exists in the importin- α :NP interaction.

It is well known now that the A/NP nuclear import is regulated by phosphorylation: phosphorylation at residues Ser-9 or Tyr-10 inhibits the nuclear import while phosphorylation at the position Tyr-296 and Thr-188 leads to a nuclear retention ^{189,421}. While the B/NP phosphorylation function has not been studied yet, it has been shown that B/NP is also phosphorylated, at positions structurally conserved with A/NP ¹³⁰. The nuclear translocation and/or retention could be regulated by SUMOylation at Lys-7 and putatively at Lys-4 ¹¹¹.

Several cellular partners have also been identified as able to sequester NP in the cytoplasm, by preventing the interaction with importins ^{60,196,418}.

The nuclear import of influenza NPs is slowly unravelled, but a number of questions remain unanswered. Along with the highly divergent NLSs exhibited by distinct NP type, it is expected that what is found for one influenza NP will likely not be transposable to the other NPs (cf. **Annexe 2**). Work is still required to completely understand this essential process in the viral cycle.

B. Heterotrimeric RNA-dependent RNA polymerase

The influenza RdRp is constituted by 3 subunits, each one requiring to be imported in the nucleus upon synthesis. The current scientific consensus is that PB2 is imported by importins- α while PA and PB1 are imported as a subcomplex by the importin- β RanBP5 (**Fig. 17**) ¹³¹.

PB2 is able to interact with several Δ IBB importins- α (importin- α 1, - α 3, - α 5 and - α 7) with a strong affinity (1-10 nM), due to its bipartite NLS ²⁴. Two regions are involved in the nuclear localization: the essential bipartite motif spanning the residues 737-759 and a longer sequence between residues 449 and 495 ²²⁴. The mutation of each residue from residue 736 to residue 739 (KRKR motif) results in a cytoplasmic localization, while the deletion of the 449-495 region leads to a perinuclear localization.

Interestingly, the bipartite NLS is localized in a folded C-terminal domain and unfold to bind efficiently to importins ³⁵¹. The NLS binds importins- α in an extended conformation.

Several mutations in PB2 are known to impact importin- α bindings. The E627K and D701N mutations are well-known in the bird-to-mammal adaptation, by improving the nuclear import and binding to the mammalian importin- α 1 ^{93,293,341}. The E627K mutation is also involved in the nuclear import, but more likely by a structural change, not affecting the binding affinity but the protein context, acting as a positive feature for the importin- α 1 and importin- α 7 specificity ¹²⁷. R702K and S714R mutations also play a part in the importin binding, increasing the association rate of the complex, thus increasing the binding affinity to importin- α 1 ²⁴.

The interaction between PA:PB1 and RanBP5 is less well understood. PA and PB1 both present NLSs but the nuclear import seems to be mediated by the PB1 NLS ^{3,237,244,245,333}. PB1 possesses a bipartite NLS on two protruding β -strands, spanning the residues 187-190 and 207-211, interacting with RanBP5 ^{57,133}. The absence of PA or the mutation of the PA linker in the PA:PB1 subcomplex leads to an impaired nuclear accumulation ^{55,85}. Getting insight in the binding affinity has not been achieved yet, but the RanBP5 binding interface has been recently determined: the PB1 NLS could bind as a secondary structure to RanBP5, through RanBP5 residues 362, 405, 486, 489, 490, 528 and 529 ³⁴⁵.

Interestingly, the use of these distinct nuclear import pathways by PA:PB1 and PB2 could very well prevent the assembly of the polymerase in the cytoplasm, where the replication and the transcription would be unproductive ^{57,128,346}.

C. Ribonucleoproteins

The nuclear import of ribonucleoproteins, and viral genomes in general, is an event still poorly understood. However, it has been shown that RNPs go through the NPC to gain access to the nucleus and use the importin- α - β cellular nuclear import machinery ^{157,205,257}. Interactions between RNPs and the NPC, and the NPC crossing are not spontaneous events, with dissociation rates ranging from 1 s to 100 s. They are also regulated, for example with the binding prevented at later infection times by M1 ¹¹. The

exact mechanism behind the RNP nuclear import is still a mystery; it is well known that the NPC permeability is variable and modified during the cell life course but whether the 7-8 RNPs are transported together to dissociate in the nucleus or the individual RNPs are translocated after dissociation is unknown. One RNP is a long and large structure but it could be small enough to pass through the NPC if constrained and kept straight; such a feat could probably not occur if RNPs are translocated as a bundle. At late infection times, the nuclear export of RNPs is facilitated by the cellular caspase activation that enlarges NPCs; mechanical stress is known to induce breaks and enlargements in NPCs, so maybe a similar mechanism or something along the line of mechanical stress exists for the nuclear import ^{82,223}.

It is currently admitted that the RNP translocation is mediated by the NP_{TAIL} NLS, which is readily accessible in the RNP context ^{7,53,106,257,350,404,405}. With the large number of NP coating the vRNA, it is estimated that a large number of NP_{TAILS} are available to interact with importins- α ³⁶². Such a high NLS density and high avidity could explain the fast nuclear accumulation. However, it is still not known precisely how the avidity plays a part in the RNP nuclear import or if multiple importins- α mediate the nuclear translocation.

Objectives of the PhD thesis

The nucleoprotein is a central protein of influenza viruses, essential for many processes, from transcription/replication to cellular trafficking, viral genome protection and viral escape. Yet, many of its features are still shrouded in mysteries.

The nuclear import of the viral genome is most likely mediated by NP that had to mediate its own trafficking by supposedly intrinsically disordered extremities. A/NP_{TAIL} has been the most studied, and yet showed a weak binding to the cellular nuclear import factors, the importins- α (K_d 1-5 μ M)^{93,215,232,239,257,294,381,403}. Similarly, B/NP_{TAIL} interacts with the importin- α 7 with an affinity in the same range (*i.e.* 844 nM) of the one of A/NP_{TAIL}¹⁷⁷. In contrast, C/NP_{TAIL} showed a functional binding to the importins- α , but the study was limited to a thorough characterization with only one importin- α , the importin- α 1 (48 nM)³⁵⁰. D/NP_{TAIL} dissection was initiated before my arrival in the team, and finalized during this PhD work. All of these NP_{TAILS} are extremely conserved within one influenza type, but highly divergent between types, suggesting that their behaviour with importins- α is a specific and an optimized feature (*cf.* **Annexe 2**). Beyond these differences in affinities and partners, another issue comes from the fact that distinct methods and techniques had been used to get these insights, making any comparison a difficult process. The metagenomics studies from these last few years showed that the understanding of the *Orthomyxoviridae* family and of the influenza viruses is in fact rather limited: the discovery of several new influenza-like viruses was an extraordinary opportunity to understand the differences, the specificities and the evolution processes at work.

The first objective of my work was to get functional data on the rates and affinities of the complexes between the D/NP_{TAIL} and the importin- α 7 with a new strategy that was planned to be set-up for all types of NP:importins- α interactions. Once done, this strategy was then extended to the interaction between all the influenza NP_{TAILS} and all the cellular partners previously identified, namely the importin- α 1, - α 3, - α 5 and α 7, and maybe later on to the other proteins of the influenza replication machinery. This allows for the first time to compare and exactly characterize which importin- α could be a preferential partner of each NP_{TAIL}, and to dissect more precisely the features, similarities and specificities of all these partners.

The second aim was to get structural and functional data on the NPs of two recently discovered influenza viruses: the influenza D virus and the Wuhan Asiatic toad

influenza virus. The influenza D virus is more closely related to the influenza C virus, infects a broad spectrum of mammals and its reservoir seems to be the cattle ⁸. The Wuhan Asiatic toad influenza virus is more closely related to influenza B viruses and has been isolated from a toad ³²⁹. Detailing firstly the nuclear import function, along with the other specific functions of the influenza NP, provide precious information on the role and behaviour of NPs, along with important insights in the evolutionary relationship within the influenza family.

The results obtained during this PhD project revealed how differently each influenza NP was behaving, more particularly in the interaction with the cellular import machinery despite their common structural and functional basis, and to what extent the importins- α were individual entities in this context, able to pick a specific, preferential cargo amongst others, in contrast with the conventional view on these adapters.

Chapter 2: Structural analysis of the influenza D nucleoprotein and characterization of its nuclear localization signal

In 2011, a new influenza virus affecting the cattle was discovered, while it was still believed to be spared by the influenza virus infections¹¹⁷. Initially thought to be a C-like influenza virus, further experiments lead to its classification as a new type of influenza virus in 2013, the influenza D virus (IDV)¹¹⁶. In the following years, most of the research on this new virus were focused on epidemiologic, pathologic and phylogenetic analyses. It was found to be distributed worldwide with a high seroprevalence in the cattle and able to infect a diversity of mammal species^{68,116,117,285}. IDV does not seem to cause any severe disease on its own, only when associated with other pathogens^{68,216}. Currently, two distinct lineages circulate⁸.

More recent studies are now focusing on the IDV proteins. The first IDV protein studied was the surface glycoprotein, HEF, followed by NS1^{251,335}.

In this paper, we carried out a biochemical and structural analysis of this novel influenza NP, using X-rays crystallography, electron microscopy, circular dichroism, SEC, SEC-MALLS analysis, thermal shift assays, fluorescence anisotropy (FA) and surface plasmon resonance (SPR). Insights in the structure, the oligomerization, the RNA- and the importin- α - binding processes were obtained and highlighted both D/NP common features with the other influenza NPs and its own specificities.

D/NP forms tetramers in solution, and, in contrast with A/NP and B/NP, its oligomeric state could not be affected by modifying the salt concentration. The crystal structure of D/NP was solved at 2.4 Å resolution. A/NP, B/NP and D/NP structures are strikingly well-conserved, with a R.M.S.D. value between 1.6 and 2.1 Å. In contrast with A/NP and B/NP, which possess a flexible, NLS-bearing, intrinsically disordered N-terminal NP_{TAIL}, D/NP shows such a disordered extension in C-terminal. D/NP_{TAIL} is responsible for the binding to the importin- α 7 with about a 10-fold stronger affinity (100 nM), compared to A/NP. D/NP_{TAIL} contains a clear bipartite nuclear localization signal with two basic patches (residues 514-516 and 531-535). The NP truncations of the C-terminal basic patch (residue 529-552), drastically impaired the *in vitro* importin- α binding as well as the *in cellulo* nuclear import while the deletion of both basic patches (residues 511-552), completely abolished the two processes.

This paper highlights some of the conserved features of influenza NPs, such as the overall conserved structure, the mechanism for the nuclear transport and the cellular

localization, along with more specific features, such as the C-terminal localization of its bipartite nuclear localization signal and its strong affinity for the importin- α 7.

Article: The structure of the nucleoprotein of Influenza D shows that all Orthomyxoviridae nucleoproteins have a similar NPcore with or without a NPtail for nuclear transport

Donchet A., Oliva J., Labaronne A., Tengo L., Miloudi M., Gerard F.C.A., Mas C., Schoehn G., Ruigrok R.W.H., Ducatez M. and Crépin T.
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The structure of the nucleoprotein of Influenza D shows that all *Orthomyxoviridae* nucleoproteins have a similar NP_{CORE}, with or without a NP_{TAIL} for nuclear transport

Amélie Donchet¹, Justine Oliva², Alice Labaronne¹, Laura Tengo¹, Myriam Miloudi¹, Francine C.A. Gerard¹, Caroline Mas³, Guy Schoehn¹, Rob W.H. Ruigrok¹, Mariette Ducatez² & Thibaut Crépin¹

This paper focuses on the nucleoprotein (NP) of the newly identified member of the *Orthomyxoviridae* family, Influenza D virus. To date several X-ray structures of NP of Influenza A (A/NP) and B (B/NP) viruses and of infectious salmon anemia (ISA/NP) virus have been solved. Here we purified, characterized and solved the X-ray structure of the tetrameric D/NP at 2.4 Å resolution. The crystal structure of its core is similar to NP of other Influenza viruses. However, unlike A/NP and B/NP which possess a flexible amino-terminal tail containing nuclear localization signals (NLS) for their nuclear import, D/NP possesses a carboxy-terminal tail (D/NP_{TAIL}). We show that D/NP_{TAIL} harbors a bipartite NLS and designed C-terminal truncated mutants to demonstrate the role of D/NP_{TAIL} for nuclear transport.

In 2011, a virus was isolated from a pig with Influenza-like symptoms in Oklahoma (USA). Electron microscopy showed features of an Influenza virus particle and real-time RT-PCR revealed that it was neither an Influenza A virus (IAV) nor an Influenza B virus (IBV). Next-generation sequencing analyses allowed the identification of RNA segments with around 50% identity to human Influenza C virus (ICV). Further serological analyses showed that antibodies against this new virus failed to cross-react with IAV, IBV or ICV (1). All these results suggested it was a new genus of the *Orthomyxoviridae*, temporarily named *C/swine/Oklahoma/1334/2011* (*C/swine/OK*) and then Influenza D virus (IDV). IDV is widely distributed around the world; it was detected in North America¹⁻⁴, Asia⁵⁻⁷, Europe⁸⁻¹⁰ and Africa¹¹. A serological study in Nebraska (USA) found a seroprevalence of 80% in cattle serum from 2003³.

The *Orthomyxoviridae* family includes different *genera*, Influenza A, B, C and D viruses, infectious salmon anemia (ISA) virus, Thogoto virus, Quaranjavirus and others. These viruses are segmented negative strand RNA viruses. Their genomes are made of a set of RNA segments coated with multiple copies of the nucleoprotein (NP) and associated to the viral heterotrimeric polymerase. The number of vRNA segments is specific to each type of Influenza viruses and related to the number of glycoproteins at the surface of the viral particle, 8 segments for IAV and IBV, 7 for IVC and IVD and 6 for Thogoto virus. These ribonucleoproteins (RNPs) are competent for both transcription and replication. To date, several X-ray structures of NP have been published, each of them without RNA. There are three structures of Influenza A nucleoprotein (A/NP): two with NP assembled as a trimer^{12,13} and one for the monomeric R416A mutant¹⁴. The X-ray structure of the tetrameric Influenza B nucleoprotein (B/NP) is also known¹⁵, whereas the structure of Isavirus NP (ISA/NP) was solved as a dimer¹⁶. The overall folds of A/NP and B/NP are very similar with a root-mean-square deviation (rmsd) of 1.6 Å.

¹Institut de Biologie Structurale (IBS), Univ. Grenoble Alpes, CEA, CNRS, 38044, Grenoble, France. ²IHAP, Université de Toulouse, INRA, ENVT, Toulouse, France. ³Integrated Structural Biology Grenoble (ISBG) - UMS 3518 (CNRS-CEA-UJF-EMBL), 38044, Grenoble, France. Correspondence and requests for materials should be addressed to T.C. (email: thibaut.crepin@ibs.fr)

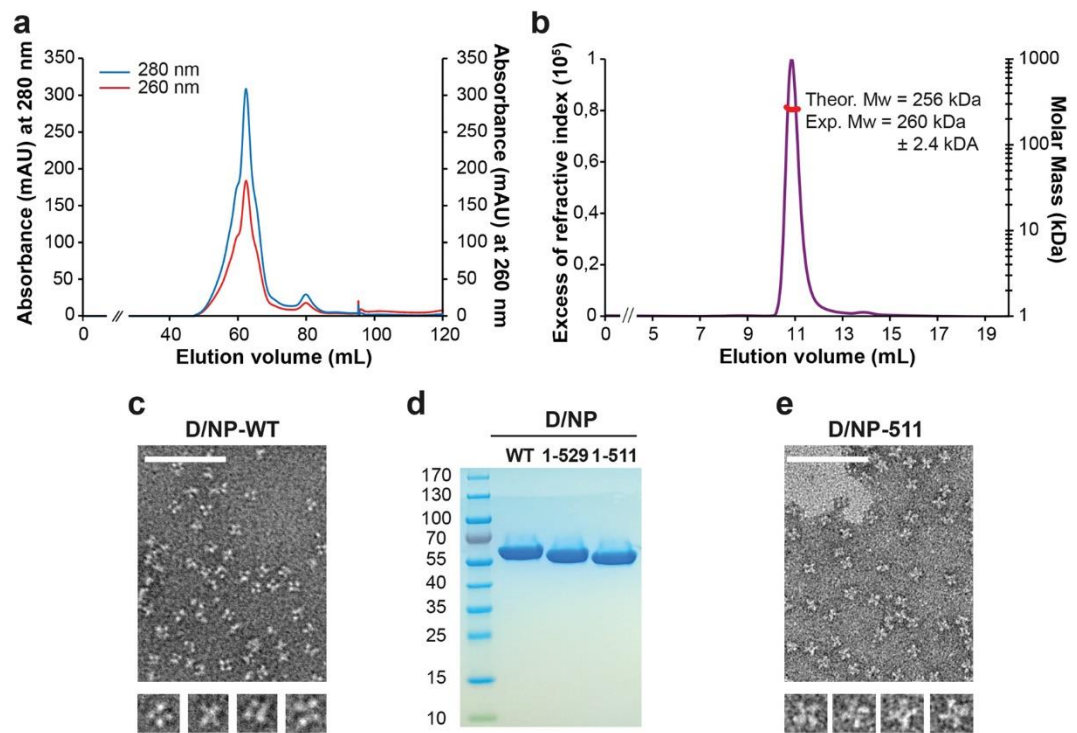


Figure 1. Purification and characterized of Influenza D nucleoprotein. **(a)** Size exclusion chromatography profile of wild-type D/NP. The sample was loaded on a Hiloal™ 16/600 S200 column equilibrated with the running buffer 20 mM Tris-HCl pH 7.5, 300 mM NaCl and 5 mM β -mercaptoethanol. **(b)** SEC-MALLS-RI analysis of D/NP. SEC was performed with a Superdex™ 200 increase 10/300 GL column equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM β -ME. The panel shows the theoretical Mw and the measured Mw. **(c)** and **(e)** Electron microscopy images of the elution peak of D/NP and D/NP-511. Samples show different oligomeric states although most oligomers are tetramers. The scale bar corresponds to 100 nm. **(d)** Coomassie blue-stained SDS-PAGE (4–20% gradient polyacrylamide) showing the purified wild-type D/NP and the two C-terminal truncated mutants (D/NP-529 and D/NP-511).

Influenza viruses transcribe and replicate in the nucleus of the infected cells and the NPs and the polymerase subunits need to interact with the nuclear transport system of the cell. For Influenza A and B viruses, several studies have shown that the nuclear localization signals (NLS) of NP recognized by the cellular importins- α , are located within the flexible N-terminal tail^{17–24}. Recently, crystal structures of the two NLSs of A/NP bound to importin- α have been solved^{25,26}.

In this paper, we characterized D/NP and solved the X-ray structure of its tetramer. The C-terminal D/NP_{TAIL} harbouring a classical bipartite nuclear localization signal (NLS) was not visible in the structure. We designed two C-terminal truncated mutants (D/NP-511 and D/NP-529) to study the interaction of D/NP with importin- α . Our biochemical experiments demonstrate that D/NP_{TAIL} is involved in the interaction with importins- α and immunofluorescence showed that the wild-type D/NP goes into the nucleus whereas the mutants stay in the cytoplasm.

Results

Recombinant D/NP forms a tetramer in solution. The DNA coding sequence of D/NP was cloned in a bacterial expression plasmid and over-expressed in *Escherichia coli* as a C-terminal His-tagged recombinant protein. D/NP was purified with a nickel affinity chromatography followed by a heparin column and a final gel filtration. Figure 1a shows a typical gel filtration elution profile using the absorbance signals at 260 and 280 nm (ratio 280/260 > 1.75). The protein could then be concentrated at 2 to 6 mg.mL⁻¹. Polyacrylamide gels and SEC-MALLS-RI experiments have confirmed the homogeneity and the molecular weight of the recombinant tetrameric D/NP (Fig. 1b,d). By electron microscopy (negative staining), we showed that D/NP forms mainly tetramers in solution (Fig. 1c). Previously, it was shown that the oligomerization of recombinant A/NP and B/NP can be modulated by the NaCl concentration^{14,18,27,28}. Starting from purified and stable oligomeric samples (trimers for A/NP and tetramers for B/NP), monomeric proteins can be obtained by decreasing stepwise the NaCl concentration. After purification at 300 mM NaCl followed by dialysis at 150 mM NaCl, D/NP was eluted from gel filtration (with a 150 mM NaCl running buffer) in the same volume, meaning that a smooth reduction of the salt concentration did not change its oligomeric state. However, a decrease to 50 mM NaCl induced an irreversible and total precipitation of D/NP, even with a stepwise reduction at 150 mM NaCl. Therefore, the experiments on D/NP and its mutants were carried out at 300 mM NaCl.

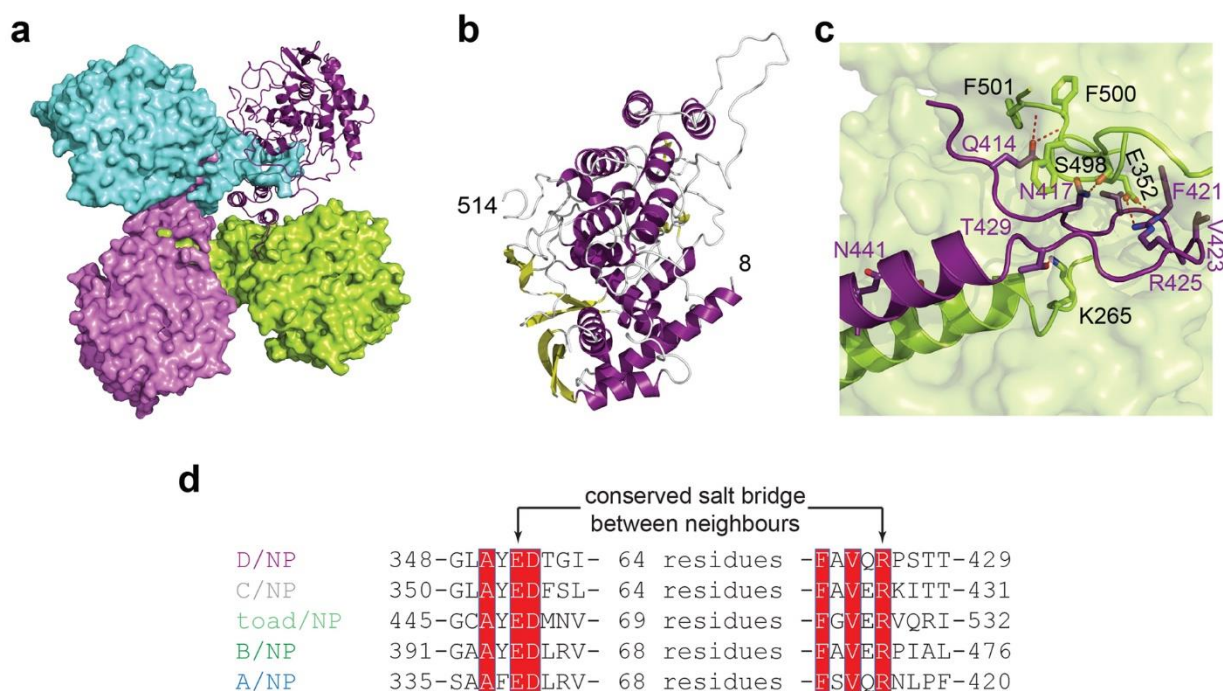


Figure 2. Structure of Influenza D nucleoprotein. **(a)** Structure of the tetrameric D/NP, with 3 protomers shown in surface (respectively in green, pink and cyan) and the fourth in cartoon (deep purple). **(b)** Cartoon representation of one monomer of D/NP with the α -helices in deep purple and β -strands in yellow. **(c)** Detail of the interactions between two protomers of the tetrameric D/NP as shown in **(a)**. The conserved R425 of one protomer (shown as deep purple cartoon) stabilized the position of the oligomerization loop at the surface of the neighbouring protomer, through its conserved E352. **(d)** Sequence alignment of the salt bridges of the oligomerization-loop of one protomer to the NP_{CORE} of the neighbour protomers. For the sequences see Table 1.

	A/NP	B/NP	toad/NP	C/NP	D/NP	ISA/NP
A/NP		1.6 Å (383 C α)	—	—	2.1 Å (356 C α)	2.9 Å (340 C α)
B/NP	38%		—	—	1.9 Å (365 C α)	2.8 Å (340 C α)
toad/NP	25%	30%			—	—
C/NP	22%	25%	24%		—	—
D/NP	24%	25%	23%	38%		2.9 Å (330 C α)
ISA/NP	20%	18%	18%	16%	20%	
Tho/NP	18%	21%	20%	18%	17%	20%
WfB/NP	20%	19%	19%	20%	18%	17%

Table 1. Nucleoproteins of Orthomyxoviruses. A/NP, Influenza A virus nucleoprotein, strain A/WSN/1933(H1N1) (Uniprot accession number B4URF1, PDB entry 2IQH); B/NP, Influenza B virus nucleoprotein, strain B/Managua/4577.01/2008 (Uniprot accession number C4LQ26, PDB entry 3TJ0); Toad/NP, Wuhan asiatic toad Influenza virus nucleoprotein (GenBank accession number AVM87634); C/NP, Influenza C virus nucleoprotein, strain C/AnnArbor/1/1950 (Uniprot accession number Q6I7C0); D/NP, Influenza D virus nucleoprotein, strain D/bovine/France/2986/2012 (Uniprot accession number A0A0E3VZU8, PDB entry 5N2U); ISA/NP, infectious salmon anemia virus nucleoprotein, isolate salmon/Norway/810/9/99 (Uniprot accession number Q8V3T7, PDB entry 4EWC); Tho/NP, Thogoto virus nucleoprotein (Uniprot accession number A0A0B6VKB5); WfB/NP, Wellfleet Bay virus nucleoprotein (Uniprot accession number A0A0A1E9N5). Sequence identities were obtained using MUSCLE⁷⁷ and structure comparisons were calculated using PDBeFold⁷⁸.

Like the nucleoproteins of A, B and ISA, D/NP bound single-stranded RNA. We measured the K_d of 14 nM with a fluorescence anisotropy assay with an RNA of 24 nucleotides of polyUC labeled in 3' with 6-fluorescein amidite (FAM)²⁷. The K_d of 14 nM can be compared with the K_d s of 7 nM of the trimer of A/NP, 31 nM of the tetramer of B/NP²⁹ and 24 nM for the dimer of ISA/NP¹⁶.

Structure of D/NP. Full length D/NP was crystallized in sodium malonate as small fine needles that diffract X-rays up to 2.4 Å resolution (Supplementary Table 1). The structure was solved by molecular replacement using a starting model of the monomer of the A/NP R416A mutant¹⁴. The automatic search gave the position for three

molecules and a fourth protomer was fitted manually after the analysis of the electron density. The molecular replacement using the tetrameric B/NP model did not give any acceptable solutions. The asymmetric unit contained four molecules arranged as a tetramer (Fig. 2a and refinement statistics in Supplementary Table 1), consistent with the observations made by electron microscopy. Based on the electron density, 87% of the model could be built, from residues 8 to 514 (Fig. 2b). Several internal loops were missing and the C-terminal 50 residues were disordered. The oligomerization-loop of one protomer plugged into a cavity of its neighbour (Fig. 2c). The N-terminal part of the loop started with a strictly conserved glutamate residue (Gln-414) that interacted with the backbone of the strictly conserved consecutive aromatic residues $_{499}\text{FFF}_{501}$. The loop was then stabilized by several kind of contacts; mainly hydrophobic (by the side chain of the strictly conserved Phe-421 and Val-423) and of several aromatic residues lying in the pocket of the neighbouring monomer but also the salt bridge between an arginine from one monomer (Arg-425) interacting with a glutamate of the neighbour (Glu-352; Fig. 2c,d). These two residues, also strictly conserved in all Influenza NPs (Fig. 2d and Supplementary Fig. 1), are well known for modulating their oligomeric state. Once mutated in alanine, the corresponding single mutants formed monomers, unable to self-assemble^{13,14,28,30–33}. Phosphorylation state of A/NP has been shown to also be a key factor of NP/NP interfaces, especially for positions Ser-165 and Ser-407^{34,35}. Sequence alignment showed that D/NP contains a threonine (Thr-161) and a serine (Ser-416) respectively, suggesting a conserved mechanism for the regulation of the oligomerization (Supplementary Fig. 1).

Core structure of all nucleoproteins (NP_{CORE}) with tails (NP_{TAIL}). The overall folds of A/NP, B/NP, ISA/NP and D/NP were very similar (Fig. 3a) with root-mean-square deviations (rmsd) between 1.6 Å (for 383 C α for the comparison A/NP with B/NP) and 2.9 Å (for 340 C α for the comparison D/NP with ISA/NP, Table 1). Including ISA/NP, a common architecture for all Influenza-like NPs can be defined based on the X-ray structures with the NP_{CORE} starting with the first α -helix for A/NP, B/NP and D/NP and finishing with three hydrophobic residues, anchored into the surface of the protein (Supplementary Fig. 1). Figure 3b shows the X-ray structures of A/NP, B/NP, D/NP and ISA/NP with a zoom on the C-terminal regions, where a superimposed patch made by three consecutive aromatic residues is found in Influenza NPs ($_{487}\text{YFF}_{489}$ for A/NP, $_{545}\text{FFF}_{547}$ for B/NP and $_{499}\text{FFF}_{501}$ for D/NP). A similar patch is also present in the ISA/NP model ($_{580}\text{GLF}_{582}$). The NP_{CORE} contains the large and shallow positively charged surface which might bind RNA (Fig. 3c), without sequence specificity (see above)^{12,13,36,37}. Twenty or 71 residues without any structure are found N-terminally before the cores of A/ and B/NP whereas for D/NP, the core starts with a very short version of an N-terminal tail (only 7 residues), which seems to be compensated by the presence of a carboxy-terminal NP_{TAIL} of 51 residues (Supplementary Fig. 1). The length of the NP_{TAILs} is variable and its location seems to be specific for each genus. Because D/NP differs from A/ and B/NP in its global organization, we decided to further analyse the C-terminal D/NP_{TAIL}.

D/NP_{TAIL} behaves as an intrinsically disordered protein. Based on the crystal structure and a disorder prediction (Fig. 4a), we designed a construct (from residues 505 to 552) for the expression in *E. coli* as an N-terminal His-tagged recombinant version. Considering that the core of D/NP ends after residue Phe-501, we have chosen to start the construct at Gly-505, in order to avoid the hydrophobicity of Phe-503 (Fig. 4a,b). D/NP_{TAIL} was eluted from gel filtration as a 40 kDa-protein (data not shown) but with a normal migration pattern on SDS-PAGE (Fig. 4b). As intrinsically disordered proteins are known to be eluted from gel filtration with an aberrant volume^{38,39}, a SEC-MALLS-RI experiment (Fig. 4c) confirmed the monodispersity of the purified sample with a Mw of 8.2 kDa for D/NP_{TAIL} (with the His-tag). A circular dichroism analysis confirmed that D/NP_{TAIL} did not contain any significant stable secondary structure in solution (Fig. 4d), even in presence of TMAO (Supplementary Fig. 2), a chemical known to force unfolded proteins to fold to native-like species with significant functional activity^{40–42}.

D/NP_{TAIL} interacts with importin- α . The N-terminal tails of A/NP and B/NP are known to be involved in the nuclear import by interacting with importins- α ^{17–19,21,23,25,43}. The sequence analysis of D/NP showed no import signals in its N-terminal part but suggested the presence of a bipartite NLS within D/NP_{TAIL} ($_{514}\text{KRR-X}_{14}\text{-KKRGR}_{535}$; Fig. 4a). We thus tested the bipartite NLS with different constructs. First, we showed that the human importin- $\alpha 7$ is co-eluted with the His-tagged D/NP_{TAIL} from gel filtration (Fig. 5a). The interaction was confirmed by thermal-shift experiments, where importin- $\alpha 7$ appeared more stable in presence of D/NP_{TAIL} (Fig. 5b). We also confirmed the interaction between the two partners using the full-length D/NP. Using surface plasmon resonance, we measured a *K_d* of 100 nM between immobilized D/NP_{TAIL} and importin- $\alpha 7$ (Fig. 5c), ten-fold higher than the affinities measured for the N-terminally A/ and B/NP_{TAILs}^{18,25}. By gel filtration, we showed that the D/NP:importin- $\alpha 7$ complex could be eluted as a single peak. A molecular weight of 474 kDa has been measured by SEC-MALLS-RI for the complex (Fig. 5d), corresponding to the interaction of four molecules of importins- $\alpha 7$ (Mw = 4 × 55 kDa) per tetramer of D/NP (Mw = 260 kDa). To confirm the role of D/NP_{TAIL} in the interaction with importin- α , we designed two C-terminal truncated constructs of D/NP, D/NP-511 and D/NP-529, ending respectively at residues 511 and 529. D/NP-511 is deprived of the whole bipartite NLS whereas D/NP-529 contains only the first part (Fig. 4a). They both behave as the wild-type protein during the purification (Fig. 1d,e). Using pull-down experiments on nickel-affinity resin, we showed that D/NP-529 still slightly retained importin- $\alpha 7$ whereas the interaction between D/NP-511 and importin- $\alpha 7$ was totally abolished (Fig. 5e). This suggests that importin- $\alpha 7$ binds to the NP_{TAIL} of D/NP.

Nuclear transport of D/NP and its mutants. First, human HEK 293T cells were infected with D/bovine/France/5920/2014 (*moi* of 5) and indirect immunofluorescence was used to localize D/NP within the cells. After 6 h, most of D/NP was observed in the nucleus (Fig. 6a). As a control, HEK 293T cells were infected with Influenza A/PR/8/34 and we found very similar results (data not shown). We then transfected HEK 293T

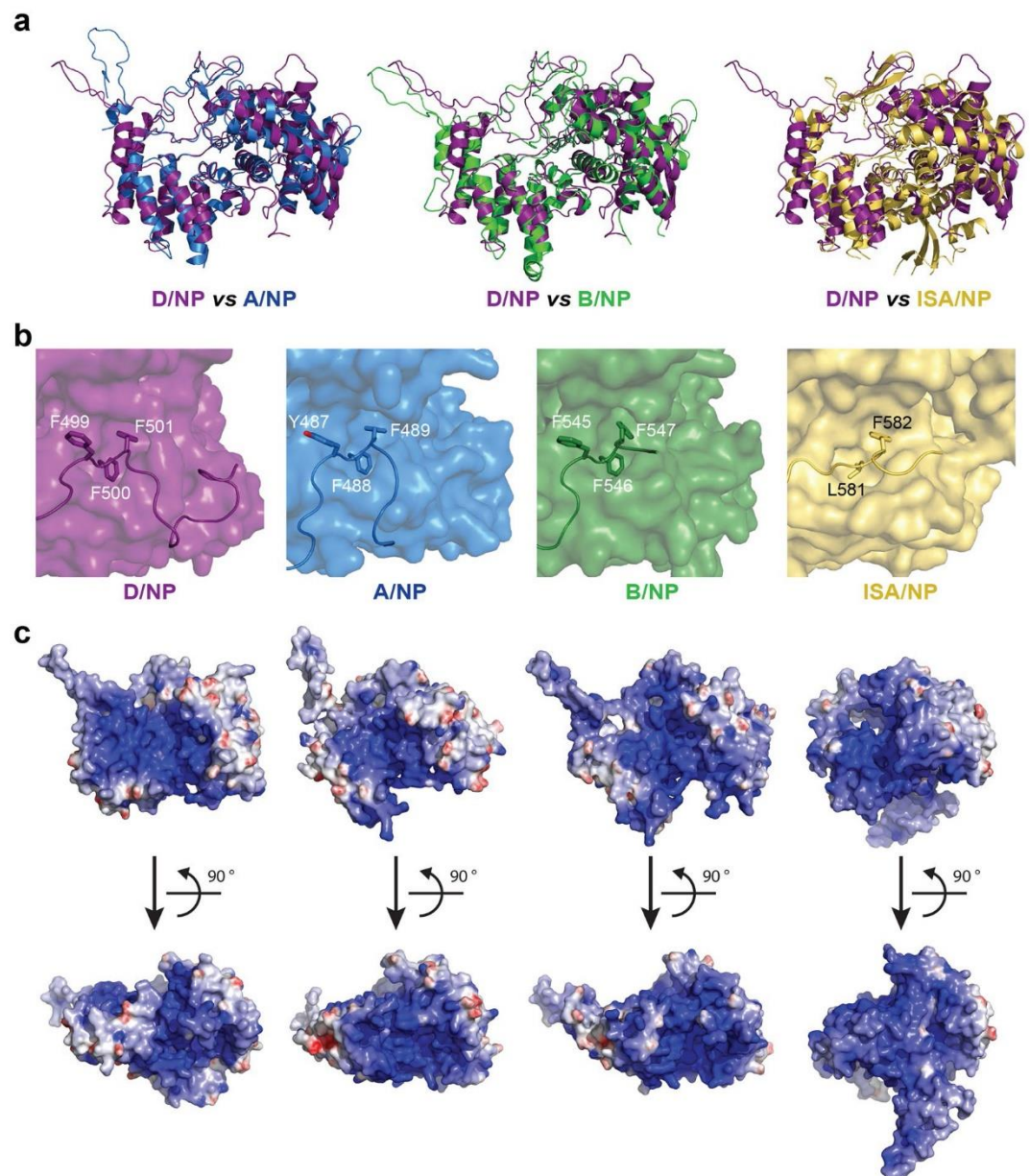


Figure 3. Comparison of D/NP with other segmented negative sRNA virus nucleoproteins. **(a)** The structure of one protomer of D/NP (deep purple) has been superimposed from left to right, with one protomer of A/NP (blue; PDB id: 2IQH), B/NP (forest; PDB id: 3TJ0) and ISA/NP (light orange; PDB id: 4EWC). The rmsd values are given in Table 1. **(b)** Anchoring of the C-terminus on NP_{CORE} by a patch of 3 consecutive aromatic residues. The panel corresponds to a zoom of the superimposed structures shown in panel A with NP_{CORE} represented in surface. **(c)** Electrostatic surface potentials of one protomer of D/NP, A/NP, B/NP and ISA/NP. The electrostatic surfaces were calculated from the crystal structures using DelPhi⁷⁵. The potential scales range from -10.0 kT/e (red) to 10.0 kT/e (blue).

cells with a plasmid containing wild-type D/NP and we found that D/NP can be localized in the cytoplasm but mainly in the nucleus (Fig. 6b). With this transfection strategy, D/NP-529 was located mainly in the cytoplasm with a little staining observed in the nucleus, whereas, D/NP-511 was located exclusively in the cytoplasm of the transfected cells (Fig. 6b), clearly showing that the cell used the bipartite NLS between residues 513 and 535 for the nuclear import of D/NP.

Discussion

In 2011, a new virus has been isolated from pigs exhibiting Influenza-like illness. Subsequent studies identified an *Orthomyxovirus* with seven RNA segments sharing 50% overall amino acids identity with the human Influenza C virus². First considered as a subtype of the Influenza C virus, it has since been officially named Influenza D virus (IDV) by the International Committee of Taxonomy of Viruses in 2016. Since its first isolation, IDV appears to

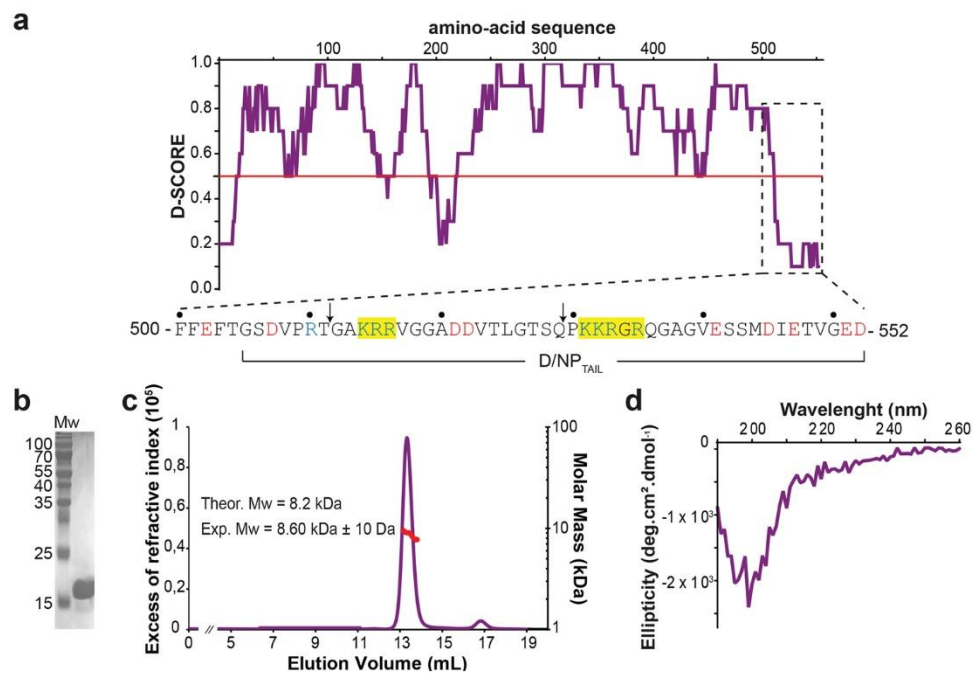


Figure 4. Biophysical characterization of D/NP_{TAIL}. **(a)** D-score (score for disorder as a function of residue) of D/NP with a zoom (below the graph) on the last 50 residues. The prediction is based on 22 predictor web servers and the D-score was calculated by adding the values for each residue and dividing by the number of used algorithms. We arbitrarily defined a threshold level at 0.50; residues with a D-score <0.50 were assigned as disordered³⁸. The yellow boxes on the sequence are to highlight the putative NLS motifs. The arrows indicate the sequences were cut for making D/NP-529 and D/NP-511. **(b)** Coomassie blue-stained SDS-PAGE (Tris-Tricine; 15% polyacrylamide) of the purified D/NP_{TAIL}. It migrates at a higher molecular weight (17 kDa approximately) than expected (8 kDa). **(c)** SEC-MALLS-RI analysis of D/NP_{TAIL} loaded on S75 10/300 GL column. For this experiment, we have chosen to keep the His-tag encoded with the pETM11 plasmid, for an optimal detection of D/NP_{TAIL} with UV. The experimental molecular weight is consistent with the expected mass. **(d)** Circular dichroism of D/NP_{TAIL}. CD is a biophysical method based on the polarization of light, used for a fast determination of the secondary structures within the proteins in solution. α -Helical proteins show negative bands at 222 nm and 208 nm and a positive band at 193 nm, proteins with well-defined antiparallel β -sheets have negative bands at 218 nm and positive bands at 195 nm and disordered proteins have very low ellipticity above 210 nm and negative bands near 195 nm.

circulate all around the world in many mammals, with cattle as a possible reservoir, and with serological data suggesting it may transmit to humans⁴⁴. The recent structure of IDV Hemagglutinin-Esterase-Fusion glycoprotein (HEF) has described an open receptor-binding cavity capable of accommodating diverse extended glycan moieties that could be one reason for its broad cell tropism⁴⁵.

With this paper, we provide a detailed characterization of IDV nucleoprotein. We determined the structure of the tetramer of D/NP and we failed to find the biochemical conditions to stabilize the monomer. For A and B/NP we had found that the monomer stays in solution at low salt, 50 mM NaCl^{14,27,28} but the D/NP protein makes aggregates in salt conditions lower than 150 mM. Post-translation changes like phosphorylation or ubiquitination may be able to stabilise the monomer, as it has been shown for A/NP^{14,34,46,47}. The phosphorylation on A and B/NP are summarized in Supplementary Fig. 1.

The comparison of the structures of the nucleoproteins of Influenza A, B, D and of infectious salmon anemia virus (ISAV) shows that of all these proteins share a common structural core (NP_{CORE}) that start with the first α -helix in the structure of A, B and D, up to three consecutive aromatic residues (Fig. 3 and Supplementary Fig. 1). Looking at the sequences of all known *Orthomyxoviruses* nucleoproteins shows that such hydrophobic patch is present in C/NP (501 FFF₅₀₃) and could be present in Tho/NP (451 YLF₄₅₃) and Wfb/NP (Wellfleet bay virus nucleoprotein; 532 VIY₅₃₄). The only structure that differs is the ISAV/NP that has a folded domain upstream of the first α -helix of the NP_{CORE} (Fig. 7). One could also see that Tho/NP is constituted only by the NP_{CORE} without any other appended sequences. NPs of IAV and IBV have an N-terminal NP_{TAIL} whereas the NPs of ICV and IDV have a C-terminal NP_{TAIL}. The NP_{TAIL} of A is only 20 residues whereas the tails of the other Influenza viruses NPs are much longer; 71 residues for IBV, 62 for ICV, 51 for IDV. Recently, new large-scale methods for finding RNA viruses in vertebrates other than mammals and birds, identified up to 240 “new” viruses⁴⁸. These authors found new Influenza viruses close to IBV, a Wuhan spiny eel Influenza virus and a Wuhan Asiatic toad Influenza virus. The NP for the toad virus (toad/NP) is very close to B/NP (identity of 30%, Table 1) with a very long N-terminal tail of 126 residues (Fig. 7 and Supplementary Fig. 1). Interestingly, toad/NP possess an alanine residue (Ala-278) aligned with A/NP Ser-165 and D/NP Thr-161 and a phenylalanine (Phe-519) aligned with A/NP Ser-407 and D/NP Ser-416.

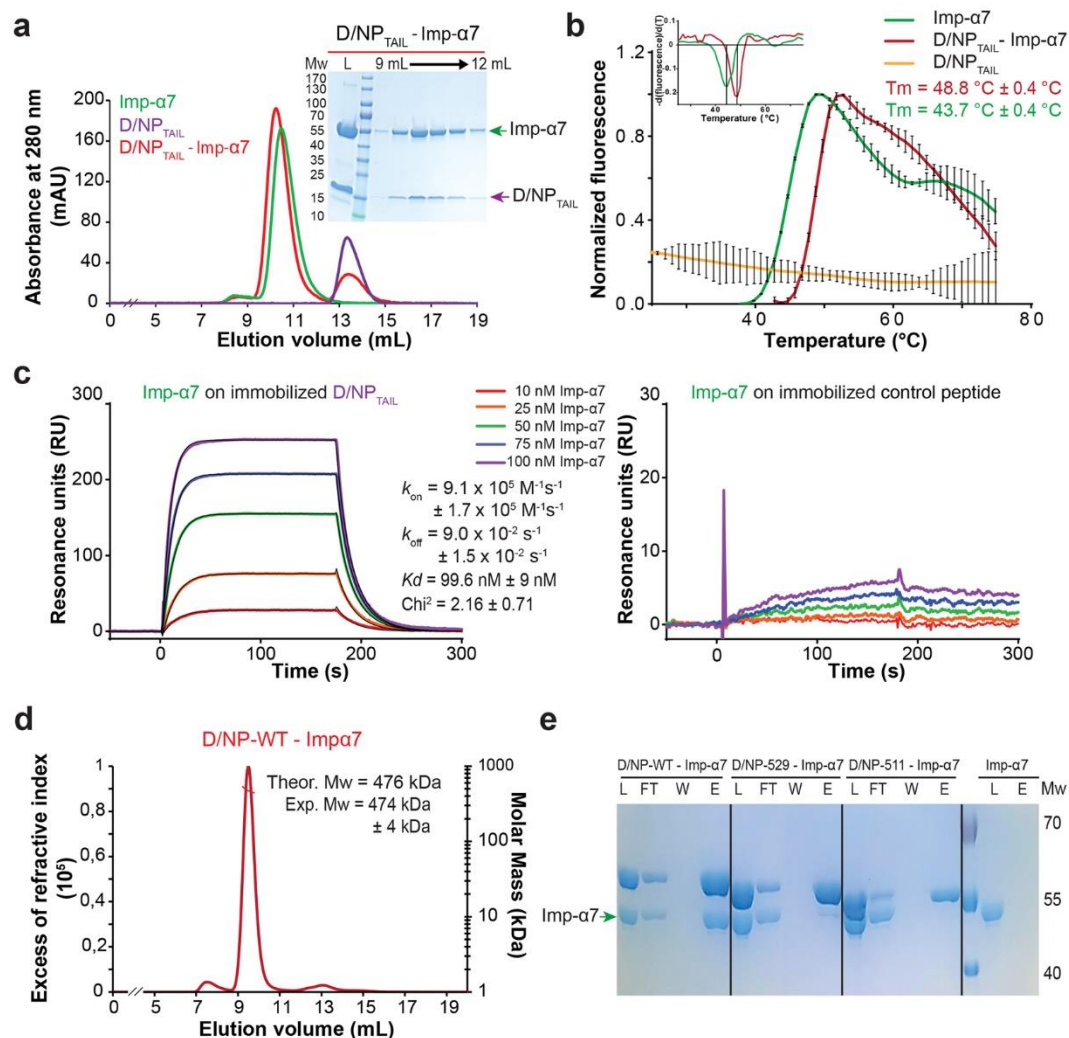


Figure 5. Interaction of D/NP and D/NP_{TAIL} with importin- α 7. **(a)** Size exclusion chromatography profile of a mixture between human importin- α 7 and D/NP_{TAIL}. The mixture (molar ratio 1 importin- α 7 for 2 D/NP_{TAIL}) was incubated 1 hour at room temperature and then loaded on a SuperdexTM75 10/300GL column equilibrated with the running buffer 20 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM β -mercaptoethanol. **(b)** Thermal stability assay of importin- α 7 in absence (green) or in presence (red) of D/NP_{TAIL} using ThermoFluor⁷⁶. In presence of D/NP_{TAIL}, the melting temperature of importin- α 7 is 5 °C higher. D/NP_{TAIL} alone using ThermoFluor did not give any denaturation signal (yellow curve). The upper insert corresponds to the derivative of the fluorescence signal for a precise measure of the melting temperature. **(c)** Affinity of importin- α 7 for D/NP_{TAIL} by measured by surface plasmon resonance (SPR). Biotinylated D/NP_{TAIL} (left) and control peptide (right) were captured on a streptavidin-coated sensor chip surface before injections of several importin- α 7 concentrations (10 nM in red, 25 nM in orange, 50 nM in green, 75 nM in blue and 100 nM in purple). The sensorgrams of the interaction between D/NP_{TAIL} and importin- α 7 were fitted under a Langmuir 1:1 binding model with mass-transfer (black line). **(d)** SEC-MALLS analysis of D/NP in complex with importin- α 7. The mixture (molar ratio 1 D/NP for 1.2 importin- α 7) was incubated 1 hour at room temperature and then loaded on a SuperdexTM 200 increase 10/300 GL. The experimental molecular weight is consistent with the expected mass of four importins- α 7 bound per D/NP tetramer. **(e)** Pull-down assays of human importin- α 7 by D/NP and the two C-terminal truncated mutants (D/NP-529 and D/NP-511). The his-tags are on D/NP. The mixtures (molar ratio 1 D/NP for 1.2 importin- α 7) were incubated 1 hour and the experience was done as described in panel (a). The figure shows the coomassie blue-stained SDS-PAGE (12% polyacrylamide) with the Load, FlowThrough, Wash and the second fractions (E2).

We demonstrated that the C-terminal D/NP_{TAIL} presents all the characteristics of an intrinsically disordered protein and that is involved in the nuclear import through its interaction with cellular importins- α . This role is not a big surprise since the presence of such NLS motifs was identified within the flexible NP_{TAILS} of viruses A and B located at the N-terminus of these proteins^{17,18,21,23,25,43,49}. Tho/NP has been shown to be imported in the nucleus⁵⁰ but this nucleoprotein does not possess any additional intrinsically disordered protein-like peptide with an NLS. Recently, a second NLS on A/NP (strain A/X-31) at the rim of the RNA binding channel was shown. A crystal structure shows that this internal NLS (₁₉₈Lys-Arg-INDRNFWRGENC-Arg-Arg-T-Arg₂₁₆) binds to mouse

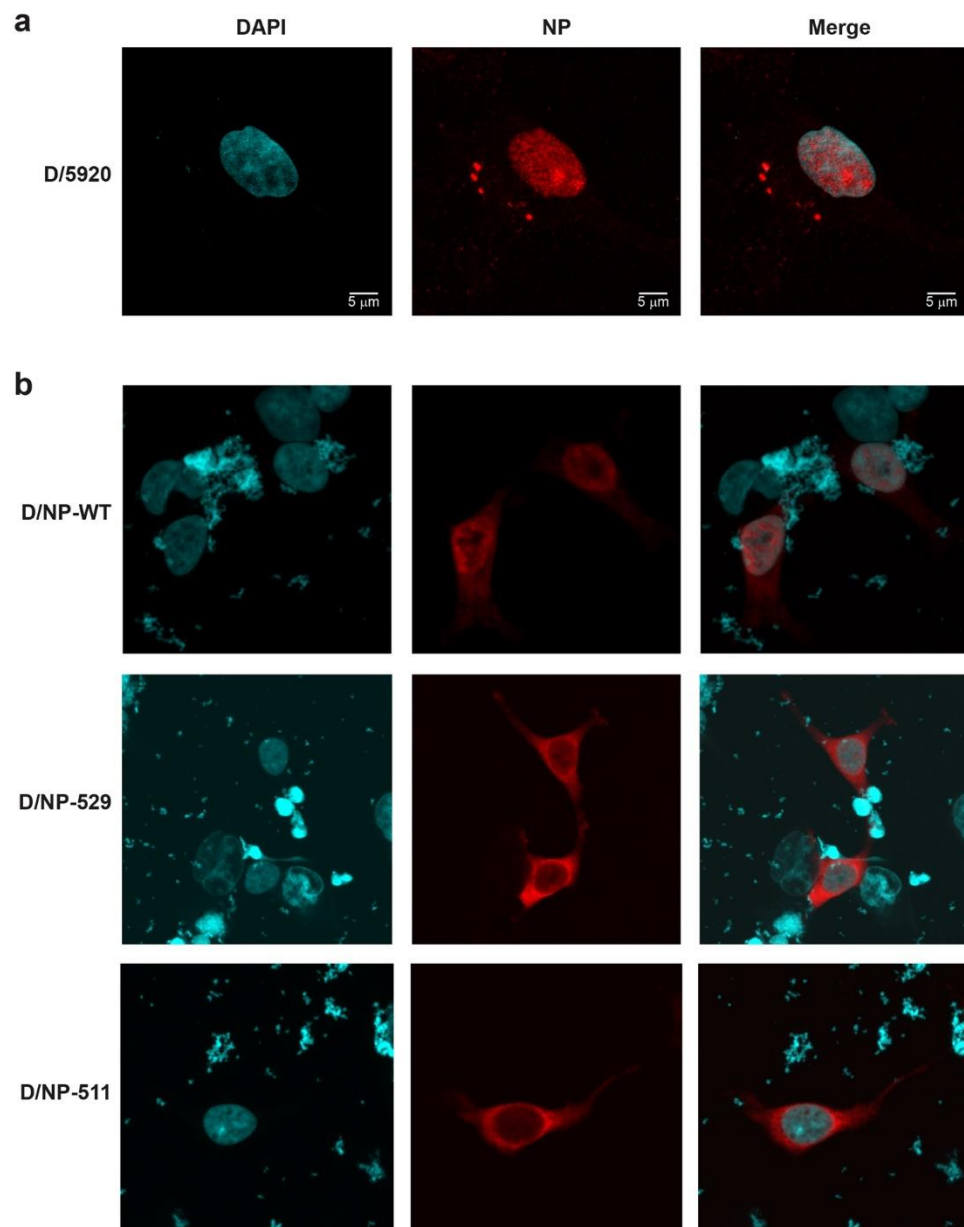


Figure 6. Nuclear transport of D/NP in HEK 293T cells. Microscopy pictures of HEK 293T cells with DAPI, NP and merged DAPI + NP staining (left, middle and right panels, respectively). **(a)** D/NP after infection of HEK 293T cells at 6 hours post infection with a *moi* of 5. **(b)** Cells were transfected with wt D/NP, D/NP-529 and D/NP-511. After 24 h cells were fixed, permeabilized and analyzed by indirect immunofluorescence. NP localization was observed using an in house rabbit hyperimmune NP-IDV serum and anti-rabbit IgG labeled Rhodamine RX (in red). Cells were mounted with DAPI-Vectashield and observed with a Leica Zeiss 710 (magnification: x63). In red: NP protein; in blue: nucleus. The bar in (A) represents 5 μm.

importin- α ¹²⁶. Likewise, for the NLSs of Tho/NP and ISA/NP, it has been suggested that the rims on the RNA binding surfaces can be used as an NLS for nuclear import for viral replication^{16,50,51}.

Finally, we have shown that the NP_{TAILs} of these nucleoproteins are intrinsically disordered proteins. The preservation of such intrinsically disordered protein-like peptide with a conserved function highlights its importance. The name for N_{TAIL} was first used for the C-terminal disordered tail of measles virus nucleoproteins⁵², which is about 125 residues long. This tail is disordered but can adopt a short α -helical structure (residue D484-A502) to promote the interaction with the phosphoproteins of these viruses which bind themselves to the corresponding RNA polymerase^{53,54}. For these viruses, the tails keep the nucleocapsids in a flexible conformation. When cleaved off with trypsin, the helical nucleocapsids become rigid^{55,56}. The tails do not change the affinity of the nucleoprotein for RNA but change the kinetics for the assembly of the nucleocapsids⁵⁷. The RNPs of Influenza A and B viruses are also very flexible⁵⁸⁻⁶¹ which makes it difficult to handle for high resolution structures. The EM structure of the measles nucleocapsid with a resolution of 3.6 Å⁶² was performed on the nucleocapsids without

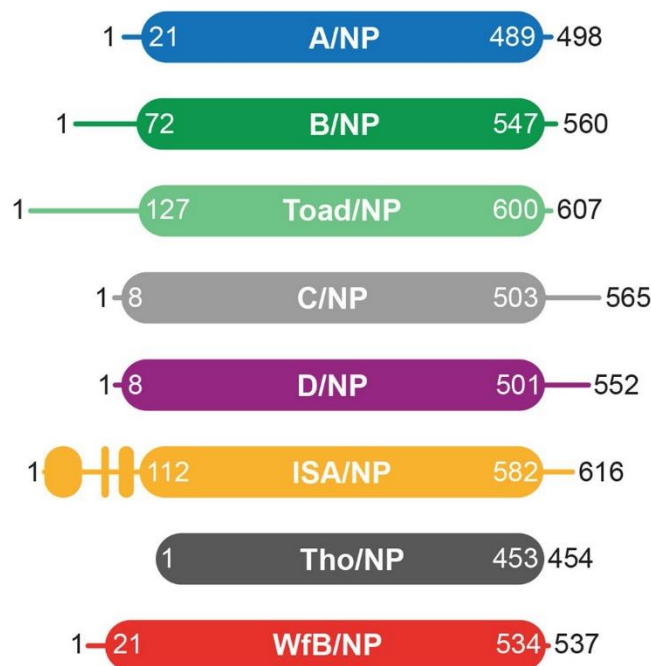


Figure 7. Schemas for nucleoproteins of *Orthomyxoviruses*. Schematic representation of the nucleoprotein based on the amino acid sequence identity and structure analysis of the protein from representative members of the *Orthomyxoviridae* family. The schema respects the size of the proteins. The protein accession numbers are the same as Table 1. The flexible tails are represented with simple lines whereas the folded parts and the cores are represented with filled boxes.

the C-terminal N_{TAIL} . If we could generate Influenza RNPs with NP without NP_{TAIL} to rigidify the RNPs, it would make it easier solving a high resolution structure to observe the RNA on the nucleoproteins.

Methods

Molecular biology for expression of nucleoprotein of Influenza D. The DNA coding sequence of D/bovine/France/2986/2012 NP was bought at GeneArt (ThermoFisher Scientific) optimized for bacterial expression. The different constructs have been PCR amplified and cloned following the supplier procedures (New England Biolabs). D/NP, D/NP-511 and D/NP-529 DNA coding sequences were cloned in pETM13 (EMBL) to express C-terminal His-tagged proteins whereas D/ NP_{TAIL} (505-D/NP-552) DNA coding sequence was cloned in pETM11 (EMBL) to express an N-terminal His-tagged fragment. The DNA coding sequence of the human importin- $\alpha 7$ (KPNA6; Uniprot accession number O60684) was cloned without its IBB domain (amino-acids 58 to 536) in pET9a (Novagen) to express an N-terminal His-tagged protein⁶³. Sequencing was performed by Eurofins.

Expression and purification of proteins. *Escherichia coli* BL21 RIL (DE3) cells (Life Technology) were transformed with the resulting plasmids. Cultures were induced 12 h by adding 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 18 °C and collected by centrifugation. The pellets were resuspended and sonicated in 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 M NDSB-201 (Sigma), 2 mM β -mercaptoethanol (β -ME) and complete protease inhibitor cocktail (Roche) for D/NP constructs and 50 mM Tris-HCl pH 8, 500 mM NaCl, 1 mM β -ME and complete protease inhibitor cocktail for importin- $\alpha 7$. All purifications were performed at room temperature. All D/NP constructs were purified by nickel affinity chromatography (Ni-NTA, Qiagen) followed, in the case of D/NP, D/NP-511 and D/NP-529, by a HiTrapTM Heparin HP column (GE-Healthcare) on a NGC system (BioRad). Elution fractions of D/NP, D/NP-511, D/NP-529 and D/ NP_{TAIL} were dialyzed against 20 mM Tris-HCl pH 7.5 at 50 mM or 300 mM NaCl, 5 mM β -ME. The last purification step was a size-exclusion chromatography using a HiloalTM 16/600 S200 column (GE-Healthcare) for D/NP, D/NP-511 and D/NP-529 or a S75 10/300 GL column (GE-Healthcare) for D/ NP_{TAIL} . Importin- $\alpha 7$ was purified by Nickel affinity chromatography and elution fraction were dialyzed with TEV (1/100) against 20 mM Tris-HCl pH 7.5 at 150 mM NaCl, 5 mM β -ME and 20 mM imidazol. Finally, a size-exclusion chromatography using SuperdexTM 200 increase 10/300 GL column (GE-Healthcare) was performed in 20 mM Tris-HCl pH 7.5 at 150 mM NaCl, 5 mM β -ME. Peak fractions were concentrated using a 10 kDa Amicon concentrator. Protein concentrations were determined using the extinction coefficients at 280 nm, $\epsilon = 44\,537\text{ M}^{-1}\cdot\text{cm}^{-1}$ for D/NP, $\epsilon = 41\,558\text{ M}^{-1}\cdot\text{cm}^{-1}$ for D/NP-529 and D/NP-511, $\epsilon = 2980\text{ M}^{-1}\cdot\text{cm}^{-1}$ for D/ NP_{TAIL} , and $\epsilon = 46\,785\text{ M}^{-1}\cdot\text{cm}^{-1}$ for importin- $\alpha 7$.

SEC-MALLS-RI analysis. Multi-angle laser light scattering (MALLS) coupled with size exclusion chromatography (SEC) and refractometry (RI) is a method for measuring the absolute molecular mass of a particle in solution that is independent of its dimensions and shape⁶⁴. SEC was performed with a column (SuperdexTM 200

increase 10/300 GL or Superdex 75 10/300 GL) equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 5 mM β -ME. Analytical runs were performed at 20 °C with a flow rate of 0.5 mL \cdot min $^{-1}$. MALLS detection was performed with a DAWN-HELEOS II detector (Wyatt Technology) using a laser emitting at 690 nm and protein concentration was measured on-line with the use of differential refractive-index measurements, with an Optilab T-rEX detector (Wyatt Technology) and a refractive-index increment, dn/dc, of 0.185 mL \cdot g $^{-1}$. Weight-average molar masses (Mw) were calculated with ASTRA (Wyatt Technology) as previously described³⁸.

Electron microscopy. Samples (concentrations around 0.05 mg \cdot mL $^{-1}$) were applied between a carbon and a mica layer. The carbon was then floated on the top of a 2% (w/v) sodium silicotungstate, pH 7.0 solution. The carbon film was covered by a copper grid. Both were fished using a small piece of journal paper and air dried before insertion in the electron microscope⁶⁵. Charge-coupled Device (CCD) frames were taken with a FEI T12 microscope operating at 120 kV and a nominal magnification of 45 000 times. The dilutions for EM were performed with the size-exclusion buffer right before preparing the grid.

Crystallisation and structure determination. D/NP was crystallised by vapor diffusion using the sitting drop method. The crystals were obtained in 2.4 M sodium malonate pH 4 with a protein concentration of 1.5 mg \cdot mL $^{-1}$. The crystals were directly flash-frozen without any cryoprotectant. Data were collected at the ESRF (beam-line ID30A-3) and processed with the XDS package⁶⁶. The structure was solved by molecular replacement using the Influenza A virus R416A monomeric nucleoprotein structure (PDB ID code 3ZDP) without the oligomerization loop (from residues 391 to 439) as a model. Model building and refinement were performed using CCP4i suite program for crystallography (PHASER, ARP/wARP, REFMAC5, COOT)^{67–71}. The final refinement was done using BUSTER⁷². The coordinates have been deposited in the Protein Data Bank under PDB ID code 5N2U. The protein structure figures were drawn using PyMOL⁷³.

Thermal shift assays. Thermal shift assays were performed following the established protocols of thermofluor experiments⁷⁴ using a fluorescent probe (SYPRO Orange). SYPRO Orange dye binds non-specifically to hydrophobic surfaces and water strongly quenches its fluorescence. When a protein unfolds with the increase of the temperature, the exposed hydrophobic surfaces bind the dye, resulting in an increase of the fluorescence by excluding water. Samples were diluted at 0.25 mg \cdot mL $^{-1}$ in 50 mM Tris-HCl pH 7.5, 300 mM NaCl and 5 mM β -ME and 5X SYPRO Orange dye (Invitrogen) in a final volume of 40 μ L. The thermal stability was measured using a real time PCR machine (Mx3005P Q-PCR, Stratagene). The dye was excited at 488 nm and the emission light was recorded at 585 nm while the temperature was increased by increments of 1 °C per minute from 25 to 75 °C. The relative fluorescence emission was then plotted against its corresponding temperature to produce the thermal shift profile curve. The melting temperatures were estimated from the derivative curves.

Circular dichroism. Circular dichroism (CD) spectroscopy is an efficient tool for rapid determination of the secondary structure and folding properties of a protein by measuring the absorption difference between left and right circularly polarized light. A JASCO J-810 CD spectropolarimeter equipped with a temperature-controller (Peltier system) was used to record the far-UV CD spectrum of D/NP_{TAIL} at 6.4 μ M in presence or in absence of trimethylamine N-oxide (TMAO). TMAO is a stabilizing chemical agent known to promote protein folding. It can be used with CD spectroscopy to assess putative folding of unfolded peptides and proteins. In addition to D/NP_{TAIL} without TMAO, final concentrations of 0.5, 1, 2 and 3 M TMAO were used in phosphate buffer (10 mM, pH 7.2) to validate the unfolded nature of D/NP_{TAIL}. Due to the high absorbance of TMAO around 200 nm, spectra were recorded up to the point before the HT rose above 700 V (190 nm without TMAO, 205 nm with 0.5 M TMAO, 207 nm with 1 M TMAO, 210 nm with 2 M TMAO and 212 nm with 3 M TMAO). Spectra were collected at 20 °C with fifteen runs using a cuvette with a path-length of 1 mm. After blank subtraction, the CD signal (in mdeg) was converted to mean molar residue ellipticity (in deg \cdot cm 2 \cdot dmol $^{-1}$).

Surface plasmon resonance (SPR) measurements and analysis. The D/NP_{TAIL} sequence was fused to the sequence encoding a biotinylation motif (NGSGGGLNDIFEA-QKIEWHE) and cloned in pETM11 (EMBL). *E. coli* BL21 RIL (DE3) were transformed and grown up to an OD of 0.6–0.8 in a medium supplemented with biotin (12.5 μ g \cdot mL $^{-1}$) in order to biotinylate the peptide *in vivo* during the expression (12 h, 18 °C, 0.3 mM IPTG). Purification was performed at room temperature in two steps, a Nickel affinity chromatography, followed by TEV cleavage and size exclusion chromatography (S75 10/300 GL, GE-Healthcare). A control biotinylated peptide (LEEMK-KGHLERECMEETCSYEEAREVFEDSEKTNEFWNK-biotin) was also used as negative control.

SPR experiments were carried out on a Biacore 3000 (GE Healthcare). Streptavidin was first immobilized on a CM5 sensor chip (GE Healthcare) surface through amine coupling at a flow rate of 5 μ L \cdot min $^{-1}$. The biotinylated NP_{TAIL} (10 μ g \cdot mL $^{-1}$) or the control biotinylated peptide (10 μ g \cdot mL $^{-1}$) were diluted in surfactant-supplemented HBS-N running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% Tween 20) (GE Healthcare) and the attachment was carried out through biotin:streptavidin interaction, at a flow rate of 5 μ L \cdot min $^{-1}$ up to 400–500 RU. A flow-cell containing immobilized streptavidin only was used as reference while the flow-cell with the streptavidin:biotinylated NP_{TAIL} complex was used as active flow-cell. For kinetic measurements, the importin- α 7 analyte was serially diluted in surfactant-supplemented HBS-N running buffer to final concentrations of 10, 25, 50, 75 and 100 nM. Each concentration was injected in triplicate in both the reference and active flow-cells. Analyte injection and following buffer injection times were set at 180 s and 150 s respectively at a flow-rate of 15 μ L \cdot min $^{-1}$. As the analyte spontaneously and completely dissociated, no regeneration was required and complete dissociation was achieved in buffer for 300 s. All sensorgrams were reference-subtracted and injection points were aligned to be processed with the analysis. Kinetic data were analysed using the Biacore 3000 Evaluation software (GE Healthcare) under a Langmuir 1:1 binding model with mass-transfer. χ^2 values for the sensorgram fits used to

determine the kinetic parameters were kept below 3, due to the obtained high signal, and T-value for rate parameters above at least 30. The residuals for the fitting were kept between 1 and -1.

Nuclear import of WT and NP mutants in HEK 293T cells. *Plasmids.* The D/NP, D/NP-511 and D/NP-529 genes were amplified by PCR, cloned into the multiple cloning site of pSC-A-amp/kan vector (Strataclone blunt kit, Agilent) and expressed into competent bacterial cells, according to the manufacturer's instructions. The NP genes in pSC-A-amp/kan vector were cloned into KpnI and BamHI restriction sites of an eukaryotic expression vector, pCDNA3.1 (+), driven by a CMV promoter. The constructs sequences were confirmed by Sanger sequencing (GATC Biotech platform, Germany).

Rabbit antibodies. To generate rabbit hyperimmune serum anti-D/NP, three 7 to 8 weeks old New Zealand white rabbits (Pôle Experimental Cunicole de Toulouse, Castanet-Tolosan) were immunized at day 0 and day 35 with 100–200 µg D/NP in incomplete Freund adjuvant. Rabbits were anesthetized and bled two weeks post boost (50 mL blood collected intra-cardiac before humane euthanasia).

Infection. Human embryonic kidney cells (HEK 293T; ATCC) were infected with D/bovine/France/5920/2014 at a multiplicity of infection of 5. Briefly, cells were washed with PBS, the inoculum was added and the cells were incubated 1 hour at 37 °C + 5% CO₂. After incubation, infection media (OPTI-MEM) was added and the cells were incubated until the fixation.

Transfection. HEK 293T cells were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) complemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Dominique DUTSCHER SAS) and incubated at 37 °C + 5% CO₂ for 24 hours. Two hours before transfection, DMEM media was removed and replaced by OPTI-MEM media (Dominique DUTSCHER SAS). Cells were transfected with 2.5 µg of the respective plasmids using Mirus-TransIT reagent (Mirus) following the manufacturers' instructions. Cells were then incubated for 24 h at 37 °C + 5% CO₂.

Immunofluorescence. Cells were pre-fixed using 4% paraformaldehyde (Bio-Rad) for 10 minutes at room temperature and then, fixed and permeabilized using ice cold ethanol:acetone for 10 minutes at room temperature. After a blocking step using PBS complemented with 5% horse serum, cells were incubated with a rabbit hyperimmune D/NP serum at a 1:100 dilution for 2 hours at room temperature. After three washing steps with PBS-triton 0.05%, cells were incubated with anti-rabbit IgG labeled Rhodamine RX (Jackson Immuno; Research, 711.296.152) at 1:200 dilution for 1 hour in the dark and at room temperature. Then, cells were washed with PBS-triton 0.05% and coverslips were mounted on glass slides using Vectashield-DAPI mounting medium (Vector Laboratories) and analyzed with a Leica Zeiss 710 at the Cell Imagery Platform in Purpan (Toulouse).

Ethics Statement. Experimentations were conducted in accordance with European and French legislations on Laboratory Animal Care and Use (French Decree 2001-464 and European Directive CEE86/609) and the animal protocol was approved by the Ethics Committee "Sciences et santé animale" number 115.

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Author Contributions

T.C. and M.D. conceived the experiments. A.D., J.O., A.L., L.T., M.M., F.C.A.G., C.M., G.S., M.D. and T.C. performed the experiments. A.D., J.O., A.L., F.C.A.G., G.S., R.W.H.R., M.D. and T.C. analysed the data. R.W.H.R., M.D. and T.C. wrote the paper.

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Chapter 3: Comparative analysis of the interaction involving each influenza nucleoprotein types and importins- α involved in influenza nuclear import

The nuclear import of the influenza replication machinery is a critical step of the viral cycle and this host:pathogen interaction is one of the tropism determinants. The initial nuclear import of the ribonucleoproteins is believed to be mediated through the NP_{TAIL} NLS, while the newly synthesized NP and RNA polymerase subunits possess their own NLS for nuclear import purposes^{28,54,57,283,346}. The nuclear translocation is a complex, highly regulated system. Small ions and molecules can passively diffuse in the nucleus, but bigger proteins such as NP and the viral RNA polymerase require an active transport in the nucleus, and more particularly by hijacking the Ran/importin- α /importin- β system^{139,156,211,263,266,306}.

A large number of studies have tried to describe and characterize the interaction between the cellular nuclear import machinery and the influenza proteins. A/NP has been the most characterized in complex with importins- α , and has been shown to interact with importin- α 1, - α 3, - α 5 and - α 7^{27,232,261,381,403}. While precious insights were gained, the affinities obtained so far is in the low micromolar, outside of the *in vitro* range of a functional NLS³⁶⁰. Similar results have been obtained for B/NP^{177,328,383,404}. In contrast C/NP and D/NP present *in vitro* affinities more consistent with a nuclear import efficiency *in cellulo*, but these studies did not thoroughly investigate all the nuclear import partners³⁵⁰. Importins- α are the direct partners of influenza NPs in the nuclear shuttling, but they were, so far, not studied extensively and comparatively. It is now well-known that importins- α are distinct entities, with their own regulatory behaviours and binding preferences^{94,283,360}.

In this paper, we performed a complete comparative analysis of the interactions between IAV, IBV, ICV, IDV NP_{TAILS} and the four human importins- α identified as partners. Using SEC, SPR, SEC-MALLS analysis and FA, we obtained affinities for all these complexes and unravelled striking specificities.

Using YFP-fused NP_{TAILS} to avoid the NP oligomerization and avidity contribution while keeping a globular context, we showed that these constructs were still able to interact with the importins *in vitro*. SPR revealed interactions with fast kinetics for A/NP_{TAIL}, C/NP_{TAIL} and D/NP_{TAIL}. D/NP_{TAIL} was able to interact with strong affinities with the importins- α 3, - α 5 and - α 7. Both A/NP_{TAIL} and C/NP_{TAIL} interact with varying affinities with these importins- α , with seemingly one preferential partner. B/NP_{TAIL}, on the other hand, showed a completely different behaviour, with even faster association and

dissociation rates and a low affinity for all importins. The contribution of previously identified key residues of B/NP_{TAIL} and D/NP_{TAIL} was analyzed by alanine scanning and resulted in a drastic decrease in affinity. The importin- α 1, showing homodimerization behaviour in solution, could not be used in the surface plasmon resonance experiment. Instead, a SEC-MALLS competition assay and fluorescence anisotropy measurements were used and revealed a strong affinity of importin- α 1 for D/NP_{TAIL} and C/NP_{TAIL}, medium-to-low affinity for A/NP_{TAIL} and poor affinity for B/NP_{TAIL}.





This paper provides insights on the specificity of these crucial interactions, for which both partners contributes equally. It also provides a strong basis for further studies aiming at dissecting this particular, essential host:pathogen interaction.

Article: Differential behaviors and preferential binding for influenza nucleoproteins and importins- α

Donchet A., Stermann E., Gerard F.C.A., Ruigrok R.W.H. and Crépin T
Viruses (2020)

Article

Differential Behaviours and Preferential Bindings of Influenza Nucleoproteins on Importins- α

Amélie Donchet, Emilie Vassal-Stermann , Francine C. A. Gérard [†] , Rob W. H. Ruigrok  and Thibaut Crépin ^{*} 

Centre National de la Recherche Scientifique (CNRS), Institut de Biologie Structurale (IBS), University Grenoble Alpes, Commissariat à l'Énergie Atomique et aux Énergies Alternatives (CEA), 38044 Grenoble, France; amelie.donchet@ibs.fr (A.D.); emilie.stermann@ibs.fr (E.V.-S.); francine.baraggia@grenoble-inp.fr (F.C.A.G.); rob.ruigrok@ibs.fr (R.W.H.R.)

* Correspondence: thibaut.crepin@ibs.fr; Tel.: +33-(0)476-209-439

† Current address: Laboratoire des Matériaux et du Génie Physique (LMGP), University Grenoble Alpes, CNRS, Institut Polytechnique de Grenoble (Grenoble INP), F-38000, Grenoble, France.

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Abstract: Influenza viruses are negative single-stranded RNA viruses with nuclear transcription and replication. They enter the nucleus by using the cellular importin- α / β nuclear import machinery. Influenza nucleoproteins from influenza A, B, C and D viruses possess a nuclear localization signal (NLS) localized on an intrinsically disordered extremity (NP_{TAIL}). In this paper, using size exclusion chromatography (SEC), SEC-multi-angle laser light scattering (SEC-MALLS) analysis, surface plasmon resonance (SPR) and fluorescence anisotropy, we provide the first comparative study designed to dissect the interaction between the four NP_{TAIL}s and four importins- α identified as partners. All interactions between NP_{TAIL}s and importins- α have high association and dissociation rates and present a distinct and specific behaviour. D/NP_{TAIL} interacts strongly with all importins- α while B/NP_{TAIL} shows weak affinity for importins- α . A/NP_{TAIL} and C/NP_{TAIL} present preferential importin- α partners. Mutations in B/NP_{TAIL} and D/NP_{TAIL} show a loss of importin- α binding, confirming key NLS residues. Taken together, our results provide essential highlights of this complex translocation mechanism.

Keywords: influenza nucleoprotein; nuclear transport; importin- α ; nuclear localization signal; influenza-host interaction; surface plasmon resonance; fluorescence anisotropy

1. Introduction

Influenza viruses are part of the *Orthomyxoviridae* family and form the genus *Influenzavirus*. Up to now, the International Committee officially recognizes four types (respectively types A, B, C and D) in its Taxonomy of Viruses (ICTV), but because of actual large-scale meta-transcriptomic approaches new specimens are regularly discovered, making this classification a constantly evolving entity. While influenza viruses were thought to be uniquely warm-blooded organism pathogens, a study has recently uncovered their existence in fish and amphibians in China [1]. A first criterion of distinction between the actual four types of *Influenzavirus* is based on their tropism. Influenza type A viruses (IAV) are viruses from water fowl but some of the viruses can also infect a range of hosts from chickens to swine and humans; type B (IBV) almost exclusively infect humans; type C (ICV) can infect both human and swine whereas type D (IDV) has a broad host tropism but limited to mammals. Influenza viruses are negative-sense single-stranded RNA viruses with segmented genomes. The RNA segments are packaged in ribonucleoproteins (RNPs), a complex architecture made by a single RNA molecule encapsidated by multiple copies of the nucleoprotein (NP) and the heterotrimeric RNA-dependent

RNA polymerase (RdRp) bound to the strictly conserved 3' and 5' end [2,3]. The number of RNPs varies depending on the type: IAV and IBV genomes consist of eight RNA segments, i.e., there are eight different RNPs, but only seven for ICV and IDV viruses. This is related to the inherent organisation of the viral spikes, respectively the haemagglutinin (HA) plus the neuraminidase (NA) for A and B or a single hemagglutinin-esterase-fusion (HEF) polypeptide with both concatenated activities for C and D.

Unlike most RNA viruses, influenza viruses transcribe and replicate their genome in the nucleus of infected cells. In general, the access to the nuclear compartment is through the highly regulated nuclear pore complex (NPC) [4–7]. Several pathways are involved in cytoplasmic-nuclear trafficking. Small molecules (< 30–60 kDa) are able to diffuse passively through the NPC but bigger molecules often require an active mechanism to be imported [8–10]. The main active transport is based on the importins- α/β (coupled to the small GTPase Ran) pathway. Importins- α and - β can both interact with the cargos but only the last ones mediate directly the translocation, and importin- α :cargo complexes must first interact with importin- β [11,12]. Cargos are recognized by importins- α/β through nuclear localization signals (NLSs), usually made of basic or hydrophobic motifs [13,14]. Additionally to the interaction with the NLS motifs, recent karyopherins- β /cargo-protein structures have demonstrated that these complexes are dependent on multiple interactions, mediated through intricate 3D interfaces which involve residues well beyond the NLS peptide stretch [15–17].

The human genome encodes for seven importins- α and at least twenty importins- β [9,18,19], meaning that the importins- α/β pathway is a difficult problem. Nuclear import of influenza virus proteins has been of a major interest for a long time [20,21]. Considering the neo-synthetized components of the replication machinery, two subunits (PA and PB1) of the heterotrimeric RdRp are imported as a sub-complex by the importin- β RanBP5 [17,22–24] whereas the third subunit (PB2) and NP interact with importins- α [25–28]. Because of its role in the RNPs formation, NP is critical for influenza replication but also shown to be the major contributor to the nuclear transport of the RNPs [29]. The NLS of NPs are located in intrinsically disordered tails of the proteins, either in N_{terminal} for A/ and B/NP or in C_{terminal} for C/ and D/NP (Figure 1). A minor putative NLS has been located in a loop of the core of A/NP, between residues 198 to 216, but its role remains unclear [30,31]. A/NP is known to interact with several importin- α isoforms, including the universal - α 1, - α 3, - α 5 and possibly - α 7 [32–36]. In recent years, our group has worked on the mechanisms of the interaction between two different NPs and the human nuclear trafficking machinery [37,38], in particular the tails containing the NLS of influenza B (B/NP_{TAIL}) and D (D/NP_{TAIL}) NPs. We showed that the NLS of B/NP_{TAIL} is highly extended from residues 30 to 71, around a single basic Lysine-Arginine (44-KR-45) patch, with a low affinity of 844 nM for the human importin- α 7. In the case of the C_{terminal} D/NP_{TAIL}, the NLS was composed of two basic KR patches, respectively 514-KR-515 and 532-KR-533. Both contribute to the nuclear trafficking and we estimated that the affinity of D/NP_{TAIL} for the human importin- α 7 was at 100 nM by surface plasmon resonance (SPR). The literature also contains several studies detailing the relation between A/NP and C/NP and importins- α [21,29,31,33,39–43] (Table 1). However, recent work highlighted that each importin- α is unique, with its own features and preferential partners [28,35,44,45].

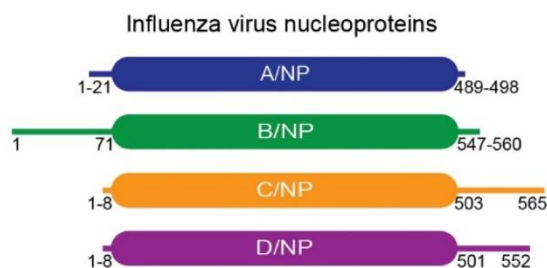


Figure 1. *Influenzavirus* nucleoproteins. The schematic representations of *Influenzavirus* nucleoproteins are based on this structural analysis [37]. The schema respects the size of the proteins. The flexible tails with the NLSs are represented with simple lines whereas the cores are represented with filled boxes.

Table 1. Binding of influenza nucleoproteins or their peptides to importins- α according to the literature. Abbreviations: ITC, isothermal titration calorimetry; SPBA, solid phase binding assay; MT, microscale thermophoresis; SPR, surface plasmon resonance.

	Construct	Importin- α	Method	K_d	Ref
A/NP	R416A	$\alpha 5$	ITC	26 nM	[41]
A/NP	2–15	$\alpha 1$	SPBA	1.7 μ M	[42]
A/NP	1–13	$\alpha 1$	ITC	5 μ M	[31]
A/NP	198–216	$\alpha 1$	ITC	72 μ M	[31]
B/NP	1–70	$\alpha 7$	ITC	844 nM	[38]
C/NP	506–565	$\alpha 1$	MT	48 nM	[43]
D/NP	505–552	$\alpha 7$	SPR	100 nM	[37]

Because we were confronted with a wide range of values (from 26 nM to 72 μ M) obtained using different techniques and a plethora of different partner constructs, we wanted to establish a systematic comparative study of the *in vitro* interaction between the flexible extremities (NP_{TAILS}) of all types of influenza NPs and the different human importin- α isoforms described in the literature. Our strategy was design, with a specific focus upon the short NLS sequences, and built with the objective of avoiding the inherent oligomerization of NPs, while guaranteeing the architecture of the tails in a unique globular environment. Using size exclusion chromatography experiments, we show that all NP_{TAILS} interact with importins- α in solution and then used surface plasmon resonance measurements to determine the affinity and binding kinetics of these interactions. In the current paper, we show that D/NP_{TAIL} interacts with all importins- α isoforms with strong affinity. Both A/NP_{TAIL} and C/NP_{TAIL} interact with varying affinities with the corresponding importins- α . On the other hand, B/NP_{TAIL} shows a completely different behaviour, with even faster association and dissociation rates and a low affinity with all the human importins- α we measured.

2. Materials and Methods

2.1. Molecular Biology and Constructs

Human importin- $\alpha 1$ (residues 69 to 529), - $\alpha 3$ (residues 64 to 521) and - $\alpha 5$ (residues 66 to 538) DNA coding sequences were bought at GENEART GmbH (Thermo Fischer Scientific, Regensburg, Germany) and subcloned into pETM11 bacterial expression vector (EMBL) without their importin- β (IBB) domains. Human importin- $\alpha 7$ (residues 58 to 536) cloning was described in [26]. NLS-bearing NP_{TAILS} from strains A/WSN/1933 (residues 1 to 21), B/Memphis/13/03 (residues 1 to 71), C/Ann-Arbor/1/50 (residues 508 to 565) and D/bovine/France/2986/2012 (residues 505 to 552) were ordered from GeneART, fused to a biotinylation sequence in C_{terminal} (Figure 2, and details of the sequences are provided in Table S1). The NP_{TAILS} were fused to the yellow fluorescent protein (YFP) either in C_{terminal} (C/NP_{TAIL} and D/NP_{TAIL}) or in N_{terminal} (A/NP_{TAIL}, B/NP_{TAIL}) according to their native localization; at the other YFP extremity, the biotinylation sequence was added (Figure 2). NP_{TAILS}-YFP constructs were subcloned into pETM11 bacterial expression vector. All constructs were expressed as N-terminally His-tagged proteins. Sequencing was performed by Eurofins Genomics (Köln, Germany). The control peptide was a synthetic peptide (GENPEP, St-Jean-de-Védas, France) corresponding to a GLA domain-derived polypeptide of the human coagulation factor X [46].

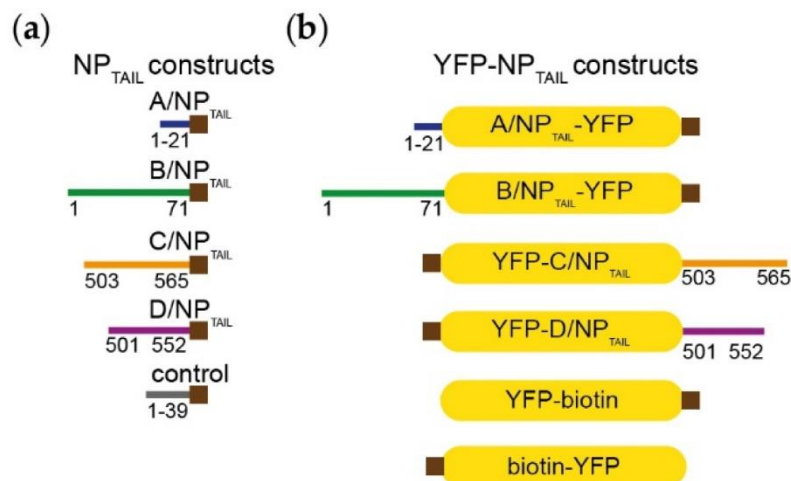


Figure 2. Constructs. (a) *Influenzavirus* nucleoproteins tails were expressed and purified independently fused with a biotinylation sequence (brown squares) in their C_{terminal} end. The details of the sequences are provided in Table S1. (b) The corresponding tails were fused to the yellow fluorescent protein (YFP) in order to integrate then in the context of a globular protein. The yellow filled boxes represent the YFP and the brown squares indicate the position of the biotinylation sequence.

2.2. Expression and Purification

Escherichia coli BL21 RIL (DE3) cells (Life Technologies, Thermo Fischer Scientific, Courtaboeuf, France) were used for protein expression. Cultures were supplemented with biotin (12.5 $\mu\text{g}\cdot\text{mL}^{-1}$, Sigma, Saint-Quentin-Fallavier, France) to biotinylate the proteins *in cellulo*, induced for 12 h by adding 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Euromedex, Souffelweyersheim, France) at 18 °C and collected by centrifugation. Pellets were resuspended in 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 2 mM β -mercaptoethanol (β -ME; Roth, Lagny-sur-Marne, France) and cComplete™, ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Meylan, France) before sonication. All purifications included a nickel affinity chromatography (resin Ni-NTA; Qiagen, Les Ulis, France), a Tobacco Etch Virus (TEV) protease cleavage, a second nickel affinity chromatography and a size-exclusion chromatography (SEC). SEC was performed on a NGC system (Bio-Rad, Marnes-La-Coquette, France) with a S75 10/300 GL column (GE-Healthcare, Dutscher, Brumath, France). The final buffer composition for all proteins was 10 mM Hepes pH 7.5, 150 mM NaCl. NP_{TAILS}-YFP protein concentrations were determined by measuring the absorbance at 280 nm and using the theoretical extinction coefficients (ϵ) 28,942 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for A/NP_{TAIL}-YFP and C/NP_{TAIL}-YFP, of 27,452 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for B/NP_{TAIL}-YFP, D/NP_{TAIL}-YFP and YFP control. ϵ at 280 nm used for Δ IBB importins- α protein concentrations were 50,607 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for importin- α 1, 42,190 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for importin- α 3, 52,285 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for importin- α 5 and 46,785 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for importin- α 7. The concentrations were then obtained using the Beer–Lambert law.

2.3. Interaction Assays by Size Exclusion Chromatography

All size exclusion chromatography (SEC) experiments were performed in the same buffer (10 mM Hepes pH 7.4, 150 mM NaCl) using a Superdex™ 200 increase 10/300GL column (GE-Healthcare) for YFP-NP_{TAIL} fusion proteins and a Superdex™ 75 10/300GL column (GE-Healthcare) for all the other NP_{TAILS}. Samples were diluted to be used at 30 μM for YFP-NP_{TAIL} fusion proteins, 60 μM for NP_{TAILS} and 25 μM importin- α 7 in 300 μL . They were incubated for one hour at room temperature before injection on a NGC system (Bio-Rad).

2.4. SEC-MALLS-RI Analysis

Size exclusion chromatography (SEC) followed by multi-angle laser light scattering (MALLS) and refractometry (RI) analysis allow the determination of the molecular mass of a protein or a complex in solution that is independent of its dimensions and shape [47]. SEC was performed with a column (Superdex™ 200 increase 10/300 GL or Superdex 75 10/300 GL) equilibrated with 10 mM Hepes pH 7.5, 150 mM NaCl. Analytical runs were performed at 20 °C with a flow rate of 0.5 mL·min⁻¹. Multi-angle laser light scattering (MALLS) detection was performed with a DAWN-HELEOS II detector (Wyatt Technology, Toulouse, France) using a laser emitting at 690 nm and protein concentration was measured on-line with the use of differential refractive-index measurements, with an Optilab T-rEX detector (Wyatt Technology) and a refractive-index increment, dn/dc of 0.185 mL·g⁻¹. Data were analyzed and weight-averaged molar masses were calculated using the ASTRA software (Wyatt Technology Corp., Santa Barbara, CA, USA).

2.5. Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were carried out at 25 °C on a Biacore T200 (GE Healthcare Life Sciences Europe GmbH, Velizy-Villacoublay, France). Avidin (50 µg·mL⁻¹ in 50 mM acetate buffer pH 4.5; Sigma) was first immobilized on a Serie S CM5 sensor chip (GE-Healthcare) surface through amine coupling chemistry according to the manufacturer instructions, until a coupling level of about 17,000 resonance units (RUs) was reached. The biotinylated NP_{TAIL}-YFP (1–10 µg·mL⁻¹) or biotinylated YFP control (1–10 µg·mL⁻¹) were then captured in surfactant-supplemented HBS-N running buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 0.05% Tween 20; GE Healthcare) at a flow rate of 30 µL·min⁻¹ until a coupling level of 100–200 RU was obtained. A flow-cell with immobilized avidin only was used as negative control while the flow-cell with the avidin: biotinylated NP_{TAIL}-YFP complex was used as active flow-cell. For kinetic measurements, importins-α were serially diluted in surfactant-supplemented HBS-N running buffer. Each concentration was injected in triplicate on both the reference and active flow-cells. Analyte injection and following buffer injection times were set between 55–180 s and 55–200 s respectively, depending on the examined partners, at a flow-rate of 30 µL·min⁻¹. Regeneration was required and achieved by pulse injection of 5 M NaCl. All sensorgrams were reference-subtracted. Data were analyzed with Steady State Analysis using the Biacore T200 Evaluation software (GE Healthcare) under a Langmuir 1:1 binding model, assuming that the binding is equivalent and independent for all binding site. Chi² values for the fitting were kept below 1 for most of the analysis with a maximum of 2.5, and T-value means for the dissociation constant above 10.

2.6. Fluorescence Anisotropy

Fluorescence anisotropy experiments were performed on a Clariostar microplate reader (BMG Labtech, Champigny-sur-Marne, France) set and dedicated to fluorescence anisotropy measurements, with excitation and emission wavelengths fixed at 482 and 530 nm, respectively. Importin-α1 was serially diluted in buffer (10 mM Hepes pH 7.4, 150 mM NaCl) and mixed in dilution series with the YFP-fused NP_{TAILS}. YFP construct final concentration was set at 25 nM. Measurements were done in 384-well plates at room temperature. Blank subtraction was done using the condition with YFP-fused NP_{TAILS} alone. Data were normalized using the equation:

$$B = \frac{(A_{exp} - A_{min})}{(A_{max} - A_{min})} \quad (1)$$

where B corresponds to the YFP-NP_{TAIL} bound fraction (value between 0 and 1), A_{exp} is the anisotropy value observed for one given importin-α1 concentration, and A_{min} and A_{max} stand for the fluorescence anisotropy values of the free YFP-NP_{TAIL} and bound YFP-NP_{TAIL}, respectively. Titrations were then fitted to the GraphPad Prism model “Single binding site with Hill slope (h)”.

3. Results

3.1. Biochemical and Biophysical Analysis of the Interaction between NP_{TAILS} and Importin- α 7

In all experiments with NP and its partners, measurements are difficult because all NPs tend to form oligomers depending on the buffer concentrations [41,48], and therefore, we used only the disordered NP_{TAILS}. We designed our study with a specific focus upon these short sequences that do not address the effect of domains beyond the NP_{TAILS}. In order to decipher the specificities of the different NP_{TAILS} for importins- α , we first use the corresponding peptides, in size exclusion chromatography (SEC) experiments combined with surface plasmon resonance (SPR) measurements to determine the affinity and binding kinetics. The first method was used to confirm the ability of the tails to interact with its partner in solution, whereas the second method was used for the determination of the kinetic parameters of the interactions. In SPR, one partner (called the ligand) is immobilized onto a biosensor surface. The interacting partner (called the analyte) diluted in a buffer is then continuously flowed across the biosensor surface, where it binds to the ligand. The binding is measured as a change in resonance units (RUs) on the biosensor surface. Measuring the increase in binding over time for a given analyte set of concentrations gives the association rate (k_{on}) up to the point where the system is at equilibrium (i.e., as many association events are observed as dissociation events). Ceasing flow and changing to buffer alone then allows the analyte to wash off the ligand. Measuring the decrease in bound partner over time gives the dissociation rate (k_{off}). The equilibrium dissociation constant (K_d) is calculated from the kinetic association and dissociation rates (k_{off}/k_{on}). When both association and dissociation rates are too fast, it is possible to use the equilibrium part of the curves to extract the dissociation constant, without relying on the rates. For the SPR experiments, we chose to immobilize the tails on the biosensor surface by using the importins- α as the analytes. The immobilization of the tails was done by fusing a biotinylation sequence in C_{terminal} (Figure 2a), with a capture strategy based on a biotin: avidin interaction. The corresponding constructs were recombinantly produced in bacteria (with 12.5 $\mu\text{g}\cdot\text{mL}^{-1}$ biotin in the growth media) and purified. We first tested their ability to interact with importin- α 7 by SEC experiments. They all formed a stable complex in solution with importin- α 7 (Figure S1). However, for SPR measurements, the kinetic parameters could not all be established because of technical issues. In particular, for B/NP_{TAIL}, the inability to stabilize the SPR signal because of a putative mass transfer phenomenon made it impossible to exploit the raw data. In consequence, we adapted our strategy.

3.2. Biochemical and Biophysical Analysis of the Interaction between NP_{TAILS} Fused to Yellow Fluorescent Protein (YFP) and Importin- α 7

We decided to increase the size of the tails by fusing them to a known protein, unrelated to the influenza virus life cycle. A larger ligand should allow the experiment to be performed with lower ligand densities, resulting in lower mass transport rates. We chose the yellow fluorescent protein because of its small size and compact folding, with an unchanged capture strategy on the biosensor surface. A YFP construct was fused in N_{terminal} for C/NP_{TAIL} and D/NP_{TAIL} and in C_{terminal} for A/NP_{TAIL} and B/NP_{TAIL}, to respect their position in the wild-type proteins (Figure 2b). The recombinant proteins were produced, purified (Figure S2) and used in SEC experiments to test their ability to interact with importin- α 7. We confirmed that adding the YFP has no visible effect on the interaction between the NP_{TAILS} and the human import factor in solution. For each tail, a peak corresponding to the complex was observed prior those of each separate samples (Figure 3). On the contrary, when the control YFP was mixed with importin- α 7, a double peak corresponding to the sum of each sample injected separately was observed (Figure S3).

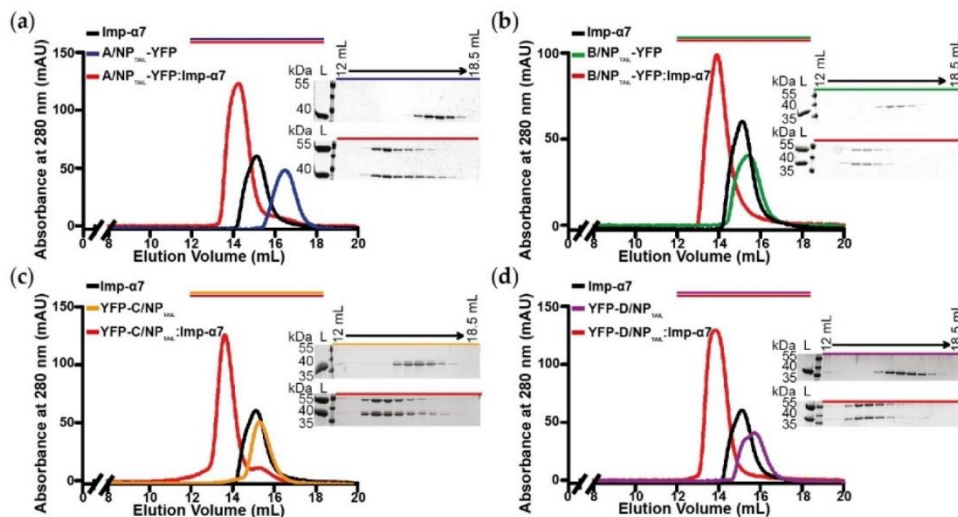


Figure 3. Analysis of the complexes between YFP- NP_{TAILs} and human importin- $\alpha 7$ in solution. Each YFP- NP_{TAIL} fusion protein ($30 \mu\text{M}$) was injected on a SuperdexTM 200 increase 10/300GL column, alone or in the presence of importin- $\alpha 7$ ($25 \mu\text{M}$). The figure shows the superimposition of the SEC profiles obtained for (a) A/ NP_{TAIL} -YFP, (b) B/ NP_{TAIL} -YFP, (c) YFP-C/ NP_{TAIL} and (d) YFP-D/ NP_{TAIL} . The colors correspond to the code used on Figure 2b with all the complexes in red and the importin- $\alpha 7$ alone in black. For each panel, the 12% SDS-PAGE corresponding to the elution of the YFP- NP_{TAIL} fusion protein alone (top) and its complex with importin- $\alpha 7$ (bottom) are shown. The control experiment is shown on Figure S3.

Using the corresponding samples, we measured the kinetic parameters of the different interactions by SPR (Figure 4; Tables 2 and 3 respectively for affinities (K_d) and kinetic parameters values (k_{on} and k_{off})). Gradient concentration of importin- $\alpha 7$ were injected into the SPR flowing channels at a speed of $30/\mu\text{L}\cdot\text{min}^{-1}$ for 50–180/s to reach equilibrium. As shown in Figure 4, a binding signal was generated immediately after injection of importin- $\alpha 7$ which dissociated from the sensor chip very quickly, suggesting that importin- $\alpha 7$ bound to the immobilized NP_{TAILs} in a quick “in and out” manner. Equilibrium analysis was then chosen to derive affinity binding constants (K_d) for the interaction between the immobilized NP_{TAILs} and the importin- $\alpha 7$ in solution. Our reference was the data obtained for the interaction between D/ NP_{TAIL} and importin- $\alpha 7$ previously measured using the same method and the same strategy [37] but with a previous generation instrument. Using a Biacore 3000, we had measured a K_d of $99 \text{ nM} \pm 9 \text{ nM}$ for this interaction and $29 \text{ nM} \pm 1 \text{ nM}$ using a new generation Biacore T200. This time, we were able to fully exploit the SPR data obtained with all NP_{TAILs} . In particular, we showed that the architecture of the NLS (mono- or bipartite NLS) has no importance for importin- $\alpha 7$; the affinity for the non-conventional monopartite A/ NP_{TAIL} was better than for the bipartite C/ NP_{TAIL} , with K_d of $73 \pm 4 \text{ nM}$ and $146 \pm 8 \text{ nM}$ respectively, but weaker than D/ NP_{TAIL} . B/ NP_{TAIL} appeared to be the lowest value with a K_d of $405 \pm 5 \text{ nM}$, but comparable to the value obtained using isothermal titration calorimetry [38]. Despite the nanomolar affinities, all of these interactions displayed a highly dynamic behaviour. Indeed, kinetics parameters show extremely high on rates, suggesting that very little energy is needed to form the interaction, but also very high off rates. Both association and dissociation rates were close to the upper detection limits of the apparatus, respectively around $10^{-6} \text{ M}^{-1}\cdot\text{s}^{-1}$ and 0.1 s^{-1} and even above in the case of the B/ NP_{TAIL} (Table 3).

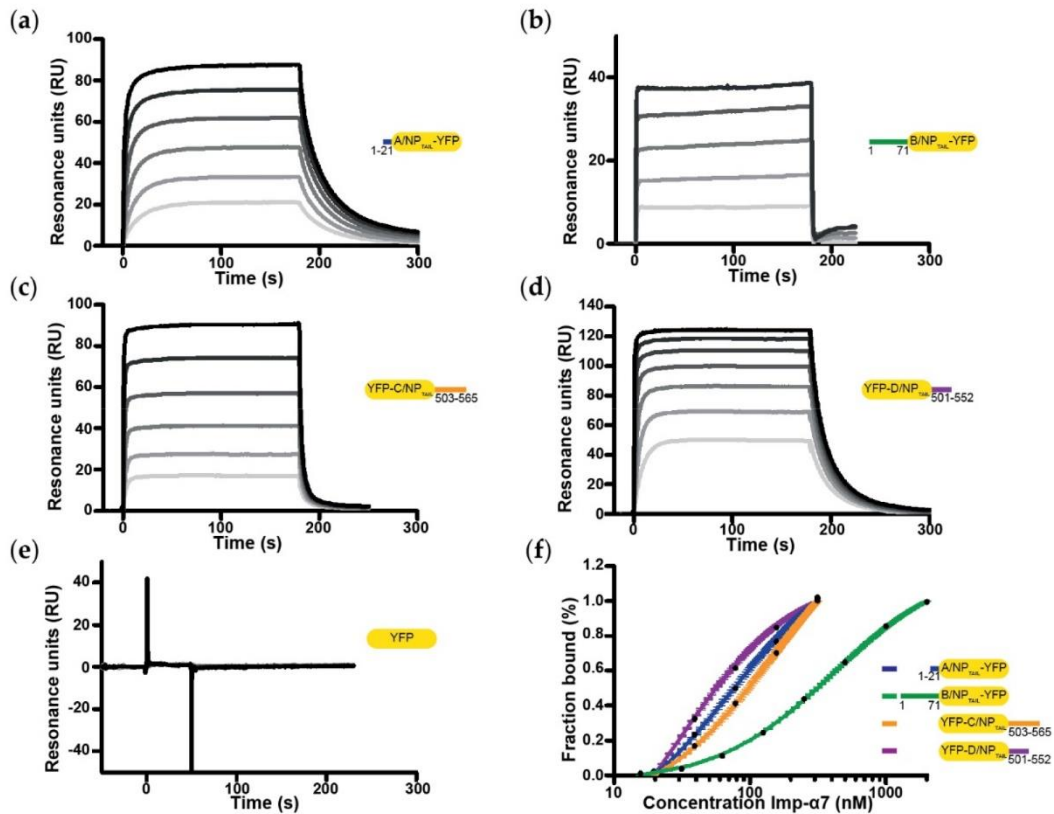


Figure 4. Affinity between YFP-NP_{TAILs} and human importin- α 7 by surface plasmon resonance (SPR). The figure shows the sensorgrams of the interaction between importin- α 7 and (a) A/NP_{TAIL}-YFP, (b) B/NP_{TAIL}-YFP, (c) YFP-C/NP_{TAIL}, (d) YFP-D/NP_{TAIL} and (e) the YFP control. For each panel, the gradation of grey represents the different concentrations of importin- α 7 used for each titration, from the lowest (light-grey) to the highest (dark-grey). The concentrations range from 19.5 nM to 625 nM for (a) and (c), from 125 nM to 2 μ M for (b), from 19.5 nM to 1.25 μ M for (d) and from 80.5 nM to 10.3 μ M for (e). (f) Steady state analysis was performed on Biacore evaluation software, the resulting fitting normalized and plotted on a log₁₀ scale.

Table 2. Affinity between wild-type influenza nucleoprotein tails and human importins- α obtained by SPR. The apparent dissociation constants (K_d) are expressed in nM; n.i. means ‘no interaction’.

	Imp- α 3	Imp- α 5	Imp- α 7
A/NP _{TAIL}	301 \pm 21	128 \pm 20	73 \pm 4
B/NP _{TAIL}	308 \pm 106	770 \pm 24	405 \pm 5
C/NP _{TAIL}	59 \pm 9	287 \pm 31	146 \pm 8
D/NP _{TAIL}	22 \pm 15	21 \pm 5	29 \pm 1
YFP control	n.i.	n.i.	n.i.

Table 3. Kinetic parameters of the interaction between wild-type influenza nucleoprotein tails and human importins- α obtained by SPR. The association rate constants (k_{on}) are expressed in M⁻¹·s⁻¹ and the dissociation rate constants (k_{off}) values are expressed in s⁻¹; n.i. means ‘no interaction’.

	Imp- α 3		Imp- α 5		Imp- α 7	
	k_{on} ($\times 10^6$)	k_{off} ($\times 10^{-1}$)	k_{on} ($\times 10^6$)	k_{off} ($\times 10^{-1}$)	k_{on} ($\times 10^6$)	k_{off} ($\times 10^{-1}$)
A/NP _{TAIL}	1.31 \pm 0.03	2.81 \pm 0.04	1.0 \pm 0.6	1.6 \pm 0.1	1.6 \pm 0.1	0.7 \pm 0.1
B/NP _{TAIL}	>10	>10	>10	>10	>10	>10
C/NP _{TAIL}	2.4 \pm 1.1	1.3 \pm 0.4	6.1 \pm 3.6	2.6 \pm 0.4	2.6 \pm 0.4	2.5 \pm 0.1
D/NP _{TAIL}	2.8 \pm 0.2	0.07 \pm 0.01	1.1 \pm 0.1	5.0 \pm 0.2	5.0 \pm 0.2	1.04 \pm 0.03
YFP control	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.

3.3. Systematic Analysis of the Interactions between NP_{TAILs} and Importins- α

Once the strategy for measuring the kinetics of all NP_{TAILs} for the importin- α 7 was established, we decided to expand it to other members of the importins- α family. The human genome encodes seven isoforms of importin- α , divided into three subfamilies known as α 1, α 2 and α 3: the α 1 subfamily contains importin- α 1 and - α 8; the α 2 subfamily contains importin- α 3 and - α 4; and the α 3 subfamily contains importin- α 5, - α 6 and - α 7 [18,49]. We chose to work with one representative isoform of each subfamily (importin- α 1 and importin- α 3, respectively for the α 1 and α 2 subfamilies) plus a second isoform for the α 3 subfamily (i.e., importin- α 5). Furthermore, all these isoforms are described in the literature regarding the influenza virus infection. We produced and purified the recombinant isoforms without their importin- β binding (IBB) domain. The case of importin- α 1 is shown below. Using SEC-MALLS-RI experiments, we first confirmed that both Δ IBB importins- α 3 and - α 5 are monomeric in solution (Figure S4), before we used them in our SPR experiments to determine their respective kinetics (Tables 2 and 3). Importin- α 5 shows a similar interaction profile as importin- α 7, with K_d D/NP_{TAIL} < A/NP_{TAIL} < C/NP_{TAIL} < B/NP_{TAIL} but with a higher amplitude in the values (from 21 to 770 nM). On the contrary, the architecture of the NLS seems essential for recognition by importin- α 3. The two bipartite NLS tails (i.e., C/NP_{TAIL} and D/NP_{TAIL}) have a higher affinity than the two monopartite (i.e., A/NP_{TAIL} and B/NP_{TAIL}) which both show identical values. Based on these results, we further deciphered the interactions by introducing point mutations in the positive charge patches of the NLSs which we had analyzed in the past (i.e., B/NP_{TAIL} and D/NP_{TAIL}) [37,38]. We show that for bipartite NLS, the second basic patch could be stronger in relation to the stability of the cargo: importin- α than the first patch (Table 4). Based on the affinities of each single patch mutants, D/NP_{TAIL} seems to present a cooperative behaviour in importin- α binding. On the other hand, importin- α 7 seems to be more prompt in compensating for mutations in the first patch whereas importin- α 3 looks more permissive to mutations in the second. The results obtained with B/NP_{TAIL} mutant confirm our nuclear magnetic resonance (NMR) observations on the major contribution of the whole tail, from residues 30 to 71, in the interaction with importins- α , and not only the regions surrounding the KR motif [38]. Regarding the B/NP_{TAIL mut1}-YFP, even if we were not able to quantify precisely the equilibrium dissociation constants from the raw data, probably because importin- α require a longer injection period to saturate all the ligand binding sites in this condition, the sensorgrams show clear concentration dependent-responses in contrast to the YFP-D/NP_{TAIL mut3} construct (Figure S5).

Table 4. Effects of mutations in B/NP_{TAIL} and D/NP_{TAIL} on the affinities for the human importins- α . The K_d obtained by SPR are expressed in nM; n.i. means ‘no interaction’.

	Imp- α 3	Imp- α 5	Imp- α 7
B/NP _{TAIL}	308 ± 106	770 ± 24	405 ± 5
B/NP _{TAIL mut1}	>5000	>5000	>5000
D/NP _{TAIL}	22 ± 15	21 ± 5	29 ± 1
D/NP _{TAIL mut1}	922 ± 77	1150 ± 154	658 ± 208
D/NP _{TAIL mut2}	2457 ± 184	>5000	>5000
D/NP _{TAIL mut3}	n.i.	n.i.	n.i.

3.4. The Case of the Importin- α 1

The SEC-MALLS-RI experiments showed a different behaviour of importin- α 1 in solution (Figure S4). Whereas the experimental molecular mass for Δ IBB importins- α 3, - α 5 and - α 7 were similar to the theoretical values, the results obtained with Δ IBB importin- α 1 suggest a propensity

for dimerization of this construct. The corresponding sample was eluted precociously from the S200 column with an apparent molecular mass of 88 kDa rather than the theoretical 50 kDa (Figure S4a). Dimerization was reported previously [50] with a monomer–dimer K_d estimated to be 8 μM and the equilibrium could be shifted to the monomeric form by adding NLS-mimicking peptides. In a second SEC-MALLS-RI set of experiments but using a Superdex 75 column, we showed that with the different NP_{TAIL} peptides the peaks corresponding to the importin- α 1 were shifted to the smaller molecular mass (Figure 5a). The largest effect is observed with D/NP_{TAIL} suggesting that a quasi-quantitative estimation of the interaction could be extracted from these results, with apparent K_d D/NP_{TAIL} < C/NP_{TAIL} < A/NP_{TAIL} < B/NP_{TAIL}.

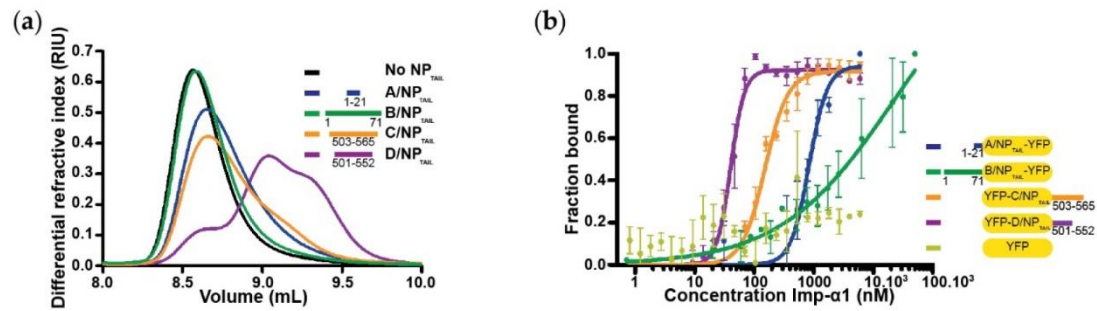


Figure 5. Binding of influenza nucleoproteins tails to importin- α 1. (a) Dissociation of importin- α 1 dimers by competition with NP_{TAIL} using a SEC-MALLS-RI experiments. Human importin- α 1 (45 μM) alone or incubated with each NP_{TAIL} (45 μM) was injected on a Superdex™ 75 increase 10/300 G. Successful competition resulted in a delayed elution volume compared to the importin- α 1 alone. Three distinct species can be identified from importin- α 1:D/NP_{TAIL} injection and two species from importin- α 1:C/NP_{TAIL} one. (b) YFP-NP_{TAIL}s: importin- α 1 affinities by fluorescence anisotropy. Titration with each NP_{TAIL} (25 nM) was carried out by fluorescence anisotropy with importin- α 1 concentrations ranging from 0.7 nM to 50 μM for B/NP_{TAIL}-YFP or from 0.8 nM to 6 μM for the other constructs. The titrations were normalized and fitted with GraphPad analysis model “specific binding with Hill slope”. The raw data are shown in Figure S6.

When we tried to measure the corresponding kinetics by SPR, we could not obtain a stable equilibrium, probably due to the competition between the monomer-dimer transition of importin- α 1 and its interaction with the immobilized tails. This experimental artifact is inherent to the nature of the analyte, which despite the purification steps may dimerize in time and significantly affect affinity measurements by SPR. We then found an alternative method; with the YFP-NP_{TAIL}s we could measure the fluorescence anisotropy. Fluorescence anisotropy is a measure of light emitted by a fluorophore, unequal along different axes of polarization. This measure depends on rotational behaviour of this fluorescent molecule in solution. Depending on the fluorophore environment, fluorescence anisotropy will vary from a low value because of its high rotational rate when the molecule is free, to a high value in case of its interaction with a partner. This feature allows the determination of an apparent binding constant when the fluorescent molecule is kept at a constant concentration upon titration of its partner. By titration of our ΔIBB importin- α 1 construct in presence of the different YFP-NP_{TAIL}s (Figure 5b), the corresponding apparent K_d was determined (Table 5). The curve of the B/NP_{TAIL} could not be estimated because the K_d of the B/NP_{TAIL} for importin- α 1 was too close to the K_d of the dimerization of importin- α 1.

Table 5. Affinity between wild-type influenza nucleoprotein tails and human importin- α 1 obtained by fluorescence anisotropy. The apparent K_d are expressed in nM; n.d. and n.i. mean ‘not determined’ and ‘no interaction’, respectively.

	A/NP _{TAIL}	B/NP _{TAIL}	C/NP _{TAIL}	D/NP _{TAIL}	YFP Control
imp- α 1	621 \pm 89	n.d.	149 \pm 1	41 \pm 12	n.i.

4. Discussion

The interaction between viral proteins and cellular factors is of growing interest for understanding the evolution mechanisms of viruses, and RNA viruses in particular. These are major potential therapeutic targets, as these interfaces are less prone to mutations. It has been decades since the interactions between importin- α and the influenza virus nucleoprotein have been studied but numerous features and mechanisms are still unknown [32,33,35,51]. Importins- α are essential actors in cells and were diversified and highly conserved through evolution (Table S2). Each importin- α shows a high sequence identity in higher eukaryotes with a specific expression pattern and behaviour but also preferential bindings to cargo and functions.

In this paper, we provide a comparative characterization of the interactions between four importin- α isoforms with the tails from A/NP, B/NP, C/NP and D/NP. The affinities are between the nanomolar and the low micromolar range, with association and dissociation rates surprisingly high. Two different behaviours were identified: (i) A/NP_{TAIL}, C/NP_{TAIL} and D/NP_{TAIL} present a dynamic complex formation and release with high-affinity preferential binding and (ii) B/NP_{TAIL} shows even faster association and dissociation rates with very low affinity.

The A/NP_{TAIL} was isolated from a human strain of influenza A virus and interacted preferentially with importin- α 7. This is consistent with the importins- α expression pattern in the human respiratory track [52] as well as of previously reported work [31]. It is of note that PB2 isolated from human strains also preferentially bind to importin- α 7, while avian PB2s tend to interact with importin- α 3 [28]. The poor binding of A/NP_{TAIL} to importin- α 3 is consistent with the fact that importin- α 3 tends not to interact well with monopartite NLS; while A/NP_{TAIL} is not a monopartite NLS *per se*, it possesses only a short NLS motif. In contrast, C/NP_{TAIL} presents a higher affinity for importin- α 3. Importin- α 3 has a low auto-inhibition and a unique intrinsic flexibility, acting as an adapter for unusual and complex NLS sequences or NLS close to globular domains [53,54]. Such NLSs could not associate with a less flexible importin- α due to steric hindrance. The NLS sequence of C/NP_{TAIL} is similar to a bipartite NLS, with two basic patches, but separated by a long linker sequence (more than 20 residues). Such a sequence could require the flexibility of importin- α 3 to achieve optimal binding. D/NP_{TAIL} is a textbook case of bipartite NLS, which binds tightly to importins- α , using both the minor and major binding pockets [55–57]. D/NP_{TAIL} strongly interacts with all tested importins with a high affinity (20–41 nM), in the same range as other bipartite NLSs ($K_d \approx 10$ nM) [28,58]. In other studies, it has been shown that the *in vitro* affinities using purified proteins and peptides were in the nanomolar range whereas the interaction between NLSs and importins- α *in cellulo* is in the micromolar range. Addition of cytosol in *in vitro* assays led to the destabilization of importin: NLS complexes, even in the case of bacterial lysate, highlighting the contribution of non-specific interaction and competition in this cellular process [44,59]. Importins cycles between both compartments have been estimated to be fast, around 30 ms; nanomolar affinity leads to a residence time of several seconds or up to several minutes, which is far too long to allow optimal release in the nucleus, while micromolar affinity put it in the millisecond range [59].

The sensorgram shapes of C/NP_{TAIL} and D/NP_{TAIL} along with the affinities resulting from D/NP_{TAIL} mutations clearly suggest that the interaction follows a 1:1 binding model with cooperativity. At the other end of the spectrum, B/NP_{TAIL} behaviour towards importins- α is still puzzling. It is possible that the B/NP_{TAIL} is not properly processed in our settings. It is well known that A/NP and B/NP nuclear import are regulated through post-translational modifications (PTM), in particular

through phosphorylation [60,61]. A discrete PTM could be an activating signal for nuclear import regulation [62–66]. The absence of the NP_{CORE} in our experiment could also be responsible for the lack of functionality of B/NP_{TAIL}: three-dimensional context could matter for importin specificity, with putative additional contacts and constrains, and for NLS docking [18,28,53,67]. Another hypothesis comes from the nuclear import receptor involved and the complex to be imported. While importin- α/β complex is largely believed to be the most common nuclear import pathway, this is still debated. Other adaptor proteins besides importin- α are known to interact with cargo and importin- β [68,69]. Co-import of neo-synthesized B/NP with another cargo is also a possibility, taking in account that RNP nuclear import functionality putatively relies on avidity rather than affinity [70,71]. Therefore, it is not be excluded that B/NP uses another receptor/complex to gain access to the nucleus. Further investigations into other nuclear import receptors could shed light on successful B/NP nuclear translocation. One interesting feature of B/NP is its long, intrinsically disordered N_{terminal} tail. Such a tail could be involved in the formation of liquid phase separation and help with protein recruitment [72,73]. If B/NP exhibits such behaviour, it could be interesting to dissect it further as nucleoporins in the nuclear pore complex channel tend themselves to be involved in phase separation [74–76].

Our work focuses on human importins- α , as three out of the four NP_{TAILS} were derived from human viruses. However, due to the high conservation of these importins through evolution (Table S2), it is likely that extrapolation to other mammal importins can be achieved to some degree. Further work would be required to validate this completely.

While a lot of work is still required to fully understand the mechanistic and specificity of the interactions between influenza nucleoproteins and importins- α , we provide here this first comparative study on the interaction of NP_{TAILS} with importins- α 1, - α 3, - α 5 and - α 7. NP_{CORE} involvement, NP oligomerization, cellular environment, IBB domain contribution, and post-translational modification of both partners are other factors which need to be investigated in the future, in the hope of fully dissecting this host-pathogen interaction and trying to target and disrupt this essential hijacking mechanism.

5. Conclusions

While the interactions between influenza NPs and importins- α have been extensively studied, this has mainly been done through the prism of A/NP, with a large number of highly different methods and sometimes without the essential focus required on importins- α . Whereas each method and study brought useful characterization insights, it is a complex task to compare affinity from widely different assays, with significant changes in conditions, partners, settings and analysis. Here, we provide the first comparative study to dissect several interactions between influenza NPs and importins- α using the same strategy and under the same conditions. We showed that A/NP_{TAIL}, C/NP_{TAIL} and D/NP_{TAIL} interact strongly with one or several importins- α . On the contrary, the human specific B/NP_{TAIL} interacts poorly with selected importins- α , raising new questions about this essential interaction in the influenza virus cycle.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4915/12/8/834/s1>, Figure S1: analysis of the complexes between NP_{TAILS} and human importin- α 7 in solution, Figure S2: SEC-MALLS-RI analysis of the different YFP-fused tails used in that work, Figure S3: absence of interaction between YFP and human importin- α 7 in solution, Figure S4: SEC-MALLS-RI analysis of the different importins- α used in that work, Figure S5: interaction between human importin- α 7 and YFP-NP_{TAILS} mutants, Figure S6: interaction between human importin- α 1 and YFP-NP_{TAILS}, Table S1: sequences, Table S2: sequence identity between the importins- α used in the studies.

Author Contributions: T.C. conceived and designed the experiments; A.D., E.V.-S. and F.C.A.G. performed the experiments; A.D., E.V.-S., F.C.A.G. and T.C. analysed the data; A.D. and T.C. wrote the original draft; A.D., E.V.-S., F.C.A.G., R.W.H.R. and T.C. edited the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Chapter 4: Structural and biochemical
characterization of the Asiatic toad
influenza NP

As a newly discovered virus in a 2018 metagenomic study, no further information are known on the Asiatic toad influenza virus (ToadV), except for the partial genome sequences released ³²⁹. Six genomic segments have been recovered so far: the three segments encoding the RdRp subunits (PA, PB1 and PB2), and the HA, NA and NP segments. With both HA and NA encoded by the genome, this virus seems structurally/genetically closer to IAV and IBV. Furthermore, the sequence alignment of ToadV nucleoprotein sequence with all other influenza nucleoproteins shows a higher percent identity with B/NP than A/NP (**Table 6**), which is surprising, considering the huge difference in term of host spectrum between these two well-known influenza viruses. This discovery is particularly interesting: the evolutionary relationship between influenza viruses is still poorly understood, and the differences between the four types of influenza viruses are at the same time extensive and enigmatic.

Table 6: Sequence identities between Toad/NP and the other influenza NPs.

The sequences were obtained from the Influenza Research Database ⁴¹⁹. The strains used for the alignment are those used in this PhD work.

	Toad/NP
A/NP	22.5 %
B/NP	28.7 %
C/NP	22.1 %
D/NP	20.1 %

Based on the characteristics established since the publication of D/NP structure ⁶³, the structure prediction of Toad/NP (**Figure 19**) revealed that, similarly to B/NP, it possesses a long N-terminal tail (\approx 130 amino acids) with a folded core and a short C-ter disordered tail (7 residues). Toad/NP N-terminal tail was almost twice as long as B/NP_{TAIL} (\approx 130 residues against 71), with a similar pattern of scattered basic residues (**Table 7**). Interestingly, Toad/NP_{TAIL} could putatively possess a folded domain, as found in ISA/NP, despite their evolutionary distance. In spite of a credible alignment of Toad/NP_{CORE} and B/NP_{CORE}, the actual N-ter edge of Toad/NP_{CORE} could not be strongly pinpointed: the B/NP Proline 72 is aligned with the Toad/NP Glutamine 132 and two Prolines at plausible positions could be found close by (P116 and P123).

affected by the salt concentration used (150 mM and 300 mM) and a 50 mM NaCl buffer lead to a degraded elution, maybe through aggregation. The NP_{TAIL} constructs were purified with a nickel affinity chromatography followed by a size exclusion chromatography with a likewise TEV protease cleavage. Toad/NP_{FULL} and Toad/NP_{CORE} final buffer was 20 mM Tris-HCl pH 7.5, 150 or 300 mM NaCl, 5 mM β -mercaptoethanol while all Toad/NP_{TAIL} constructs were kept in 10 mM Hepes pH 7.5, 150 mM NaCl. The ratio 280/260 was always close to two, indicative of the absence of a nucleic acid contamination (**Fig. 20**).

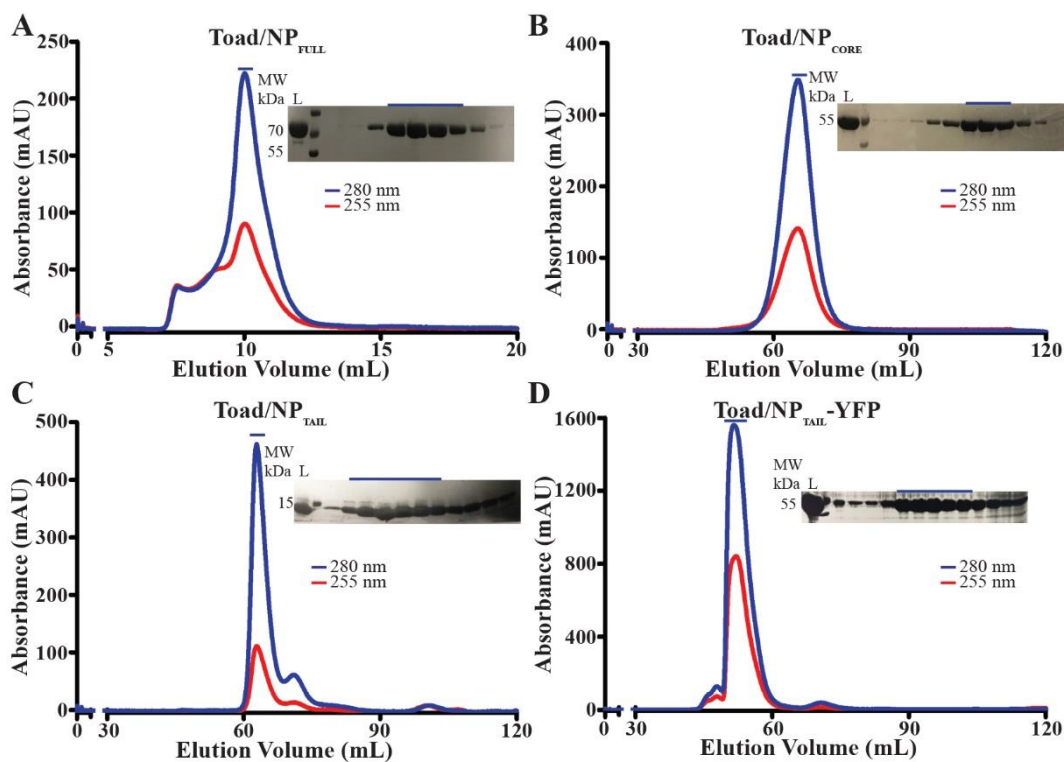


Figure 20: Profiles of the SEC purification step for each Toad/NP constructs.

Gel filtration profiles and the corresponding SDS-PAGE of (A) Toad/NP_{FULL} on a SuperdexTM 200 10/300 GL column (B) Toad/NP_{CORE} on a HiLoadTM 16/600 SuperdexTM 200 column, (C) Toad/NP_{TAIL} and (D) Toad/NP_{TAIL}-YFP on a HiLoadTM 16/600 SuperdexTM 75 column. L and MW stand for Load and Molecular Weight respectively.

I. Characterization of the interaction with importins- α

It was suspected that, similarly to other NPs, Toad/NP should access to the nucleus to participate to the transcription and the replication processes, and probably has a crucial role in the initial import of the RNPs in the nucleus. Therefore, as Toad/NP_{TAIL}

presented several basic patches, the first aim was to determine if it was responsible for an interaction with importins- α , similarly to other influenza NPs, but knowing that the importins- α we had used in initial experiments were human paralogs, potentially different despite their high sequence identities from homologous found in batrachians (**Annexe 1**).

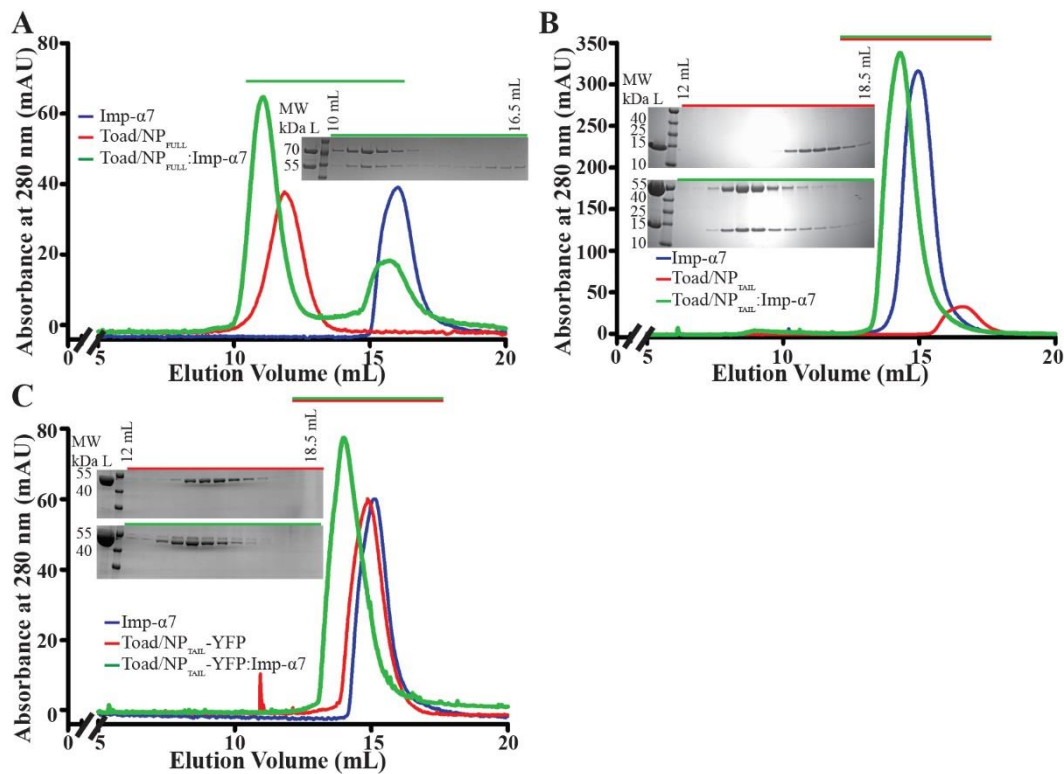


Figure 21: Interaction assays between Toad/NP constructs and human importin- α 7 by size exclusion chromatography.

Interaction profiles between the importin- α 7 and (A) Toad/NP_{FULL} (30 μ M), (B) Toad/NP_{TAIL} (70 μ M), (C) Toad/NP_{TAIL}-YFP (30 μ M). The importin- α 7 was used at a concentration of (A) (C) 25 μ M or (B) 70 μ M. For each panel, the 4-20% SDS-PAGE of the complex elution, with or without the elution of the Toad/NP construct alone is associated. L and MW stand for Load and Molecular Weight respectively.

An interaction assay by gel filtration was achieved as previously^{63,64,177}. Briefly, after a 1h incubation, three samples were successively injected on a SEC column (Superdex™ 200 increase 10/300 GL): the NP constructs alone, the importin- α 7 alone and a mix NP:importin- α 7 (**Fig. 21**). Toad/NP_{FULL} and Toad/NP_{TAIL} (fused or not with the YFP) were shown to be eluted in a complex with the importin- α 7. The experiment was done with Toad/NP_{CORE} and in that case, it did not seem to interact with the importin- α 7. The experiment should be redone in the future, for validation.

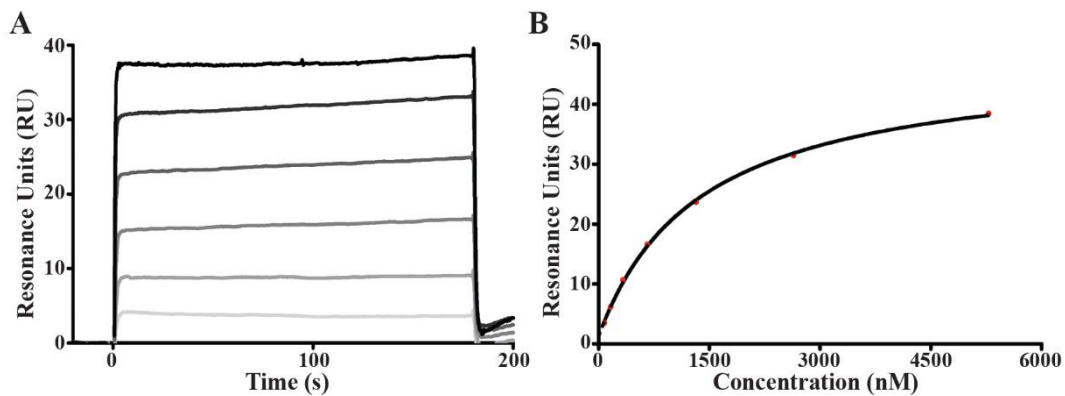


Figure 22: Characterization of the interaction between Toad/NP_{TAIL}-YFP and the human importin- α 7 by SPR.

(A) Sensorgrams and (B) associated steady state fit determined on the Biacore evaluation software of the interaction between Toad/NP_{TAIL}-YFP and the importin- α 7. Each shade of gray represents one concentration, ranging from 83 nM to 5282 nM (light gray to dark gray).

Toad/NP_{TAIL} fused to YFP was then used for a Biacore characterization of the interaction with the importins- α 3, - α 5 and - α 7. The attachment of Toad/NP_{TAIL}-YFP on the sensor chip surface was done by the biotin in C-terminal, through the avidin:biotin interaction, up to 200 resonance units. For the kinetics, the importin- α 7 was injected at several concentrations (from 83 nM to up to 5 μ M), in a serially diluted manner (Fig. 22). The first interesting feature came from the rates of association and dissociation. Similarly to B/NP_{TAIL} and in clear contrast with A/NP_{TAIL}, C/NP_{TAIL} and D/NP_{TAIL}, they were extremely high, outside of the range of detection of the apparatus used (cf. Chapter 3). The second feature concerned the affinities. Toad/NP_{TAIL} showed a low affinity for the importin- α 5 and - α 7 but a high affinity for the importin- α 3 (Fig. 22 and Table 8), similar to the affinities obtained for other NP_{TAILS}⁶⁴. The trend in affinity between the two related NP (*i.e.* B/NP and Toad/NP) was well conserved, with a wider range for Toad/NP_{TAIL}. While these low affinities and this trend are still puzzling, the affinity between Toad/NP_{TAIL} and the importin- α 3 seemed to suggest a putative preference for this partner.

Table 8: Biacore steady state affinities between Toad/NP_{TAIL} or B/NP_{TAIL} and human Δ IBB importins- α .

K_d values are expressed in nM. χ^2 values represent the goodness of the fit, the lower the better. Values for B/NP_{TAIL} come from the Chapter 3.

	Imp α 3		Imp α 5		Imp α 7	
	K_d	χ^2	K_d	χ^2	K_d	χ^2
Toad/NP _{TAIL} -YFP	155.8 \pm 23.1	0.02	1217 \pm 63	0.7	1024 \pm 341	0.14
B/NP _{TAIL} -YFP	308 \pm 106	0.5	770 \pm 24	0.003	405 \pm 5	0.1

Further work is although required to determine more clearly the residues involved in this interaction, the *in cellulo* kinetics of the nuclear localization and whether other interfaces exist.

II. Characterization of the interaction with RNAs

One of the main features of influenza nucleoproteins is their essential role in the viral/complementary RNA bindings, for protection and replication/transcription purposes. A/NP and B/NP RNA binding activities have been extensively studied, highlighting a nanomolar affinity with an important impact of the salt concentration^{13,27,71,178,242,353}. It is actually not known exactly how many nucleotides a single NP binds, but supposedly ranging from 12 to 24 nucleotides. ISA/NP, a non-influenza NP belonging to the *Orthomyxoviridae* family, is known to bind to 20-mer, 24-mer and 28-mer RNAs with nanomolar affinities, a monomer binding to about 12 nucleotides⁴²². In order to decipher its RNA binding activity and to dissect its specificities/similarities regarding others NPs, the affinities of both full length Toad/NP_{FULL} and Toad/NP_{CORE} for several RNAs were measured by fluorescence anisotropy.

Briefly, fluorescence anisotropy relies on the fact that a fluorescent molecule in solution is in rotation and therefore, upon excitation, polarizes the light. This movement depends on several parameters, and more particularly on the size of the molecule. The bigger the molecule is, the slower it diffuses and the more it polarizes the light. In our experiments, FAM-labelled RNAs (5 nM) were mixed with serially diluted Toad/NP_{FULL} and/or Toad/NP_{CORE}. The corresponding complex, far bigger in size, polarizes the light. This feature allows the determination of an apparent dissociation constant by titrating the RNAs from a fully free state to a fully bound state.

Measurements were done at 150 mM and 300 mM NaCl. In these salt concentrations, the constructs seem to be mainly tetrameric. Three sizes of RNAs were used: 6-mer, 12-mer and 24-mer RNA labelled in 3' with fluorescein (FAM), with either a polyA or a polyUC sequence. A short RNA (6-mer) is able to bind to the RNA-binding groove without saturating it and usually clearly highlights an impaired binding ability for any conditions or constructs. A 12-mer RNA could represent the size fitting the NP RNA binding groove ^{178,422}. Finally, the 24-mer RNA is the standard binding size used in influenza NP:RNA interactions.

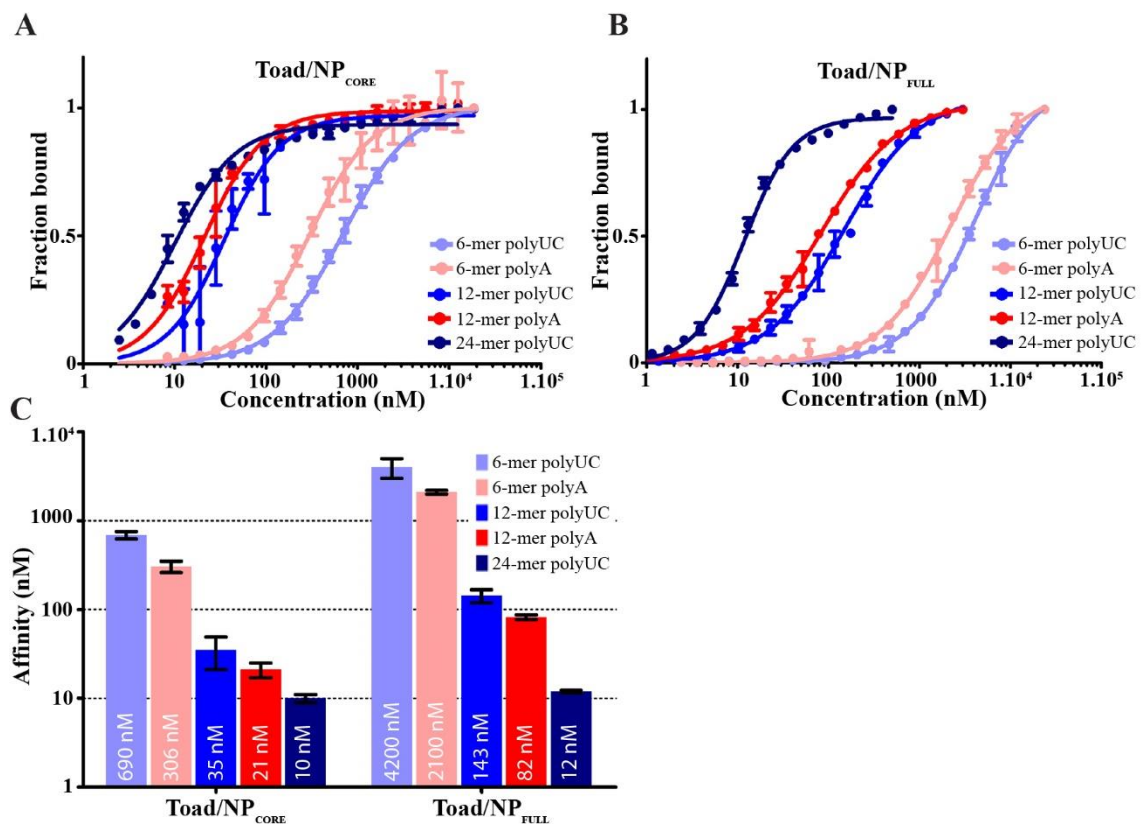


Figure 23: Titration against FAM-labelled RNAs of Toad/NP_{FULL} and Toad/NP_{CORE} by fluorescence anisotropy at 150 mM NaCl.

Titration measurements against FAM-labelled RNAs with (A) Toad/NP_{CORE} or (B) Toad/NP_{FULL}. (C) Affinities of the full length Toad/NP or its core for RNAs at 150 mM NaCl by fluorescence anisotropy. The fitting was done using the GraphPad Prism 5 equation “One site – Specific Binding with Hill slope”.

Toad/NP was serially diluted and mixed with 5 nM of FAM-labelled RNA in a 384-well microplate. The measurements and analyses were done as previously detailed (cf. **Chapter 3**), on a CLARIOstar microplate reader, with excitation and emission wavelengths fixed at 482 and 530 nm respectively, after a 10-min incubation at room

temperature (**Fig. 23, Table 9**). After blank subtraction and normalization, the fitting were represented using GraphPad Prism 5, with the equation “One site – specific binding with Hill slope”. The Hill slope coefficient was consistent with the cooperative features of the binding in all conditions: the longer the RNA, the higher the factor, although this was more marked for Toad/NP_{CORE} than for Toad/NP_{FULL}.

Table 9: Toad/NP:RNA apparent dissociation constants determined by fluorescence anisotropy.

NaCl Concentration	150 mM NaCl		300 mM NaCl
	Toad/NP _{FULL}	Toad/NP _{CORE}	Toad/NP _{CORE}
6-mer polyA	2.1 $\mu\text{M} \pm 0.1 \mu\text{M}$	306 nM ± 44 nM	7.8 $\mu\text{M} \pm 1.5 \mu\text{M}$
6-mer polyUC	4.2 $\mu\text{M} \pm 0.6 \mu\text{M}$	690 nM ± 64 nM	12.8 $\mu\text{M} \pm 1.6 \mu\text{M}$
12-mer polyA	82.2 nM ± 4.9 nM	21 nM ± 4 nM	751 nM ± 13 nM
12-mer polyUC	143 nM ± 24 nM	35 nM ± 14 nM	1.2 $\mu\text{M} \pm 0.1 \mu\text{M}$
24-mer polyUC	11.9 nM ± 0.4 nM	10 nM ± 1 nM	106 nM ± 7 nM

At 150 mM NaCl, a concentration close to the intracellular KCl concentration, Toad/NP_{FULL} and Toad/NP_{CORE} did not show any strong preference for a polyA or a polyUC sequence, with only a 2-fold difference in term of affinity for polyA RNA molecules. Both Toad/NP_{FULL} and Toad/NP_{CORE} interacted with a low nanomolar affinity (10 nM) with a 24-mer polyUC RNA, saturating the binding sites. Toad/NP_{CORE} showed similar affinities for 24-mer polyUC, 12-mer polyA and 12-mer polyUC, suggesting that the binding pattern was identical and putatively saturated the binding groove. These results are in agreement with the hypothesis that 12 nucleotides are strongly bound to one NP monomer, while the others are more loosely interacting, maybe responsible for the recruitment or interaction with other NPs. In contrast, the affinity of Toad/NP_{FULL} for both 12-mer RNA molecules is lower (7- and 12-fold decrease in affinity). A sharp decrease in affinity was observed for the two constructs when the 6-mer RNAs were used. This pattern of decreasing affinities for decreasing RNA sizes is similar to those observed for A/NP and B/NP, and expected for cooperative binding with a binding groove that may be able to accommodate about 12 nucleotides¹⁷⁸.

The most striking feature of these titrations came from the the comparison between Toad/NP_{FULL} and Toad/NP_{CORE}. Interestingly, when Toad/NP was deprived of its long N-terminal tail, the affinities are 4- to 7-fold higher than when the full-length protein was used, suggesting a putative regulatory role, direct or indirect, of the tail in the RNA-

binding activity. The RNA titrations with Toad/NP_{TAIL} were done, to verify any RNA-binding activity, but the tail did not seem to bind RNAs specifically, with affinities determined for both 24-mer and 12-mer RNAs above 230 μ M. At 300 mM NaCl, Toad/NP_{CORE} was clearly impaired in its global RNA binding activity in comparison to 150 mM NaCl, with more than a 10-fold decrease towards all RNAs. However, it still showed this pattern of decreasing affinity for shorter RNAs, with again a 2-fold difference between polyA and polyUC. This characterization should be continued, firstly with measurements with Toad/NP_{FULL} at 300 mM NaCl. It could also be interesting to go into details of the oligomerization state/process of both Toad/NP, with and without RNAs, to better understand what the role of Toad/NP_{TAIL} is and how Toad/NP interacts with RNAs and package the genome into RNPs.

III. Structural characterization of Toad/NP

The structural analysis of this new influenza NP was conducted in parallel of the biochemical/biophysics experiments. In order to gain evidences on the propensity of Toad/NP_{TAIL} to fold in secondary structure elements, a measurement of the ¹⁵N-labelled Toad/NP_{TAIL} by NMR was done on the ISBG NMR platform (**Fig. 24**). The spectra showed that, while Toad/NP_{TAIL} contained an intrinsically disordered region (narrow dispersion of the peaks in the middle of the spectra, $\omega_{2-1}^1\text{H} = 8 - 8.6$ ppm) but also, a part of the protein looked folded (proton chemical shifts present in the other parts of the spectra).

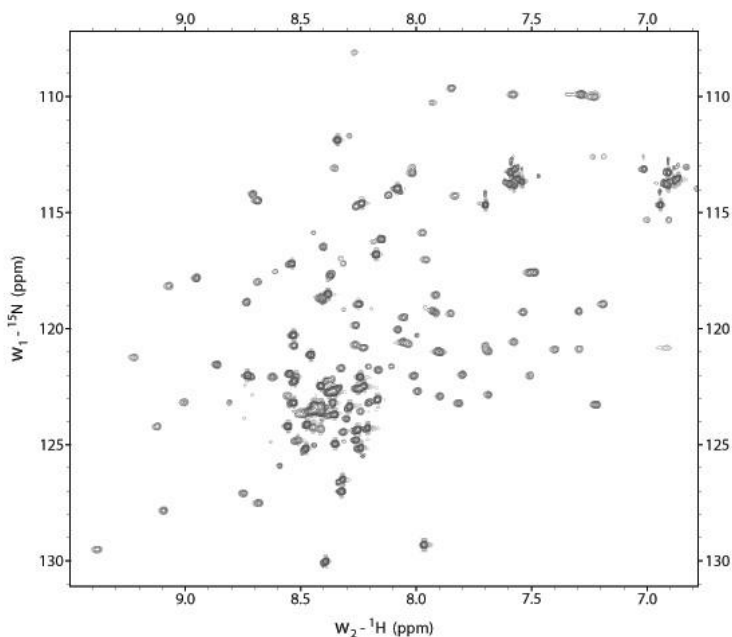


Figure 24: ^1H - ^{15}N HSQC spectra of ^{15}N -labelled Toad/NP_{TAIL}.

Therefore, based on the secondary structure predictions, a new NP_{TAIL} construct, consisting in the first 98 N-ter residues, was engineered, produced and purified similarly to the full length NP_{TAIL}. The sample was submitted to crystallization on the HTX platform (EMBL) and an initial crystallization condition using ammonium sulfate was identified and reproduced manually. The crystallization was carried out with 1:1 protein:crystallization solution ratio. The protein crystallized in 3 M ammonium sulfate in combination with various buffers. Crystals obtained in 0.1 M Tris-HCl pH 7.25, 3 M ammonium sulfate were analysed on PROXIMA-1 (synchrotron SOLEIL) and a native dataset was collected at 2.2 Å resolution. However, no structural homolog could be found to solve the structure by molecular replacement. Selenomethionine-labelled Toad/NP_{TAIL} crystals were then produced (crystals grown in 0.1 M Tris pH 7.5-7.75, 2.85-2.95 M ammonium sulfate). Diffraction data were collected on PROXIMA-1 with the multiple-wavelength anomalous dispersion (MAD) technique and processed with XDS¹⁵⁰. The structure was solved by Pierre Legrand (PROXIMA-1; SOLEIL) using the CCP4i programs for crystallography³⁹⁸. The final model was built using COOT⁷³ and refined at 2.2 Å resolution ($R_{\text{fact}} = 20\%$; $R_{\text{free}} = 24\%$) using REFMAC5 of the CCP4 suite (**Fig. 25**).

The asymmetric unit contained six molecules, with little difference in term of folding, flexibility and number of residues within the electron density. Residue 1 up to residue 77 could be assigned in the electron density map and residues 4 to 68 were folded in three distinct antiparallel α -helices (**Fig. 25**). This domain (residues 4-68) was compact, highly charged (pHi of 9.44 with 34% of charged residues (Arg, Lys, Glu and Asp)) and stabilized by many electrostatic interactions between side chains. The C-terminal part of the tail (residues 78 to 98) was not visible in the electron density maps, supposedly intrinsically disordered. Interestingly, residues 80 to 98 were composed for half of the sequence in glutamate residues.

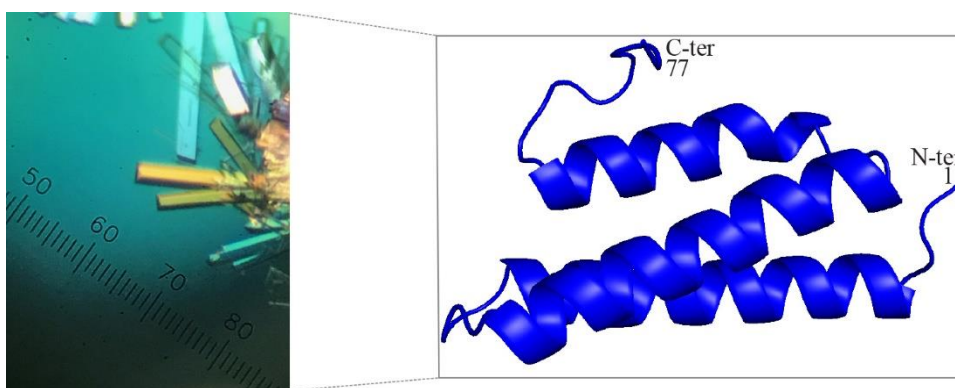


Figure 25: Crystals of Selenomethionine-labelled Toad/NP_{TAIL} and Toad/NP_{TAIL} solved structure.

One small graduation represents 10 μm .

Toad/NP_{FULL} were also crystallized using the HTX platform. Crystals were obtained in 0.1 M Bis Tris pH 7.5, 18-20 % PEG 3350, 0.2-0.25 M potassium thiocyanate. However, the packing seemed faulty: a five seconds-long exposure time was required to obtain a visible diffraction pattern at 8 \AA resolution on a single image. The space group was P4 with cell parameters $a = b = 256.4 \text{ \AA}$, $c = 215.3 \text{ \AA}$ and $\alpha = \beta = \gamma = 90^\circ$. As the long, flexible N-terminal tail could negatively affect the crystal packing, Toad/NP_{CORE} (residues 124 to 607) was crystallized to avoid such an issue. Toad/NP_{CORE} crystals were obtained in 0.1 M Bis-Tris pH 7.5, 14% PEG 3350, 0.2-0.25 M potassium thiocyanate. These crystals diffracted at 3.5 \AA (space group P2₁2₁2₁ with the following cell parameters $a = 46.5 \text{ \AA}$, $b = 56.8 \text{ \AA}$, $c = 357.4 \text{ \AA}$ and $\alpha = \beta = \gamma = 90^\circ$, suggesting two molecules in the asymmetric unit. The molecular replacement did not

offer any satisfying solution, thus crystals of selenomethionine-labelled Toad/NP_{CORE} were reproduced (0.1 M Bis-Tris pH 7.5, 12 % PEG 3350, 0.2 M potassium thiocyanate) for future synchrotron experiments (**Fig. 26**).

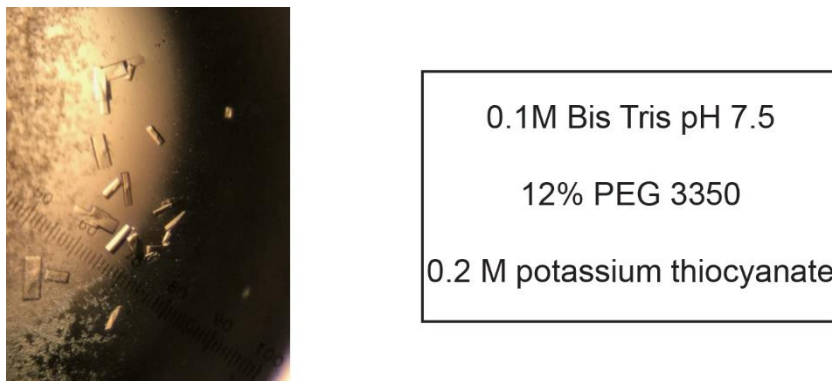


Figure 26: Crystals of Selenomethionine-labelled Toad/NP_{CORE} and its crystallization condition.

Two small graduations represent 10 μm .

IV. Discussion

ToadV has shown its phylogenetic proximity with IBV, its NP sharing a part of B/NP features but has also its own, very specific behaviour and structures.

Toad/NP seems to have a tendency to form tetramers in solution. The salt concentration in the buffer (150 mM or 300 mM NaCl) does not have a clear effect on the oligomerization state, similarly to what was observed for D/NP and in contrast to B/NP^{63,178}. Toad/NP possesses a long N-terminal tail, with several scattered basic residues, a pattern highly similar to the B/NP_{TAIL} NLS. A long stretch of residues (amino acids 30 to 71) of B/NP_{TAIL} is involved in the importins- α binding; it could be interesting to know if a similar behaviour is also found in Toad/NP_{TAIL}¹⁷⁷. Furthermore, the exact NLS motif of the tail is not known yet and further investigation are required to highlight the molecular basis of this particular interaction. Toad/NP_{TAIL} interactions with importins- α follow the same pattern as B/NP_{TAIL}, with high association and dissociation rates (outside of the detection range of the apparatus) and low affinities for the importin- α 5 and importin- α 7 (K_d of 1217 nM and 1024 nM respectively). Interestingly, Toad/NP_{TAIL} affinities are even weaker for these importins- α than B/NP_{TAIL} affinities, in the micromolar range and putatively outside of the functional range. In contrast, it

interacts more strongly with the importin- $\alpha 3$ than B/NP_{TAIL}, with an affinity (156 nM) suggesting that they could form a functional complex. However it is of note that human importins- α were used in this work. While the importin- $\alpha 3$, - $\alpha 5$, and - $\alpha 7$ from human and frog share a high sequence identity (94%, 94% and 90% respectively, **Annexe 1**), it is a possibility that these importins, and this nuclear import pathway as a whole, possess some species-specificities. The extrapolation, while possible, should be cautious.

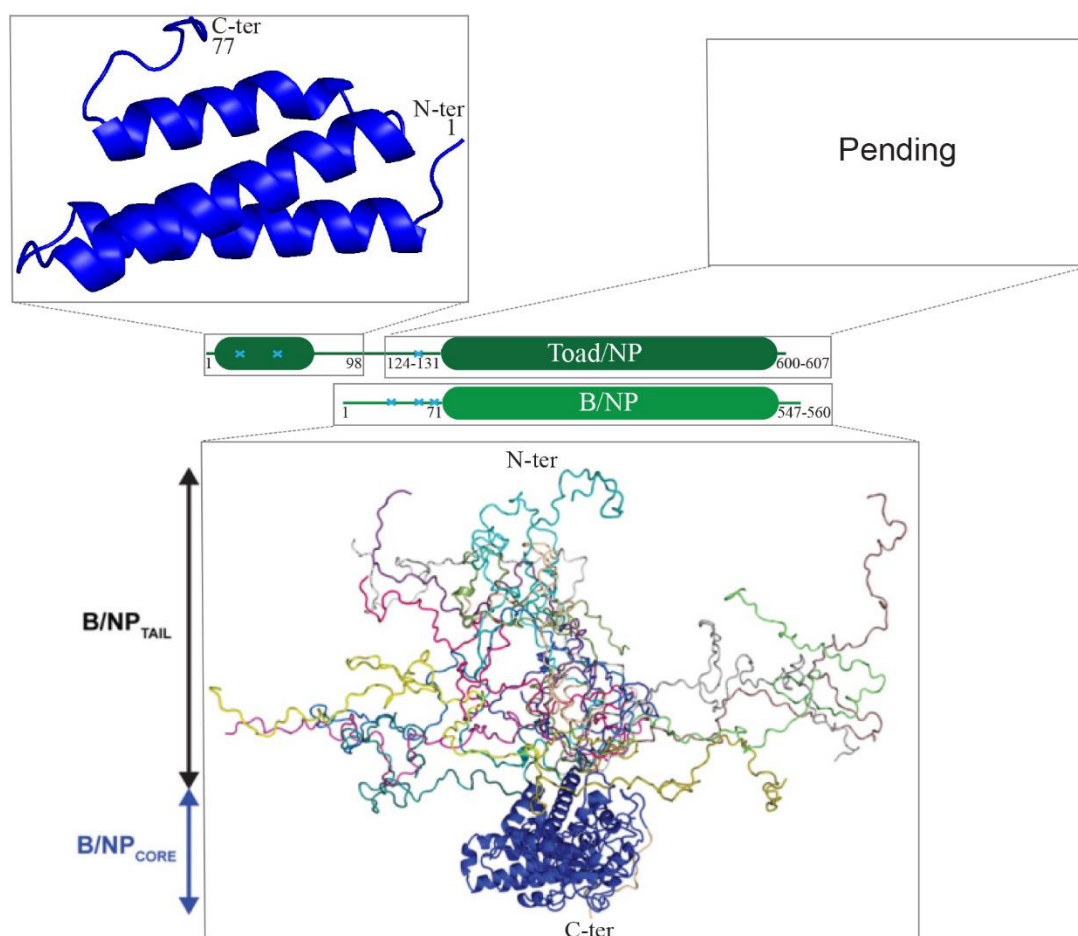


Figure 27: Structural features and comparison between Toad/NP and B/NP. Toad/NP N-terminal structure is a result of this PhD work whereas B/NP modelling was published by ¹⁷⁷.

Intrinsically disordered parts are represented as lines while folded parts are represented as rounded squares. Blue crosses represent potential NLS.

Toad/NP interacts with RNAs with a pattern similar to other influenza NPs, seemingly without sequence specificity, with cooperativity, with usually a nanomolar

affinity^{13,70,71,178,407}. One point was particularly interesting: while Toad/NP behaves similarly to B/NP, the Toad/NP_{TAIL} seems to play a unique role in RNA binding regulation, maybe through its Glutamate-rich region between residues 80 and 98. Interestingly, a negatively charged region is also found in C-terminal of influenza NPs and ISA/NP with several glutamate and aspartate, suspected to regulate the RNA-binding activity. Only ISA/NP shows such a Glutamate-rich pattern, which has been shown to impact RNA binding, but this motif is localized in the last 16 C-terminal residues. As Toad/NP does not show such an acidic C-terminal patch, it is possible to speculate about a change in localization of this particular region. As the Toad/NP C-terminal disordered tail is the shortest (8 residues) and its N-terminal one the longest, it is possible that both N-ter and C-ter tail functions could be found on the sole N-terminal Toad/NP_{TAIL}.

While the structure of Toad/NP_{CORE} is not known yet, the structure in the N-terminal tail was solved. It is neatly packed in three long antiparallel α -helices (14 - 20 residues), which do not seem too flexible, as no alternate conformation was found in the six molecules from the asymmetric unit. Regarding influenza NPs, this is the first domain found in a disordered N/C-terminal tail. B/NP, the Toad/NP closest relative, has a long N-terminal extremity that is entirely disordered. The contrast is striking between this folded domain within Toad/NP_{TAIL} and modelled B/NP_{TAIL}, extensively flexible and occupying a large volume (**Fig. 27**). This structural insight is particularly interesting for two points: firstly, the basic patches detected within the full length NP_{TAIL} are involved in importins- α binding and secondly, Glu-rich regions and low-complexity-regions are well known for their role as molecular chaperones or in protein:protein, protein:nucleic acid interactions^{45,368}. Both the folded N-terminal domain and the disordered segment require additional work to detail their roles.

Outside of this specific domain, while Toad/NP_{CORE} structure is still pending, it is highly plausible that it will be highly similar to the one of other NPs: their size is closely matched, their sequence relatively conserved and all *Orthomyxoviridae* NPs have so far revealed a structurally conserved core (**Fig. 27**).

Table 10: Sequence identities between influenza NP types.

Sequences were recovered from the Influenza Research Database ⁴¹⁹. The strains used for alignment are those used in this work. Alignment was realized using Clustal Omega ¹⁹⁹. Sala, AIEa and Salm stands for Salamander, Algae Eater and Salmon respectively.

	A NP	B NP	Toad NP	Sala NP	AIEa NP	Salm NP	Eel NP	Hagfish NP	C NP	D NP	Cane Toad NP	Frog NP
A NP	100	37.2	22.5	37.9	41.2	38.0	38.2	21.0	21.8	21.1	21.4	22.2
B NP		100	28.7	47.6	41.5	43.0	53.5	23.2	23.1	23.8	25.9	23.5
Toad NP			100	27.4	25.2	30.4	26.2	21.2	22.1	20.1	23.6	23.8
Sala NP				100	38.0	39.7	46.3	21.5	22.4	26.2	23.4	25.4
AIEa NP					100	37.8	39.1	22.2	21.9	21.1	23.6	22.3
Salm NP						100	41.2	19.7	22.8	22.4	24.7	24.1
Eel NP							100	21.6	23.7	23.8	23.4	22.2
Hagfish NP								100	21.4	20.1	20.8	22.3
C NP									100	37.5	38.3	38.7
D NP										100	47.3	51.2
Cane Toad NP											100	51.1
Frog NP												100

In September 2020, when the characterization of Toad/NP was still underway, a new study identified 5 new influenza-like viruses from extremely diverse organisms ²⁶⁸. Three of them turned out to be more closely related to IBV (one salamander-, one algae eater- and one salmon-infecting virus), and two, for the first time, were identified to be more closely related to IDV (infecting either a cane toad or a frog). Preliminary predictive structural analyses can be done with these new NPs sequences, based on the work on Toad/NP. The more surprising point was the high sequence identities found between influenza NPs and influenza-like NPs. B/NP shared between 42% and 54% sequence identities with Salamander/NP, Algae Eater/NP, Salmon/NP and Eel/NP (**Table 10**). Toad/NP was more divergent, probably branching out earlier, while still relatively close to B/NP. Similarly, D/NP and D-like/NPs shared high sequence identities (between 47 and 51%).

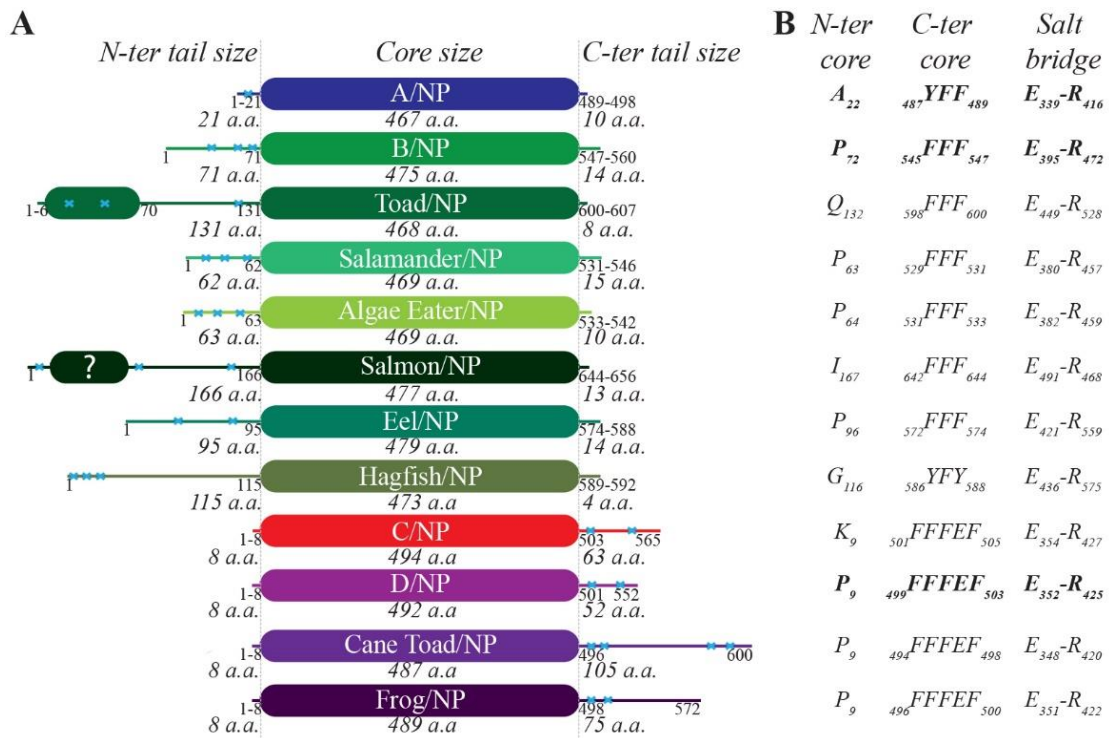


Figure 28: Diversity of newly identified NPs.

Structural predictions were performed using PsiPred¹⁴⁶. Intrinsically disordered parts are represented as lines while folded parts are represented as rounded squares. Blue crosses represent potential NLS. In (B), data coming from the crystal structures are in bold, in contrast with the predictive analyses.

All B-like/NPs seem to possess a structural organization similar to B/NP: a long intrinsically disordered N-terminal tail, a folded core, and a short C-ter tail (**Fig. 27A**). The N-terminal intrinsically disordered tail (NP_{TAIL}) was highly variable in size. Interestingly, all NP_{TAILS} possessed basic patches that could be NLSs, and one basic patch, while not conserved in sequence, was always found within the last 20-30 residues of NP_{TAILS} . A similar result came from the comparison of the organization of D/NP and D-like/NPs (**Fig. 27A**). Other hallmark features seemed to be conserved (**Fig. 27B**). The salt bridge essential for the oligomerization process (salt bridge between Glu-339 and Arg-416 in A/NP), the aromatic motif in C-terminal of the folded core and to some extent the N-terminal residue of the core could be identified for B-like and D-like NPs.

Now, considering Toad/NP and Cane Toad/NP, one intriguing feature appeared: they both were infecting a toad and their structural organization was mirrored: a NLS-bearing N-terminal NP_{TAIL} of 127 residues or a NLS-bearing C-ter NP_{TAIL} of 105 residues

respectively, a similarly sized core and the opposite terminal extremity made of 8 amino acids. Furthermore, a part of the Cane Toad/NP C-terminal tail is predicted potentially folded in one or two α -helices.

These predictive analyses confirm the judicious choice of characterizing Toad/NP, as it seems to have a unique position in the growing influenza family. This opens up many new avenues to probe at the evolutionary history and functionality of influenza viruses.

Conclusions

A large number of studies tried to dissect the IAV, IBV and ICV replication machineries function through the years, along with its essential interactions with the host cell machinery. Host: pathogen interactions are one of the more crucial interfaces, in term of function, host specificity, adaptation and therapeutic targets. The interaction of the influenza replication machinery with the cellular nuclear import system is one of them, yet little is known despite the number of publications on this interaction. The discoveries in 2011 of the influenza D virus and, during the course of this PhD, of the Asiatic toad-infecting influenza virus in 2018 were game-changing news, as the influenza world was by the past limited to three genera since the discovery of the influenza C virus in 1947, more than half a century ago. It is well known that our understanding of viruses, viral diversity and viral evolution is largely biased, but these two viruses managed to be surprising. IDV is more closely related to one of the influenza family member with a strongly restricted host spectrum (ICV) instead of the other genus with a broader host spectrum (IAV). Similarly, ToadV was phylogenetically closer to IBV than any other influenza genera.

During this PhD thesis, with a focus on the importin- α :NP_{TAIL} interaction, structural and functional insights have been gained on influenza NPs. Firstly, the structural and functional basis of D/NP initiated by Alice Labaronne, a previous PhD candidate in the lab, was finalised. In spite of its low sequence identity with A/NP and B/NP, the folded core of D/NP is structurally well conserved, with R.M.S.D. values of 1.9-2.1 Å. It presents the key conserved features of influenza NP. Firstly, the oligomerization mechanism is conserved, relying on a conserved salt bridge between a glutamate (*i.e.* E352, similar to A/NP E339) and an arginine (*i.e.* R425, similar to A/NP R416), along with other stabilizing contacts, more particularly hydrophobic contacts. The conserved hydrophobic aromatic motif localized at the C-terminal edge of the folded core (₄₈₇YFF₄₈₉ and ₅₄₅FFF₆₄₇ in A/NP and B/NP respectively) is also present in D/NP (₄₉₉FFF₅₀₁). Like B/NP, the N-terminal edge of the core is a Proline, at position 9. In contrast with A/NP and B/NP, only the 8 first residues are intrinsically disordered at the N-terminal extremity, while the last 52 amino acids are intrinsically disordered. This C-terminal NP_{TAIL} shows a clear bipartite NLS, responsible for the nuclear localization and the interaction with importins- α , with a strong affinity ($K_d = 100$ nM) for the importin- $\alpha 7$ ⁶³.

The huge differences between the tail localization, the NLS sequences, the importins- α studied and the affinities determined so far, along with the diversity of methods and techniques lead to the characterization and the comparison of all importin- α :NP_{TAIL} complexes within the same settings, in order to get a broader, more precise view of this interaction. As the A/NP_{TAIL}, B/NP_{TAIL} and C/NP_{TAIL} sequences came from human strains, it has been chosen to use human importins- α as partners; these proteins being highly conserved in higher eukaryotes, it should be possible to extrapolate the results to some extent. A SPR strategy has been set up to work on representatives of all NP types, so insights in the affinities and rates of these interactions were gained. In agreement with the cellular process, both the association and the dissociation rates are fast for such a strong *in vitro* interaction. Interestingly, each NP_{TAIL} type showed a specific, preferential binding, with a clear contribution of the importin- α paralog. D/NP_{TAIL}, as a bipartite NLS, interacted strongly with all importins- α without clear specificity, with a dissociation constant about 20-40 nM. C/NP_{TAIL}, while also a bipartite NLS, was a bit more versatile in its interactions with the importins, with a K_d ranging from 60 to 290 nM, importin- α 3 being the best candidate for the interaction. A/NP_{TAIL}, with its unclassical monopartite NLS, presented an affinity range similar to C/NP_{TAIL} (K_d 70-620 nM). The best “match” was this time the importin- α 7:A/NP_{TAIL} one. These results are particularly interesting and consistent, as the importin- α 3 and - α 7 seem to be the importins- α most expressed in the mammalian respiratory tract. B/NP_{TAIL} interaction features were the most puzzling fact, with faster rates and low affinity to all partners tested (no better than 310 nM), raising questions about its true importin partner, its state of post-translational modifications and its translocation process. These results provide a strong basis for the interaction between importins- α and influenza NPs, and highlight specificities from both NPs and importins not foreseen before. The major experienced drawback was the dimerization tendency of the importin- α 1, requiring another strategy to get insights on its behaviour towards NP_{TAILS}. These results are essential in the process of understand how this complex, tightly regulated interaction and translocation process works⁶⁴.

In the meantime, structural and functional investigations of Toad/NP were initiated, due to its surprising evolutionary proximity to B/NP and its tropism towards a cold-blooded animal. Integrated to our SPR strategy, the long N-terminal Toad/NP_{TAIL} shows a strikingly similar behaviour to B/NP_{TAIL}, with fast rates and low affinities. However, the

affinities range, depending on the importins- α , is more important, and the affinity of the Toad/NP_{TAIL}:importin- α 3 complex suggests a potential preferential interaction (K_d 160 nM). Several basic patches are identified within this tail, either in the intrinsically disordered part or in the α -helices. It is not known yet which basic patch is responsible for the interaction. Regarding Toad/NP_{CORE} interaction with RNAs, its behaviour matches the A/NP and B/NP ones determined by the team at 150 mM NaCl: it seems that a 12-mer RNA is sufficient to saturate the RNA-binding groove of the NP. The most particular feature comes from Toad/NP_{TAIL}: the removal of the long tail positively affects the binding activity, suggesting a regulatory role, putatively carried out by the Glutamate-rich sequence found in the region 80-98. While the C-terminal extremity of influenza NPs has been shown to negatively regulate the RNA-binding activity, it is the first time another potential mechanism has been found at work. In term of structural organization and in contrast with other influenza NPs, Toad/NP_{TAIL} showed a folded domain within the N-terminal tail, packed in three long α -helices. While ISA/NP in the *Orthomyxoviridae* family also present a folded domain in N-terminal, this latter is organized around 3 β -strands and 2 α -helices. So far, the function(s) of these domains is(are) not known. Structural insights on Toad/NP_{CORE} has not been gained yet, but are expected in the future, as crystals of selenomethionine NP_{CORE} has been obtained.

Globally, while this PhD work did not apply the SPR strategy to the polymerase subunits as was initially envisaged, it allowed the successful characterization of all NP_{TAILS}:importins- α , which is a crucial interaction both at the NP and the RNP levels. Furthermore, the characterization of the newly discovered D/NP and Toad/NP revealed interesting features and specificities, highlighting how differently influenza NPs could behave and be organized, while still retaining a strong common structural and functional basis.

Annexes

Annexe 1: Sequence identities between importins- α

Table S1: Sequence identities between importins- α paralogs of distinct species of interest.

Sequences and accession numbers were recovered from NCBI/Uniprot databases for human (*Homo sapiens*) importins- α . Each BLAST was done separately, using one human importin sequence against reference protein sequences from *Sus scrofa* (swine), *Bos Taurus* (bovine), *Gallus gallus* (chicken), *Anas platyrhynchos* (duck) and *Xenopus tropicalis* (frog). As no duck importin- α 7 was identified in the NCBI database, the corresponding sequence was recovered from Uniprot database and aligned with a BLAST against reference protein sequences from *Homo sapiens* (bold). 99+ corresponds to a sequence identity between 99.5 and 100 %. No importin- α 6 was found for *Xenopus tropicalis*. Importins- α from the same subfamily are highlighted in the same colour.

	Subfamily Member	Human						
		α 1		α 2		α 3		
		Imp- α 1	Imp- α 8	Imp- α 3	Imp- α 4	Imp- α 5	Imp- α 6	Imp- α 7
Human	Imp- α 1	100						
	Imp- α 8	55	100					
	Imp- α 3	51	45	100				
	Imp- α 4	50	46	86	100			
	Imp- α 5	46	42	47	48	100		
	Imp- α 6	48	42	48	49	80	100	
	Imp- α 7	48	42	49	48	82	84	100
Swine	Imp- α 1	97	54	51	49	45	48	47
	Imp- α 8	55	75	46	48	41	42	41
	Imp- α 3	52	45	99	86	47	48	48
	Imp- α 4	50	46	86	99	49	49	49
	Imp- α 5	46	42	46	48	99	80	81
	Imp- α 6	49	43	48	49	79	97	84
	Imp- α 7	48	42	48	48	81	84	99
Bovine	Imp- α 1	95	54	52	51	46	48	47
	Imp- α 8	55	75	46	46	42	42	41
	Imp- α 3	51	45	99+	86	47	48	48
	Imp- α 4	50	46	86	99	49	49	49
	Imp- α 5	46	42	46	48	99	80	81
	Imp- α 6	48	42	48	49	80	98	84
	Imp- α 7	48	42	48	49	81	84	99
Chicken	Imp- α 1	83	55	51	51	46	47	47
	Imp- α 8	60	61	49	50	43	45	44
	Imp- α 3	51	45	99	86	47	48	48
	Imp- α 4	50	46	86	98	49	49	49
	Imp- α 5	46	42	46	49	96	79	81
	Imp- α 6	49	44	48	49	81	94	84
	Imp- α 7	48	43	48	49	81	84	94
Duck	Imp- α 1	84	22	51	51	46	47	46
	Imp- α 8	60	60	49	48	42	44	43
	Imp- α 3	51	45	98	86	48	48	48
	Imp- α 4	51	46	86	99	49	49	48
	Imp- α 5	46	42	46	48	96	79	81
	Imp- α 6	48	44	47	49	81	94	84
	Imp- α 7	48	42	49	49	82	85	95
Frog	Imp- α 1	77	53	52	51	46	47	47
	Imp- α 8	62	60	49	49	43	44	44
	Imp- α 3	51	45	94	84	46	47	47
	Imp- α 4	50	45	85	95	48	50	49
	Imp- α 5	46	43	46	48	94	79	81
	Imp- α 7	49	44	48	51	79	83	90

Annexe 2: Conservation of NP_{TAIL} within each influenza type

Table S2: Consensus residues and frequencies of A/NP_{TAIL}.

The sequences were recovered from the Influenza Research Database and processed with the Sequence Variation (SNP) tool. The frequency was determined manually.

Position	Consensus (57,774 seq)	Frequency	Polymorphism (> 10%)
1	Met	100 %	
2	Ala	99.9 %	
3	Ser	99.4 %	
4	Gln	99.9 %	
5	Gly	99.97 %	
6	Thr	99.97 %	
7	Lys	99.97 %	
8	Arg	99.99 %	
9	Ser	99.3 %	
10	Tyr	99.7 %	
11	Del	99.997 %	
12	Glu	99.98 %	
13	Gln	99.9 %	
14	Met	99.99 %	
15	Glu	99.9 %	
16	Thr	99.9 %	
17	Gly	66%	Asp (33 %)
18	Gly	99.9%	
19	Glu	72%	Asp (28 %)
20	Arg	99.95%	
21	Gln	99.9%	

Table S3: Consensus residues and frequencies of B/NP_{TAIL}.

The sequences were recovered from the Influenza Research Database and processed with the Sequence Variation (SNP) tool. The frequency was determined manually.

Position	Consensus (9,209 seq)	Frequency	Polymorphism (> 10%)	Position	Consensus (9,209 seq)	Frequency	Polymorphism (> 10%)	Position	Consensus (9,209 seq)	Frequency	Polymorphism (> 10%)	
1	Met	100 %		25	Thr	98 %		49	Pro	100 %		
2	Ser	100 %		26	Ser	99.6 %		50	Ser	100 %		
3	Asn	99.99 %		27	Gly	99.97 %		51	Pro	99.8 %		
4	Met	100 %		28	Thr	99 %		52	Glu	99.9 %		
5	Asp	99.99 %		29	Thr	94.9 %		53	Arg	99.5 %		
6	Ile	99.96 %		30	Arg	99.97 %		54	Ala	99.6 %		
7	Asp	100 %		31	Pro	99.95 %		55	Thr	99.8 %		
8	Gly	99.5 %		32	Ile	99.9 %		56	Thr	99.8 %		
9	Ile	69 %		Met (30 %)	33	Ile		99.98 %	57	Ser		96 %
10	Asn	99.7 %			34	Arg		99.8 %	58	Ser		96 %
11	Thr	99.5 %	35		Pro	99.99 %	59	Glu	99.9 %			
12	Gly	99.9 %	36		Ala	99.98 %	60	Asp	92 %			
13	Thr	99.7 %	37		Thr	99.95 %	61	Asp	99.5 %			
14	Ile	99.7 %	38		Leu	100 %	62	Val	90 %			
15	Asp	99.8 %	39		Ala	99.9 %	63	Gly	98.95 %			
16	Lys	99.9 %	40		Pro	99.97 %	64	Arg	99 %			
17	Thr	94.7 %	41		Pro	99.95 %	65	Lys	99 %			
18	Pro	98.9 %	42		Ser	99.6 %	66	Thr	72 %			
19	Glu	99.95 %	43		Asn	99.9 %	67	Gln	99.8 %			
20	Glu	99.98 %	44	Lys	99.98 %	68	Lys	99.7 %				
21	Ile	99 %	45	Arg	100 %	69	Lys	99.95 %				
22	Thr	99 %	46	Thr	99.7 %	70	Gln	100 %				
23	Ser	99.7 %	47	Arg	99.99 %	71	Thr	99.99 %				
24	Gly	99.9 %	48	Asn	99.9 %							

Table S4: Consensus residues and frequencies of C/NP_{TAIL}.

The sequences were recovered from the Influenza Research Database and processed with the Sequence Variation (SNP) tool. The frequency was determined manually.

Position	Consensus (50 seq)	Frequency	Polymorphism (> 10%)	Position	Consensus (50 seq)	Frequency	Polymorphism (> 10%)
503	Phe	100%		535	Gly	100%	
504	Glu	100%		536	Met	100%	
505	Phe	100%		537	Asp	100%	
506	Asp	100%		538	Glu	100%	
507	Pro	100%		539	Asn	100%	
508	Asp	100%		540	Ser	88%	Pro (12%)
509	Tyr	100%		541	Glu	100%	
510	Asn	100%		542	Ile	98%	
511	Pro	100%		543	Gly	100%	
512	Ile	100%		544	Gln	100%	
513	Arg	100%		545	Ala	100%	
514	Val	94%		546	Lys	100%	
515	Lys	100%		547	Lys	100%	
516	Arg	100%		548	Met	100%	
517	Pro	100%		549	Lys	100%	
518	Lys	100%		550	Pro	100%	
519	Lys	98%		551	Leu	98%	
520	Pro	100%		552	Asp	98%	
521	Ile	98%		553	Gln	98%	
522	Ala	94%		554	Leu	96%	
523	Lys	100%		555	Ala	98%	
524	Arg	100%		556	Ser	98%	
525	Asn	98%		557	Thr	100%	
526	Ser	100%		558	Ser	100%	
527	Asn	100%		559	Ser	98%	
528	Ile	100%		560	Asn	100%	
529	Ser	100%		561	Ile	100%	
530	Arg	100%		562	Pro	100%	
531	Leu	100%		563	Gly	100%	
532	Glu	100%		564	Glu	94%	
533	Glu	100%		565	Asn	100%	
534	Glu	100%					

Table S5: Consensus residues and frequencies of D/NP_{TAIL}.

The sequences were recovered from the Influenza Research Database and processed with the Sequence Variation (SNP) tool. The frequency was determined manually.

Position	Consensus (34 seq)	Frequency	Position	Consensus (34 seq)	Frequency
501	Phe	100 %	527	Thr	100 %
502	Glu	100 %	528	Ser	100 %
503	Phe	100 %	529	Gln	94 %
504	Thr	100 %	530	Pro	100 %
505	Gly	100 %	531	Lys	100 %
506	Ser	100 %	532	Lys	100 %
507	Asp	100 %	533	Arg	100 %
508	Val	100 %	534	Gly	100 %
509	Pro	100 %	535	Arg	100 %
510	Arg	100 %	536	Gln	100 %
511	Thr	100 %	537	Gly	100 %
512	Gly	100 %	538	Ala	100 %
513	Ala	100 %	539	Gly	100 %
514	Lys	100 %	540	Ala	94 %
515	Arg	100 %	541	Glu	100 %
516	Arg	100 %	542	Ser	100 %
517	Val	94 %	543	Ser	100 %
518	Gly	100 %	544	Met	100 %
519	Gly	91 %	545	Asp	100 %
520	Ala	100 %	546	Ile	100 %
521	Asp	100 %	547	Glu	100 %
522	Asp	97 %	548	Thr	100 %
523	Val	91 %	549	Val	100 %
524	Thr	100 %	550	Gly	91 %
525	Pro	94 %	551	Glu	100 %
526	Gly	100 %	552	Asp	97 %

Annexe 3: Protein accession numbers

Table S6: Protein accession numbers.

		NCBI	Uniprot
Human (<i>Homo sapiens</i>)	Imp-α1	NP_001307540	P52292
	Imp-α8	NP_001139187.1	A9QM74
	Imp-α3	NP_002259.1	O00629
	Imp-α4	NP_002258.2	O00505
	Imp-α5	NP_002255.3	P52294
	Imp-α6	NP_001353233.1	O15131
	Imp-α7	NP_036448.1	O60684
Swine (<i>Sus scrofa</i>)	Imp-α1	NP_001156876.1	
	Imp-α8	NP_001156883.1	
	Imp-α3	XP_020925394.1	
	Imp-α4	NP_001177159.1	
	Imp-α5	NP_001156877.1	
	Imp-α6	XP_005654449.1	
	Imp-α7	XP_020951465.1	
Bovine (<i>Bos Taurus</i>)	Imp-α1	NP_001029621.1	
	Imp-α8	NP_001157419.1	
	Imp-α3	NP_001152788.1	
	Imp-α4	NP_001179702.3	
	Imp-α5	NP_001075202.1	
	Imp-α6	XP_002690143.2	
	Imp-α7	NP_001069422.1	
Chicken (<i>Gallus gallus</i>)	Imp-α1	NP_001006209.1	
	Imp-α8	XP_414795.3	
	Imp-α3	NP_001007964.1	
	Imp-α4	NP_001180504.1	
	Imp-α5	NP_001025945.1	
	Imp-α6	XP_419770.3	
	Imp-α7	NP_001012859.2	
Duck (<i>Anas platyrhynchos</i>)	Imp-α1	XP_005012369.2	
	Imp-α8	XP_012955615.2	
	Imp-α3	XP_027320524.1	
	Imp-α4	XP_027327221.1	
	Imp-α5	XP_027305514.1	
	Imp-α6	XP_027310463.1	
	Imp-α7		U3IY73
Frog (<i>Xenopus tropicalis</i>)	Imp-α1	NP_001008155.1	
	Imp-α8	NP_001135557.1	
	Imp-α3	XP_002933281.2	
	Imp-α4	NP_001016060.1	
	Imp-α5	NP_989192.1	
	Imp-α7	NP_001008018.1	
	Influenza viruses	A/NP	5TJW6_A
B/NP		AAU94830	
C/NP		YP_089656	
D/NP		CEE50063	
Toad/NP		AVM87634	
Eel/NP		AVM87623	
Hagfish/NP		AVM87638	
Salamander/NP		QOE76812	
Salmon/NP		QOE76828	
Algae Eater/NP		QOE76804	
Cane Toad/NP	QOE76821		
Frog/NP	QOE76796		

Annexe 4: Supplementary Data Chapter 2

The structure of the nucleoprotein of Influenza D shows that all *Orthomyxoviridae* nucleoproteins have a similar NP_{CORE}, with or without a NP_{TAIL} for nuclear transport

Amélie DONCHET¹, Justine OLIVA², Alice LABARONNE¹, Laura TENGO¹, Myriam MILOUDI¹, Francine C. A. GERARD¹, Caroline MAS³, Guy SCHOEHN¹, Rob W. H. RUIGROK¹, Mariette DUCATEZ² and Thibaut CREPIN^{1*}

¹ Institut de Biologie Structurale (IBS), Univ. Grenoble Alpes, CEA, CNRS, 38044 Grenoble, France

² IHAP, Université de Toulouse, INRA, ENVT, Toulouse, France

³ Integrated Structural Biology Grenoble (ISBG) - UMS 3518 (CNRS-CEA-UJF-EMBL), 38044 Grenoble, France

Supplementary information

Supplementary Figure 1

Sequence alignment of NP from representative Influenza strains. The sequences used are the same as in Table 1. The figure has been drawn using ESPript ¹. The upper and lower secondary structures correspond to the X-ray structures of D/NP (PDB entry 5N2U) and A/NP (PDB entry 2IQH), respectively. The stars indicate the phosphorylation sites identified for A/NP (closed symbol) and B/NP (open symbol) by Hutchinson *et al.* (red)², Li *et al.* (green)³ and Monda *et al.* (blue)⁴.

Supplementary Figure 2

Effect of trimethylamine N-oxide (TMAO) on the folding of D/NP_{TAIL}. Far UV-circular dichroism spectra of either D/NP_{TAIL} alone (purple) or in presence of TMAO (0.5 M in yellow, 1M in red, 2M in blue and 3 M in green) were collected. Because of the high background induced by the presence of TMAO around 200 nm, spectra were cut when the voltage (right bottom insert) became too high (> 700 V).

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Supplementary Table 1: Crystallographic data collection and structure refinement statistics. Values in parentheses are for highest resolution shell.

Crystal Parameters	D/NP
Data collection	
Beamline	ID30A3 - ESRF
Wavelength (Å)	0.9677
Space group	P1
Cell Dimensions a, b, c (Å)	75.17, 85.17, 103.39
α, β, γ (°)	91.20, 101.94, 101.02
Molecules per asymmetric unit	4
Wilson B-factor (Å ²)	51.4
Resolution (Å)	40 - 2.4 (2.48 - 2.4)
Observed Reflections	191 888 (18 695)
Unique Reflections	87 824 (8 505)
Completeness (%)	91.3 (94.1)
$R_{\text{sym}}(I)^{(a)}$ (%)	9.3 (84.7)
$\langle I/\sigma(I) \rangle$	7.78 (1.05)
Model quality indicators	
R_{cryst} (%)	20.2 (21.9)
R_{free} (%)	25.2 (23.8)
Non-hydrogen atoms	14 646
Water atoms	235
Average B-factors (Å ²)	55.7
B-factors per chains (Å ²)	59.8, 54.3, 52.8, 56.3
B-factors water (Å ²)	49.5
Ramachandran favoured / allowed (%)	94.0 / 5.1
rms deviations, bonds (Å) / angles (°)	0.009 / 1.15
Clashscore ^(b)	4.31
MolProbity score ^(b)	2.28

^(a) $R_{\text{sym}} = \frac{\sum_{hkl} \sum_j |I_{hkl,j} - \langle I_{hkl} \rangle|}{\sum_{hkl} \sum_j I_{hkl,j}}$

^(b) calculated using MolProbity ⁵

D/NP
 C/NP
 toad/NP MATQEQA...
 B/NP
 A/NP
 MSNMDIDGINTGTIDKAPE

1 10 20 30
 D/NP MDSTKAC...
 C/NP MSDRRQNRKT...
 toad/NP EERDENMSEED...
 B/NP EITSGTSGTTRP...
 A/NP MATKGT...
 * * *

40 50 60 70 80 90 100
 D/NP VSE...
 C/NP LNG...
 toad/NP LATA...
 B/NP LND...
 A/NP LSY...
 * * *

110 120 130 140 150 160 170 180
 D/NP VEAL...
 C/NP IVQ...
 toad/NP LMD...
 B/NP VAE...
 A/NP LRI...
 * * * A/NP Ser-165 * * *

190 200 210 220 230 240 250
 D/NP VAS...
 C/NP IAD...
 toad/NP ASRA...
 B/NP LARS...
 A/NP VME...
 * * *

260 270 280 290 300 310 320 330
 D/NP MLD...
 C/NP IAE...
 toad/NP IAQ...
 B/NP LARS...
 A/NP LAR...
 * * *

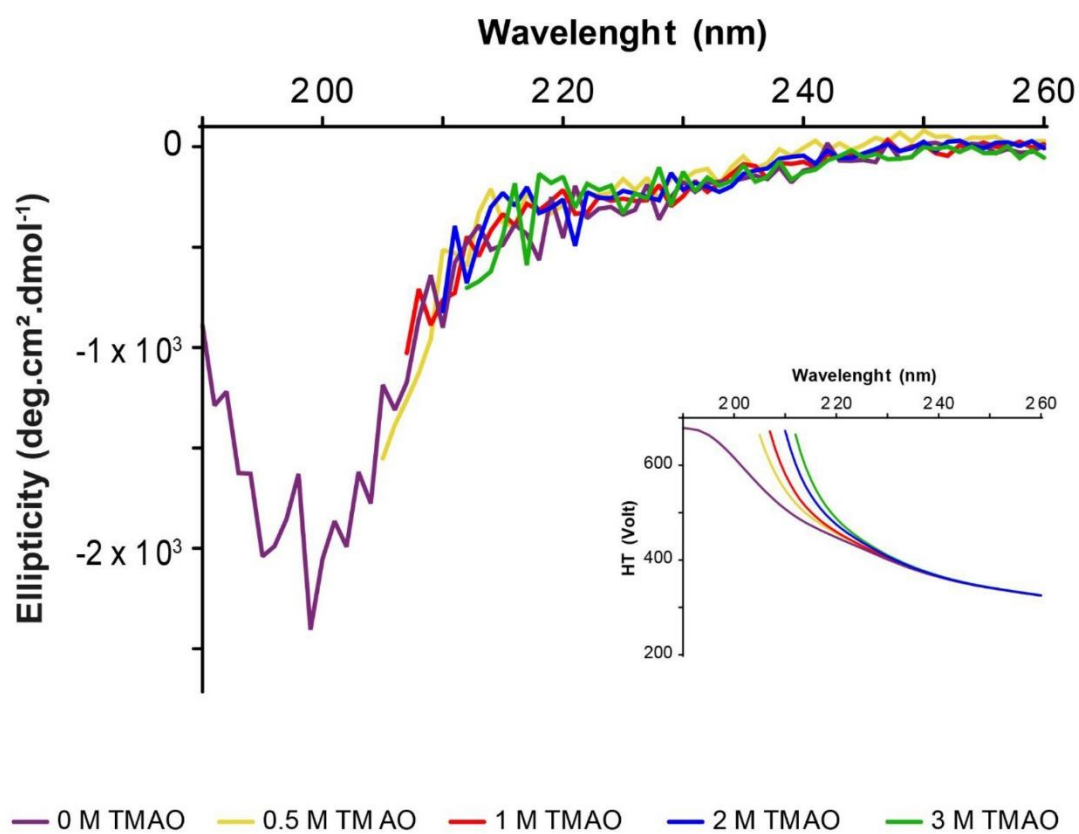
340 350 360 370 380 390 400
 D/NP NDK...
 C/NP EDK...
 toad/NP NDK...
 B/NP DKRS...
 A/NP AHS...
 * * *

exchange domain

410 420 430 440 450 460 470 480
 D/NP TGE...
 C/NP SRE...
 toad/NP DER...
 B/NP DGG...
 A/NP RAS...
 * * * A/NP Ser-407 * * *

490 500 510 520 530 540 550
 D/NP NPK...
 C/NP NPN...
 toad/NP RPP...
 B/NP NPE...
 A/NP SPV...
 * * *

Supplementary Figure 2



Annexe 5: Supplementary Data Chapter 3

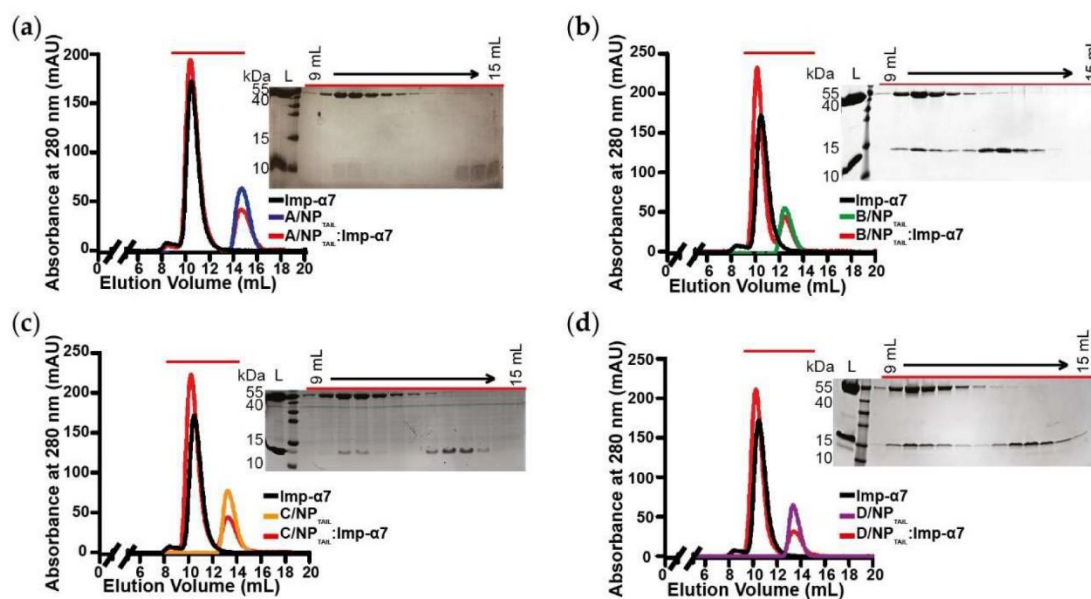


Figure S1. Analysis of the complexes between NP_{TAIL} and human importin- α 7 in solution. Each NP_{TAIL} (60 μ M) was injected on a SuperdexTM 75 10/300GL column, alone or in presence of importin- α 7 (25 μ M). The figure shows the superimposition of the size exclusion chromatography profiles obtained for (a) A/NP_{TAIL}, (b) B/NP_{TAIL}, (c) C/NP_{TAIL} and (d) D/NP_{TAIL}. The colors correspond to the code used on Figure 1, with the complexes in red and the importin- α 7 alone in black. For each panel, the 18 % SDS-PAGE corresponding to the elution of the complex is shown.

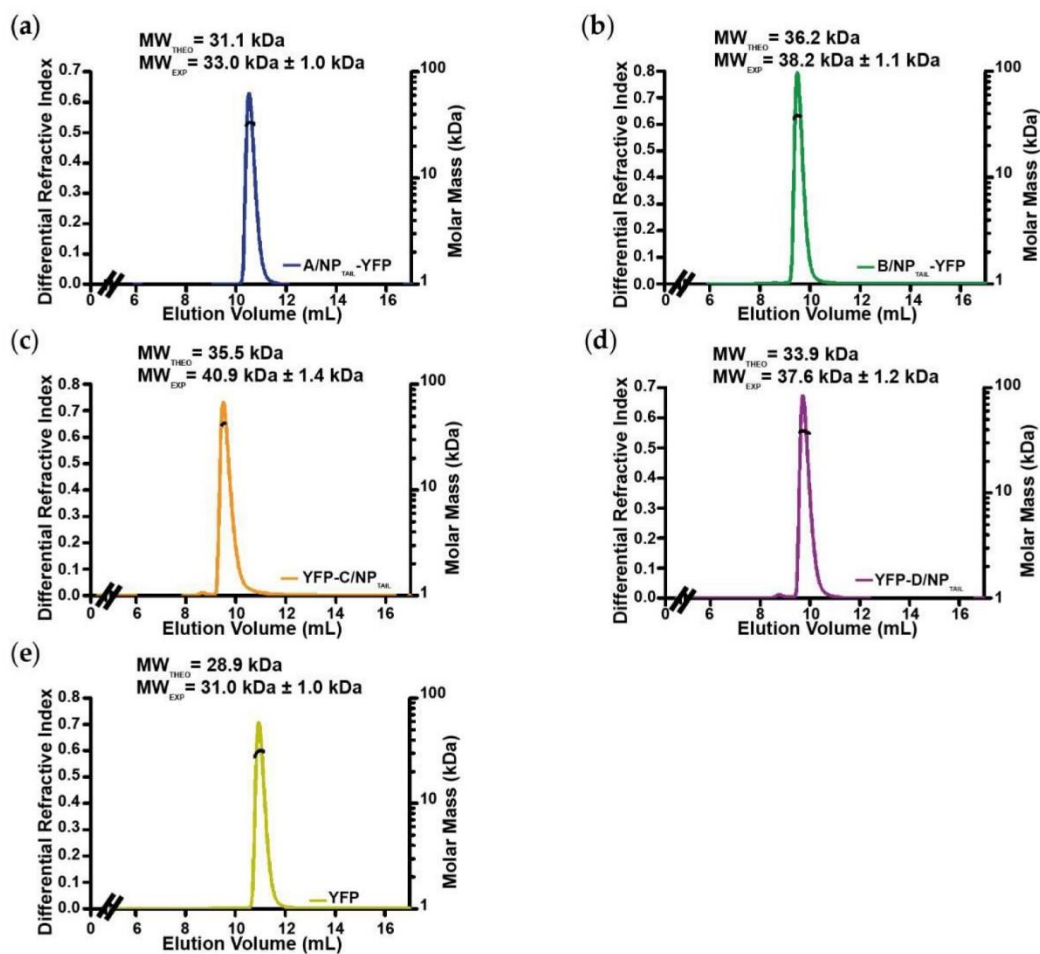


Figure S2. SEC-MALLS-RI analysis of the different YFP-fused tails used in that work. All the samples were injected on a Superdex™ 75 10/300GL column. Each panel indicates the theoretical (top) and experimental (bottom) molecular weights of (a) A/NP_{TAIL}-YFP, (b) B/NP_{TAIL}-YFP, (c) YFP-C/NP_{TAIL}, (d) YFP-D/NP_{TAIL} and (e) the control YFP. The black lines indicate the molecular weight as estimated below the peak.

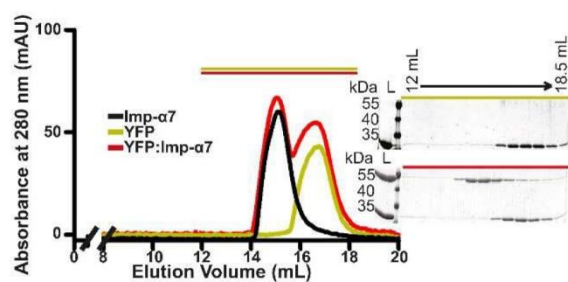


Figure S3. Absence of interaction between YFP and human importin- α 7 in solution. The YFP (30 μ M) was injected on a SuperdexTM 200 increase 10/300GL column, alone or in presence of importin- α 7 (25 μ M). The 12 % SDS-PAGE corresponding to the elution of the YFP alone (top) and the sample containing both YFP and importin- α 7 (bottom) are shown.

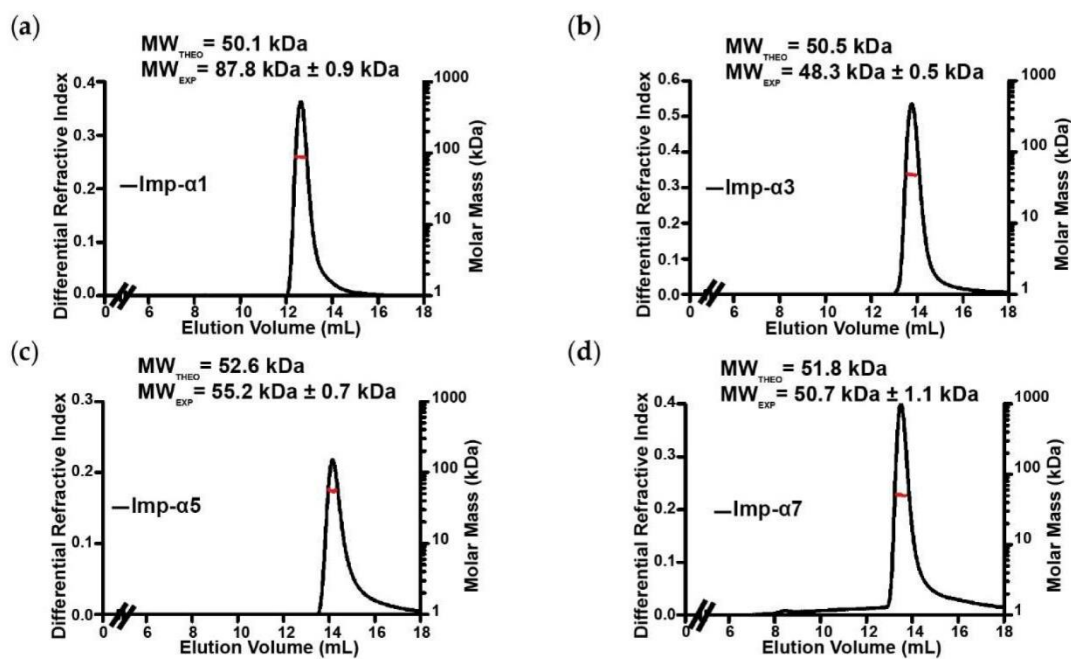


Figure S4. SEC-MALLS-RI analysis of the different importins- α used in that work. All the samples were injected on a Superdex™ 200 increase 10/300GL column. Each panel indicates the theoretical (top) and experimental (bottom) molecular weights of (a) importin- α 1, (b) importin- α 3, (c) importin- α 5 and (d) importin- α 7. The red lines indicate the molecular weight as estimated below the peak.

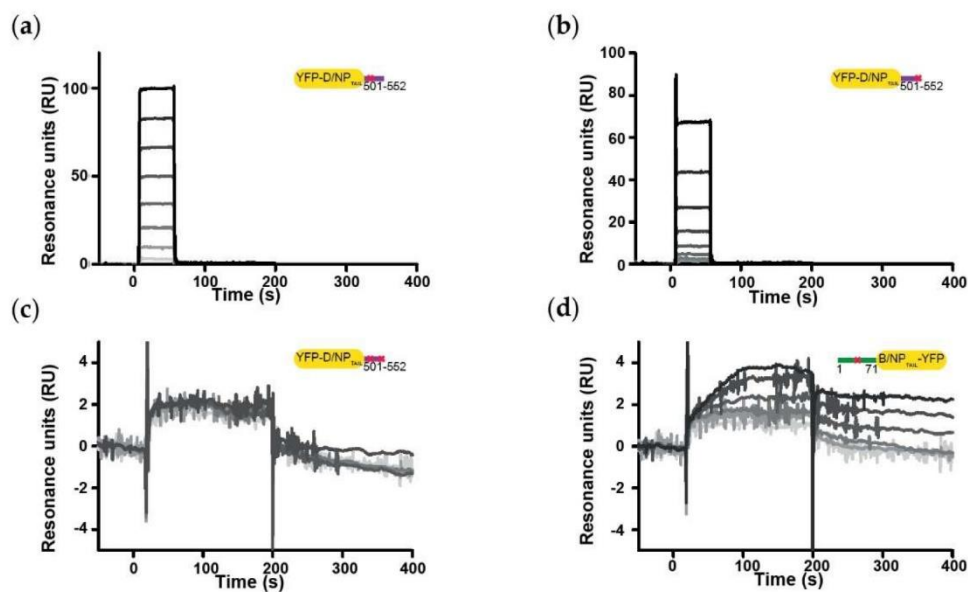


Figure S5. Interaction between human importin- α 7 and YFP-NP_{TAILS} mutants. The figure shows the sensorgrams of the interaction between importin- α 7 and (a) YFP-D/NP_{TAILS} mut1, (b) YFP-D/NP_{TAILS} mut2, (c) YFP-D/NP_{TAILS} mut3, and (d) YFP-B/NP_{TAILS} mut1. For each panel, the gradation of grey represents the different concentrations of importin- α 7 used for the titrations, from the lowest (light-grey) to the highest (dark-grey). The concentrations range from 80 nM to 10.3 μ M for (a) and (c) and from 41 nM to 4 μ M for (c) and (d). For a more accurate comparison, (c) and (d) sensorgrams were recovered from the same kinetic assay.

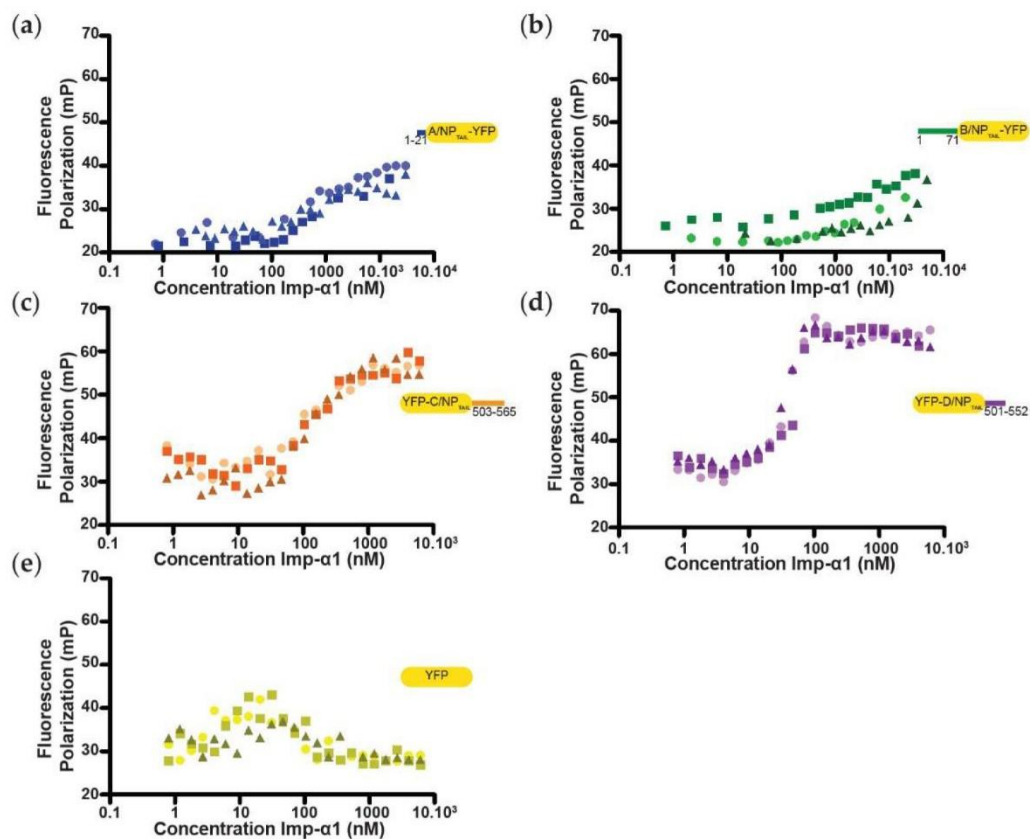


Figure S6. Interaction between human importin- α 1 and YFP-NP_{TAILS}. The figure shows the fluorescence polarization raw data of the interaction between importin- α 1 and (a) A/NP_{TAIL}-YFP, (b) B/NP_{TAIL}-YFP, (c) YFP-C/NP_{TAIL}, (d) YFP-D/NP_{TAIL} and (e) YFP control. For each panel, one shade of colour with one specific symbol represent one of the three replicate.

Table S1: Sequences. The table details the sequences of the nucleoproteins tails and biotinylation motif used in this work. The basic residues of the tails are shown in blue and the corresponding mutations in red. Biotinylated lysine are in green.

A/NP_{TAIL}	
WT	1MATKGT KRS YEQMETDGERQN ₂₁
B/NP_{TAIL}	
WT	1MSNMDIDGINTGTIDKAP EE ITSGTSGTTRPIIRPATLAPPSN KR TRNPSPERATTISEADV GRKTQKKQT ₇₁
B/NP _{TAIL} . mut1	1MSNMDIDGINTGTIDKAP EE ITSGTSGTTRPIIRPATLAPPSN AA TRNPSPERATTISEADV GRKTQKKQT ₇₁
C/NP_{TAIL}	
WT	503FEFDPDYNPIRV KRP KKPIA KR NSNISRLEEEGMDENSEIGQA KK MKPLDQLTSTSSNIPGKN ₅₆₅
D/NP_{TAIL}	
WT	501FEFTGSDVPRTGA KRR VGGADDVTLGTSQP KKR GRQGAGVESSMDIETVGED ₅₅₂
D/NP _{TAIL} . mut1	501FEFTGSDVPRTGA AA RVGGADDVTLGTSQP KKR GRQGAGVESSMDIETVGED ₅₅₂
D/NP _{TAIL} . mut2	501FEFTGSDVPRTGA KRR VGGADDVTLGTSQP AA AGAQGAGVESSMDIETVGED ₅₅₂
D/NP _{TAIL} . mut3	501FEFTGSDVPRTGA AA RVGGADDVTLGTSQP AA AGAQGAGVESSMDIETVGED ₅₅₂
Synthetic peptide	
control	1LEEM KK GHLE RE CMEETCSYEEA RE VFEDSEKTNEFWN K ₃₉
Biotinylation sequences	
N-terminal	DIFEAQ K IEWHEGGNGSGGGLN
C-terminal	NGSGGGLNDIFEAQ K IEWHE

Table S2: Sequence identity between the importins- α used in the studies. Sequences and accession numbers were recovered from NCBI/Uniprot databases for human (*Homo sapiens*) importin- α 1 (NP_001307540 / P52292), importin- α 3 (NP_002259.1 / O00629), importin- α 5 (NP_002255.3 / P52294) and importin- α 7 (NP_036448.1 / O60684). Each BLAST was done separately, using one human importin sequence against reference protein sequences from *Sus scrofa* (swine), *Bos Taurus* (bovine), *Gallus gallus* (chicken) and *Anas platyrhynchos* (duck). As no duck importin- α 7 was identified in the NCBI database, the corresponding sequence was recovered from Uniprot database (Uniprot accession number U3IY73) and aligned with each human importin alpha to get a sequence identity by global alignment (bold), 99+ corresponds to a sequence identity between 99.5 and 100 %. Importins- α from the same subfamily are highlighted in the same colour.

	Subfamily Member	Human			
		sub- α 1 Imp- α 1	sub- α 2 Imp- α 3	sub- α 3 Imp- α 5 Imp- α 7	
Human	Imp- α 1	100	51	46	48
	Imp- α 3		100	47	49
	Imp- α 5			100	82
	Imp- α 7				100
Swine	Imp- α 1	97	50	45	47
	Imp- α 3	52	99+	47	48
	Imp- α 5	46	46	99	81
	Imp- α 7	48	48	81	99
Bovine	Imp- α 1	95	82	46	47
	Imp- α 3	51	99+	47	48
	Imp- α 5	46	46	99	81
	Imp- α 7	48	48	81	99
Chicken	Imp- α 1	83	51	46	47
	Imp- α 3	51	99	47	48
	Imp- α 5	46	46	96	81
	Imp- α 7	48	48	81	94
Duck	Imp- α 1	84	51	46	46
	Imp- α 3	51	98	47	48
	Imp- α 5	46	46	96	81
	Imp- α 7	46	48	82	94

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Abstract

In 2014, American and Chinese studies have identified the influenza D virus (IDV). This new member of the Orthomyxoviridae family shares only 50% sequence identity with the already well characterized influenza C virus and less than 30% sequence identity with influenza A and B viruses. IDV has been detected in pigs and cattle, and has been shown to replicate in the ferret, the main animal model for influenza virus studies in humans, suggesting a possible transmission. In 2018, a new study identified the first influenza viruses infecting cold-blooded animals. A phylogenetic comparison highlighted that these viruses, and more particularly a toad-infecting virus (ToadV), were closer to influenza B viruses than influenza A viruses. This relationship is surprising, taking into account the influenza B restricted host spectrum in comparison to the influenza A one.

After the work on influenza A and B viruses and in line with it, the team collaborates now with Mariette Ducatez (INRA - ENV Toulouse) to detail the IDV replication, but also the replication machinery of ToadV. The replication machinery comprises various proteins, including the RNA polymerase (RdRp) and the nucleoprotein (NP). The RdRp is composed of three subunits (PA, PB1 and PB2), binds both the highly conserved 3'- and 5'-ends of the vRNA segment which is covered by multiple copies of NP. This macromolecular complex replicates the viral genome in the nucleus of infected cells and makes many contacts with cellular partners for its assembly. We have gained evidences that IDV NP uses the importins- α system, similar to influenza A and B nucleoproteins. By using advanced techniques of modern biology and physics (X-ray, electron microscopy, surface plasmon resonance, fluorescence anisotropy, ...), the aims of this project are 1/ to provide structural and functional data on IDV and ToadV nucleoproteins and also on their interactions with host partners, and 2/ to highlight both the common and specific features of all types of influenza NPs, giving precious insights on the evolution processes occurring within this growing family of viruses.

Résumé

En 2014, des études chinoises et américaines ont identifié le virus de la grippe de type D (IDV). Ce nouveau membre de la famille des Orthomyxoviridae partage seulement 50% d'identité de séquence avec le déjà bien caractérisé virus de type C et partage moins de 30% d'identité de séquence avec les virus de grippaux de type A et B. IDV a été détecté chez les cochons et les bovins et il a été montré qu'il était capable de se répliquer chez le furet, le principal modèle animal pour l'étude du virus de la grippe chez l'homme, suggérant une possible transmission. En 2018, une nouvelle étude a identifié les premiers virus grippaux infectant des animaux à sang froid. Une comparaison phylogénétique a mis en lumière que ces virus, notamment un virus isolé chez un crapaud (ToadV), sont plus proches des virus de type B que des virus de type A. Cette relation évolutive est surprenante, considérant le tropisme restreint du virus de type B comparé à celui des virus de type A. En droite ligne de ses travaux sur les virus grippaux de type A et B, l'équipe collabore avec Mariette Ducatez (INRA - ENV Toulouse) pour détailler la réplication d'IDV, mais également la machinerie répliquative de ToadV. La machinerie de réplication comprend de nombreuses protéines, incluant l'ARN polymérase (RdRp) et la nucléoprotéine (NP). La RdRp, composée de 3 sous-unités (PA, PB1 et PB2), lie les extrémités hautement conservées 3'- et 5'- du segment d'ARN viral qui est encapsidé par de multiples copies de NP. Ce complexe macromoléculaire réplique le génome viral dans le noyau des cellules infectées et interagit avec de nombreux partenaires cellulaires pour son assemblage. Nous avons obtenu des preuves que la NP d'IDV utilise le système d'import nucléaire basé sur les importines α , de façon similaire aux NP des virus de type A et B. En utilisant des techniques avancées de biologie et de physique (rayons X, microscopie électronique, résonance des plasmons de surface, anisotropie de fluorescence,...), les buts de ce projet sont 1/ d'obtenir des données structurales et fonctionnelles sur les nucléoprotéines d'IDV et de ToadV ainsi que sur leurs interactions avec les partenaires cellulaires, et 2/ de mettre en lumière aussi bien les traits communs que les spécificités de tous les types de NP grippales, ce qui permettra également de comprendre les processus évolutifs en action au sein de cette grandissante famille de virus.