Georg Kochs
Institute for VIROLOGY
UNIVERSITY OF FREIBURG

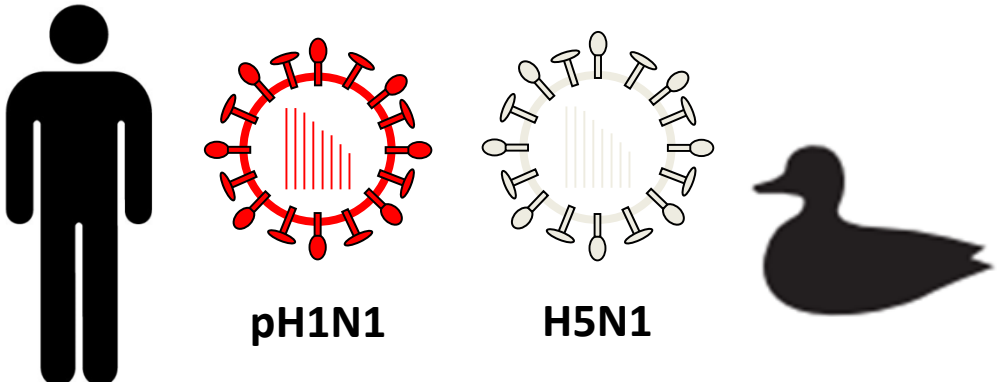
Influenza A virus & the antiviral action of Mx proteins

GEIG-Paris-03.04.14-30 min

Susceptibilité genetique de l‘hote a l‘infection grippale !

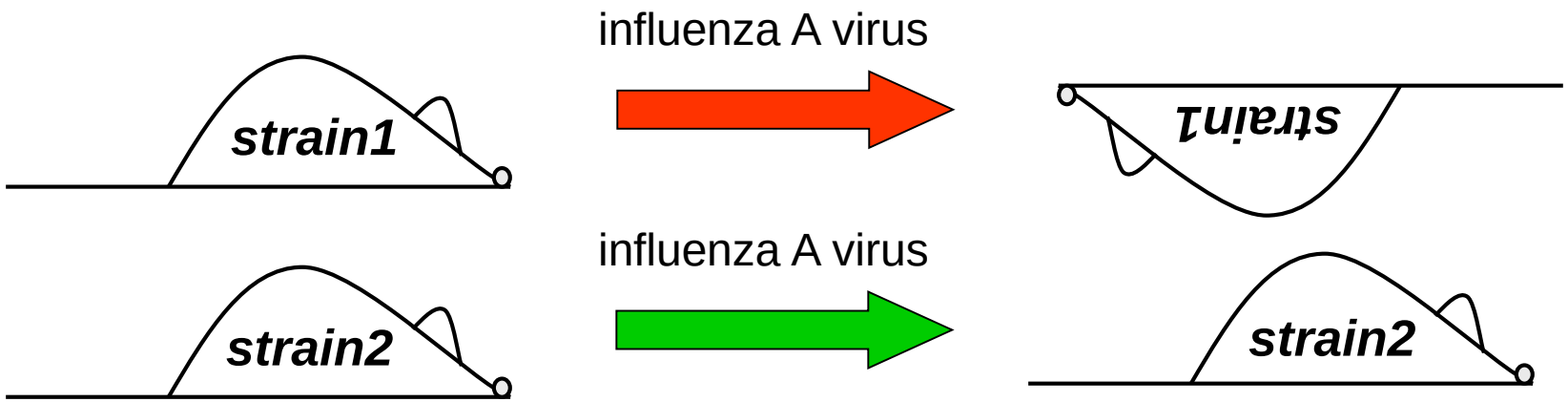
Why some individuals get severe influenza, mild influenza, or do not develope a disease upon virus infection ?

=> Host-Virus-interaction determines the mild or fatal outcome of an infection.



viral genetics has also an effect.

What means: *Host genetic susceptibility to influenza A virus infection ?*



<u>Analysis of different inbred mouse strains:</u>

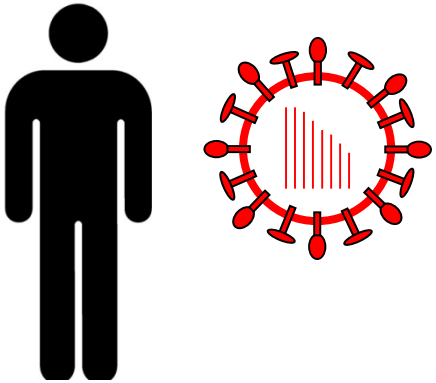
- *Large differences in the susceptibility to pathogenic influenza virus infection, indicating a clear influence of host genetics on susceptibility.*
- *A strong, non-restricted replication of the virus leads to elevated inflammatory response (cytokine storm) and severe lung pathology.*
- *Host heritable resistance is polygenic - not linked to one specific gene - !*

Human genetic susceptibility to influenza A virus infection !

Host genetic susceptibility to severe influenza in humans remains unanswered.

- Need of coordinated efforts to define and assemble cohorts of severe influenza cases -

(Peter Horby et al., (2012) Plos One 7, e33180)

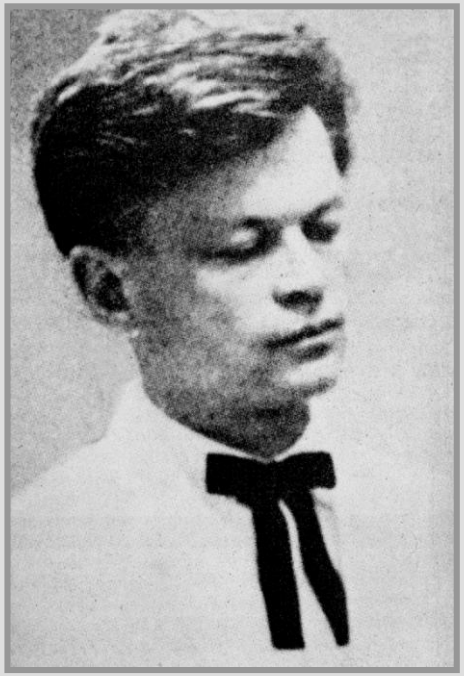


- How to identify candidates that determine host susceptibility ?

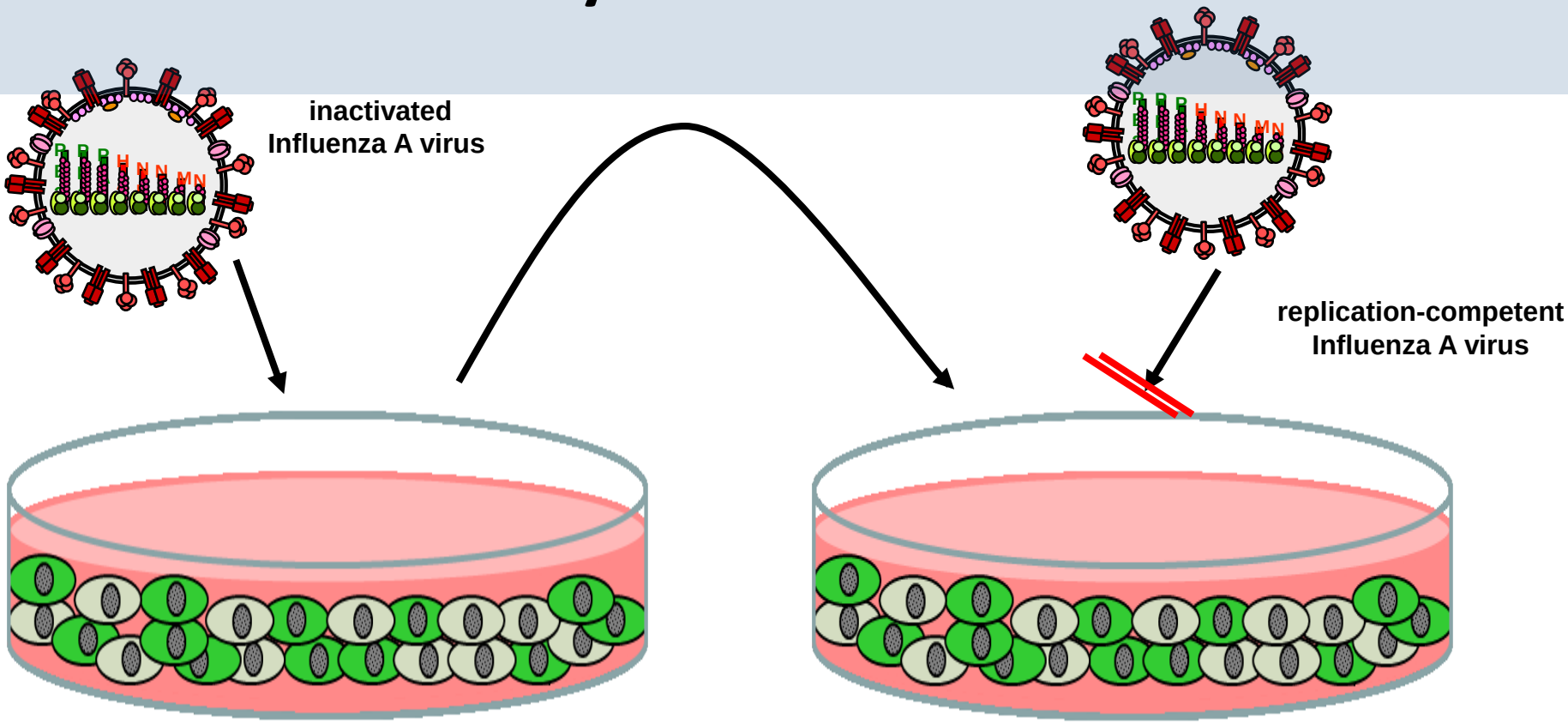
1957 – Discovery of Interferon

Alick Isaacs & Jean Lindenmann

NIMR-Mill Hill-London



1957 – Discovery of Interferon

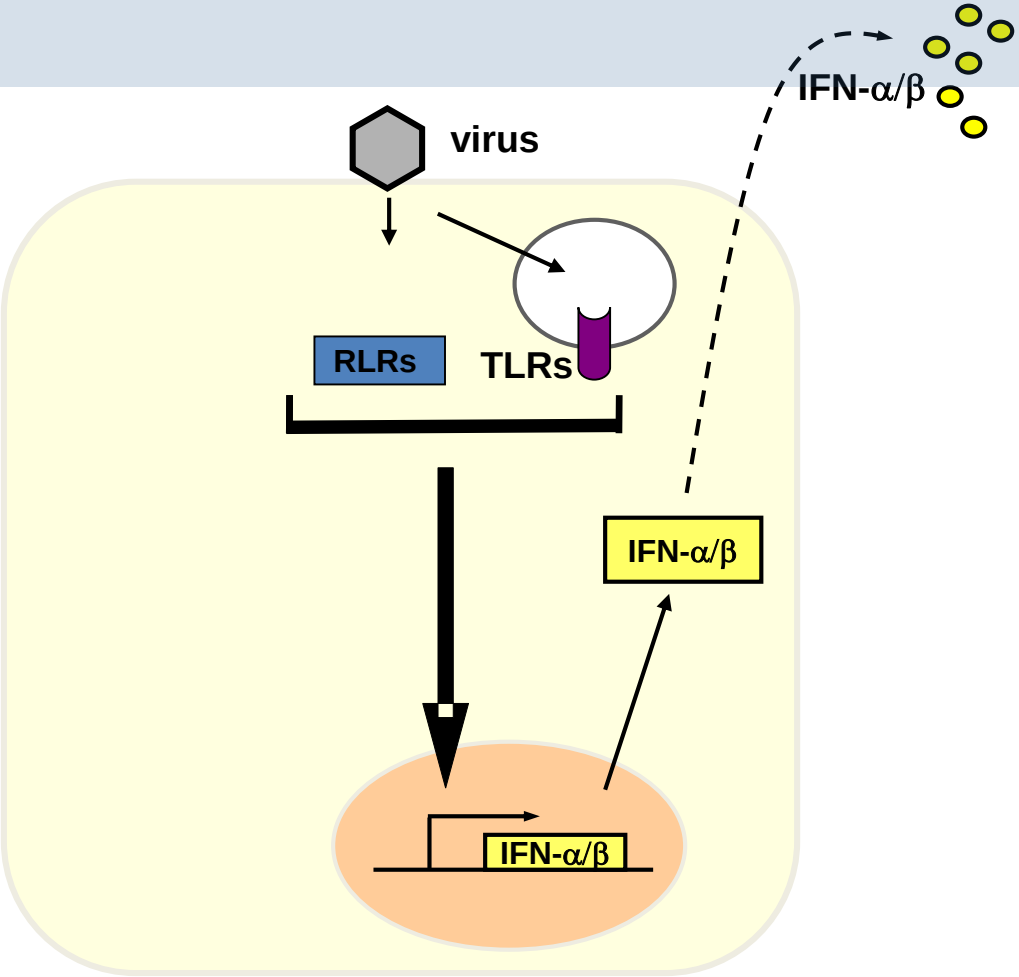


primary chicken fibroblasts

primary chicken fibroblasts

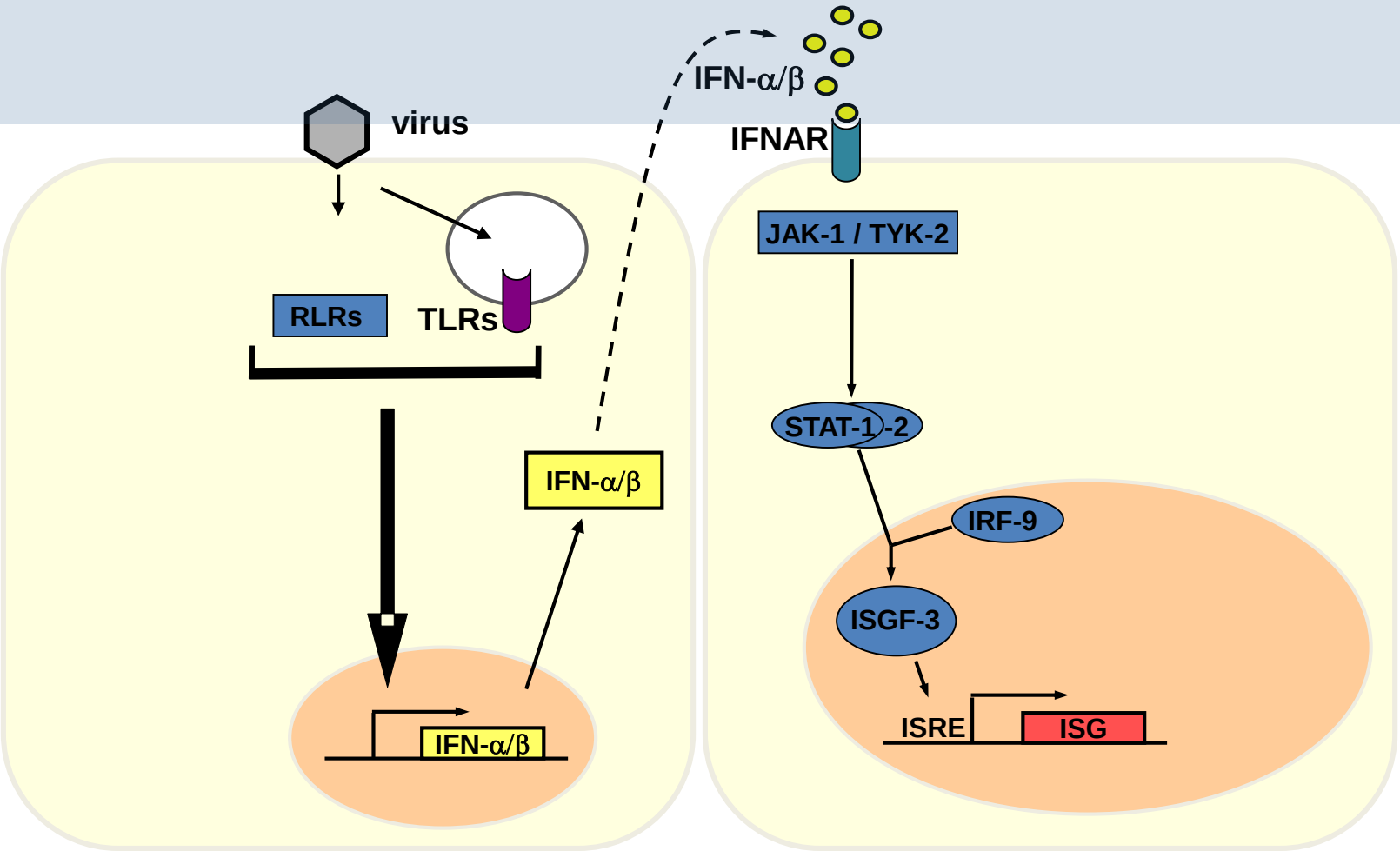
virus-inhibiting factor = <u>Interferon (IFN)</u>
- anti-viral state against influenza A virus

Influenza A virus induces Interferons

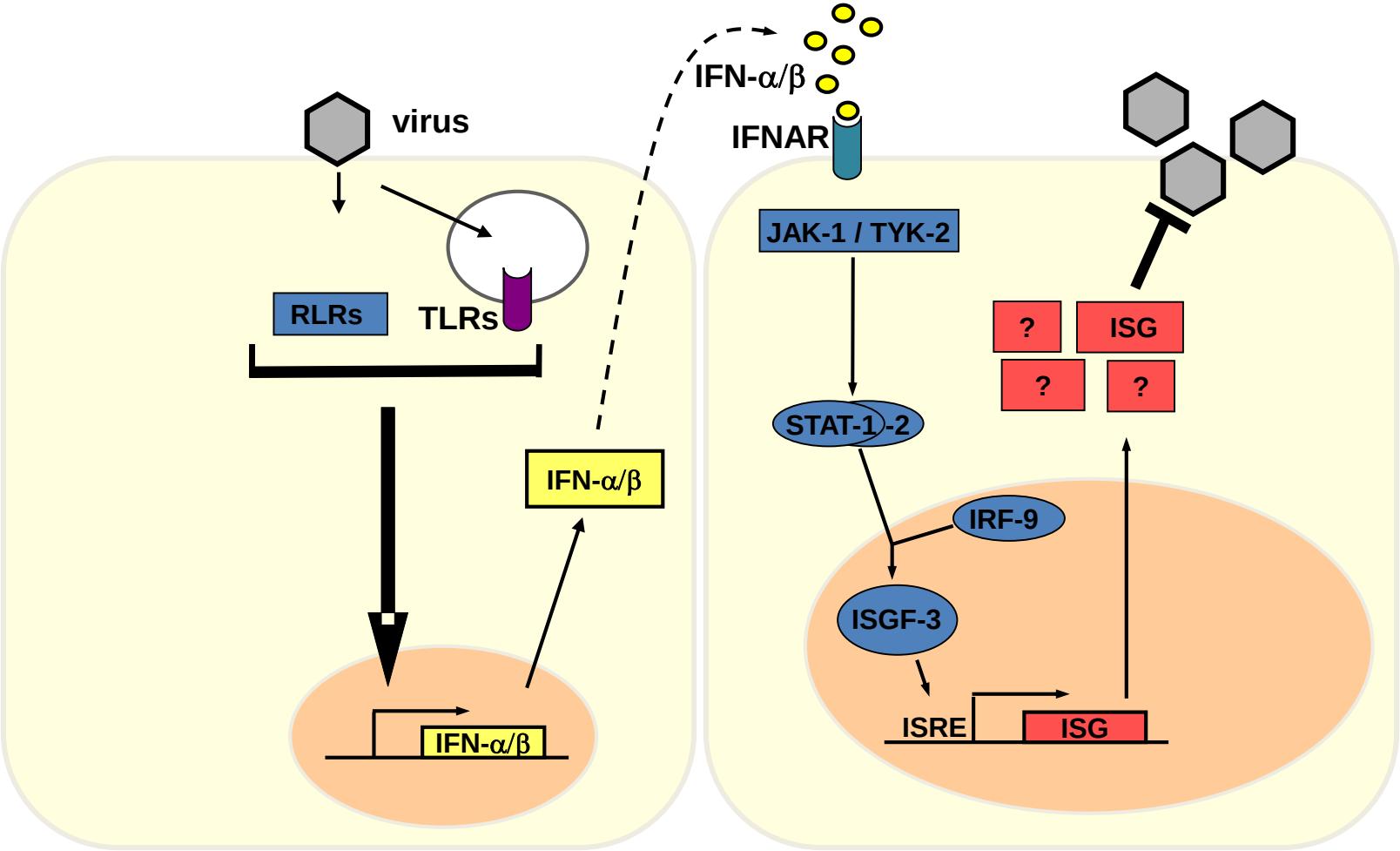


RLR – Rig-I-like receptors
TLR – Toll-like receptors

IFN induces an antiviral state



IRF – IFN-regulatory factor
JAK – Janus Kinase
STAT – Signal transducer and activator of transcription
ISGF3 – IFN-stimulated gene factor
ISRE – IFN-stimulated response element
ISG - IFN-stimulated genes


virus
RLRs
TLRs
IFN-α/β
IFN-α/β
IFN-α/β
IFNAR
JAK-1 / TYK-2
STAT-1 -2
IRF-9
ISGF-3
ISRE
ISG
?
ISG
?
?

2011 – Nobel prize in medicine

Ralph Steinman

Steinman&Cohn (1973)
JEM. 137, 1142-1162.

First description of **Dendritic cells** as important components of the host defence.

Jules Hoffmann

Lemaitre, B. et al. (1996)
Cell 86, 973-983.

First description of **Toll- receptors** in Drosophila as components of the immune system

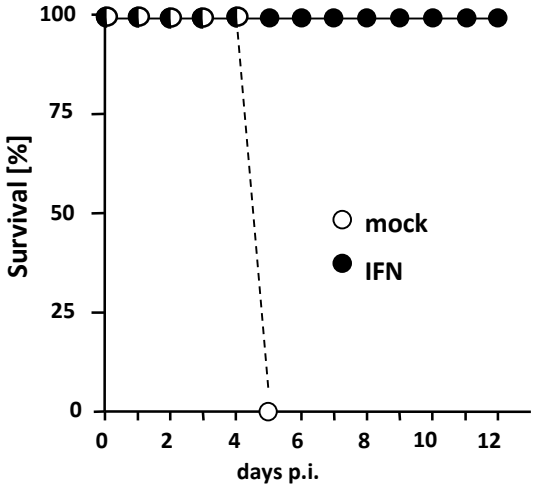
Bruce Beutler

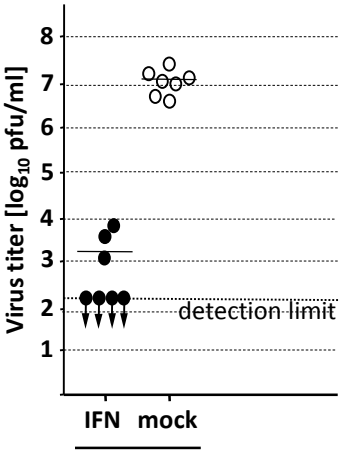
Poltorak, A. et al. (1998)
Science 282, 2085-2088.

First description of **Toll-like receptors** in mice as a component of LPS sensing. Identification of other components of **PAMP** recognition and innate Immunity, via RIG-I.

Is the IFN system of any relevance for infections in vivo ?

Antiviral effect of IFN in vivo !





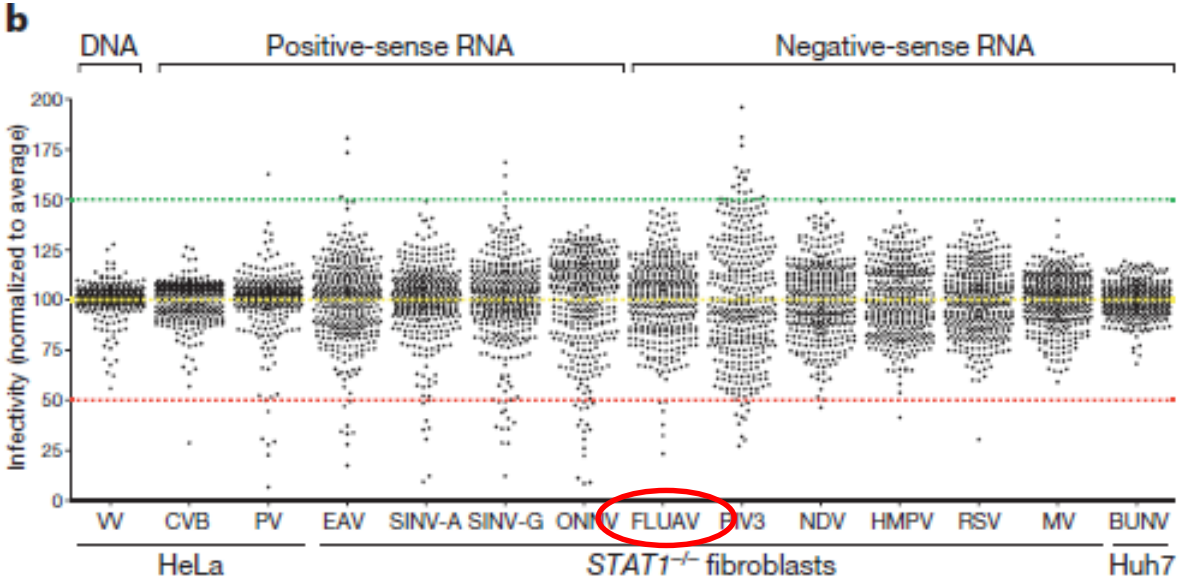
mice were treated i.n. with IFN-α 10h,
before intranasal infection with 100 LD50 of hvPR8.
(Grimm et al., (2007) PNAS 104, 6806-6811)

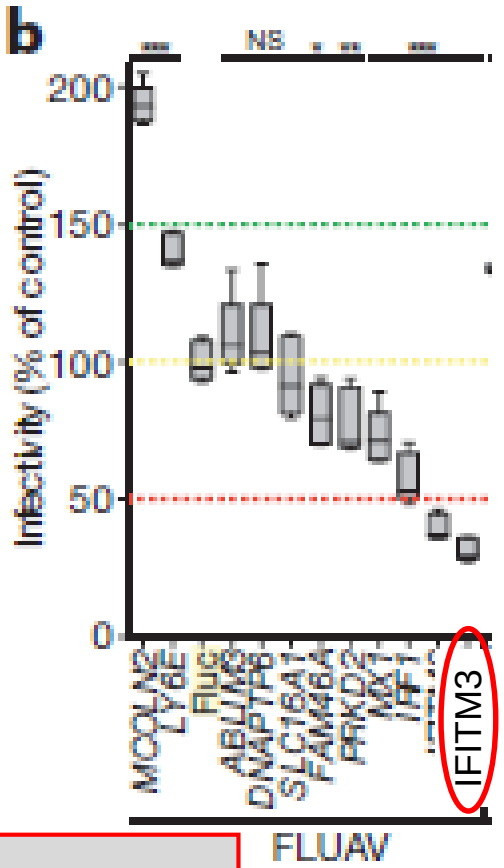
IFN induces an antiviral state against Flu,

Search for effector molecules !

IFN induces an antiviral state

Search for effector molecules !





<u>Pan-viral specificity of ISGs.</u>

Expression of 350 human ISGs for inhibition of 14 *gfp*-virus

Schoggins et al. (Rice-Rockefeller), (2014) Nature 505, 691-696.

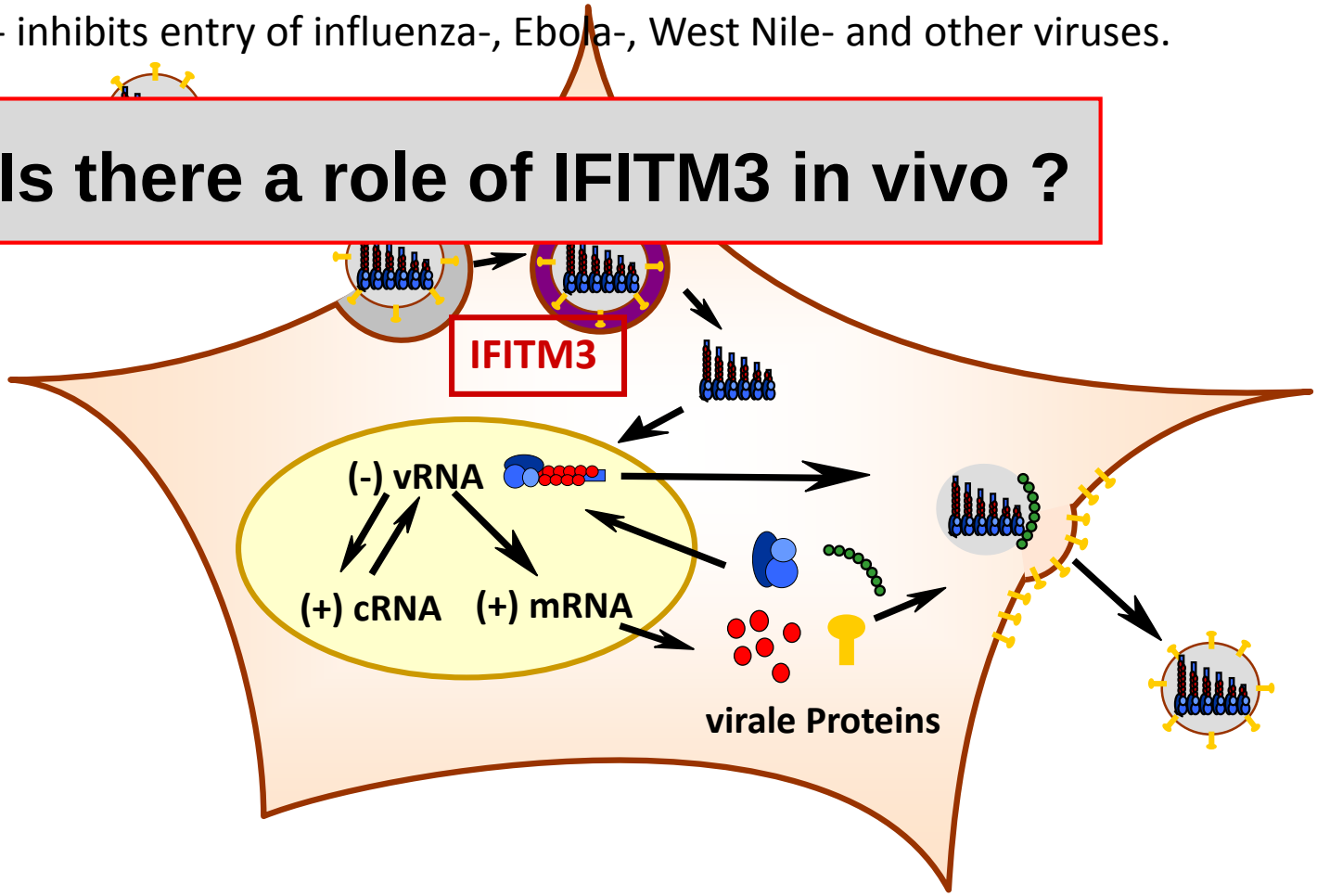
<u>IFITM3 is a potent inhibitor of influenza virus replication</u> !

What is IFITM3 ?

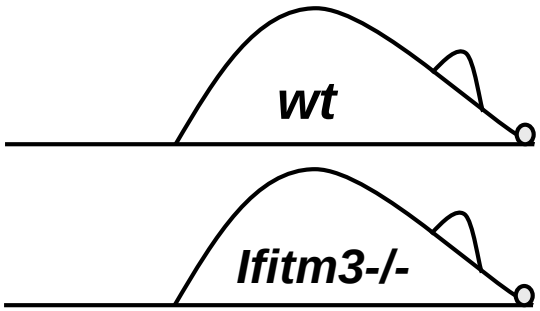
Interferon-induced transmembrane protein-3.

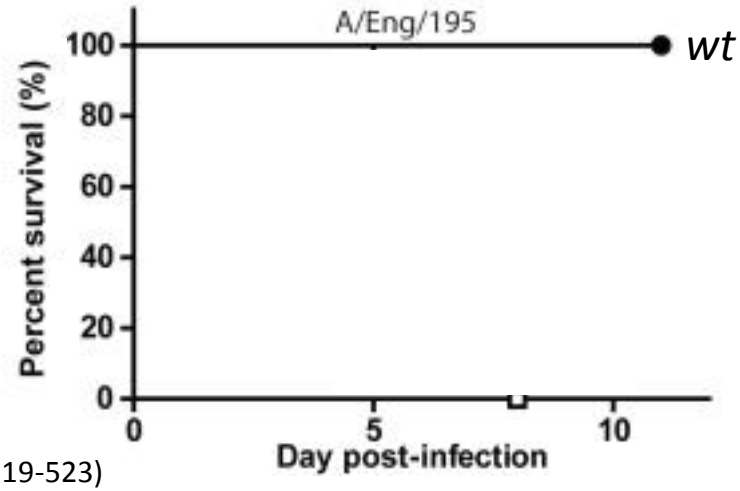
- trans-membrane protein of the endosomal compartment.
- alters membrane fluidity.
- inhibits entry of influenza-, Ebola-, West Nile- and other viruses.

Is there a role of IFITM3 in vivo ?



Antiviral action of IFITM3 in vivo ?





mice were intranasal infected with
A sublethal dose of pH1N1/09.
(Everitt et al., Kellam&Brass-Lab, UCL&MIT (2012) Nature 484, 519-523)

- IFITM3 is a potent inhibitor of influenza virus replication in vivo.

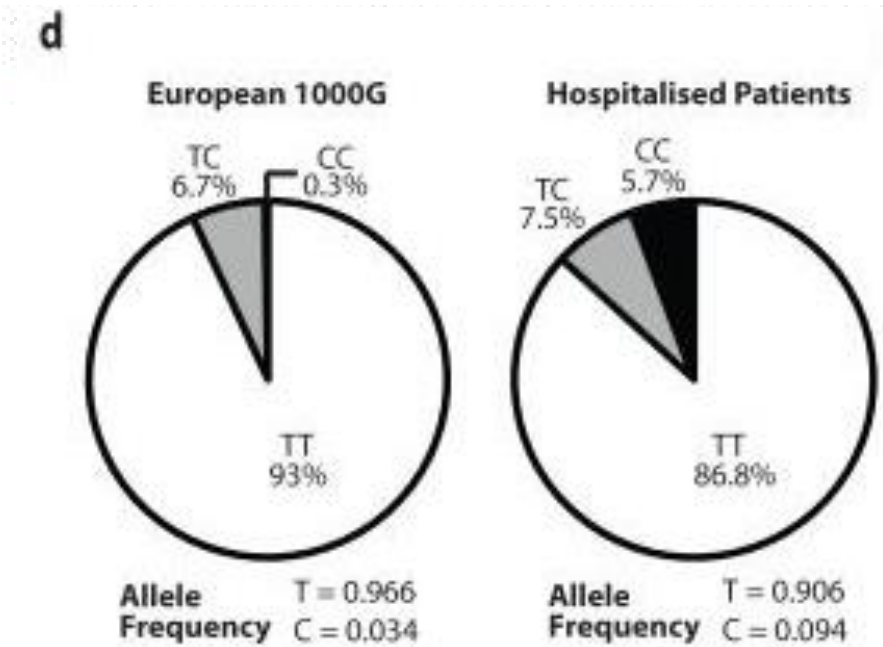
? Is there a link to severe influenza in humans ?

Role of IFITM3 in human influenza ?

- Hospitalized patients with severe Flu symptoms in 2009, n=53 in England and Scotland.
- Analysis for genotypic differences in *IFITM3* gene: 10-fold higher frequency of C-allele.

Everitt et al., Kellam&Brass-Lab, UCL&MIT-Harvard (2012) Nature 484, 519-523.
Zhang et al., (2013) Nature Communication 4, 1418-1423.

VAVLGAPHNPAPPTSTVIHIRSETSVP **T-allel/93%**
VAVLGAPHNPAPPTSTVIHIRSETSVP **C-allel/0.3%**



What is the effect of C/C for Flu ?

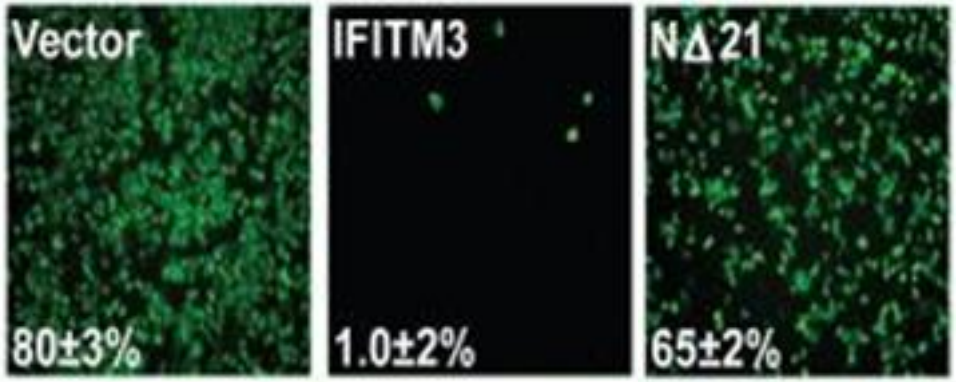
Role of IFITM3 in human influenza ?

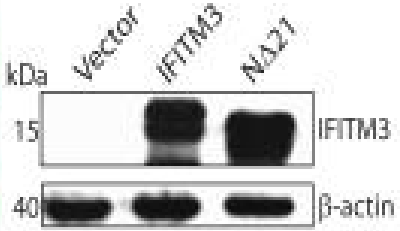
- Hospitalized patients with severe Flu symptoms in 2009, n=53 in England and Scotland.
- Analysis for genotypic differences in *IFITM3* gene: 10-fold higher frequency of C-allele.

Everitt et al., Kellam&Brass-Lab, UCL&MIT-Harvard (2012) Nature 484, 519-523.
Zhang et al., (2012) Nature Communication 4, 1418-1423.

IFITM3: MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVP **T-allel/93%**
NΔ21: MLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVP **C-allel/0.3%**

Cells expressing IFITM3 cDNAs, infected with pH1N1 for 12 h, stained for HA expression.





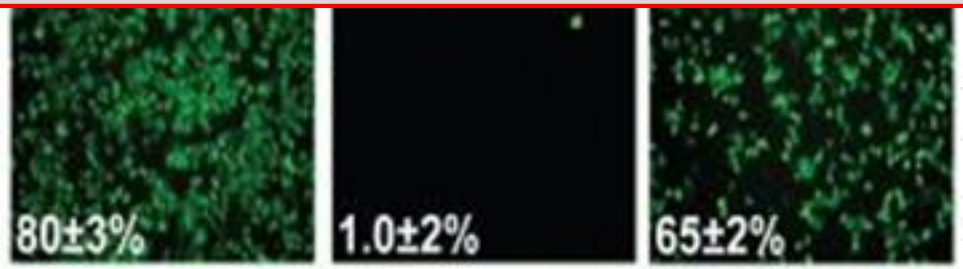
Role of IFITM3 in human influenza ?

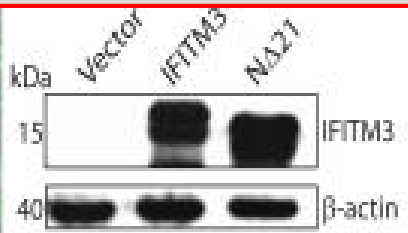
- Hospitalized patients with severe Flu symptoms in 2009, n=53 in England and Scotland.
- Analysis for genotypic differences in *IFITM3* gene: 10-fold higher frequency of C-allele.

Everitt et al., Kellam&Brass-Lab, UCL&MIT-Harvard (2012) Nature 484, 519-523.
Zhang et al., (2012) Nature Communication 4, 1418-1423.

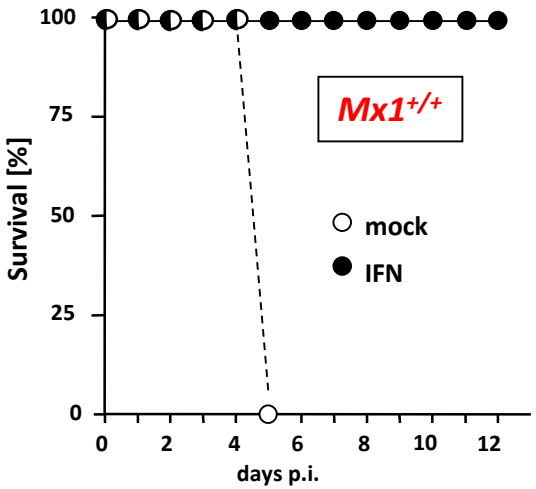
IFITM3: MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVP **T-allel/93%**
NΔ21: MLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSVP **C-allel/0.3%**

- IFITM3 is antivirally active against Flu in cell culture and in vivo.
- Changes in IFITM3 expression correlate with 6-fold greater risk for severe influenza.



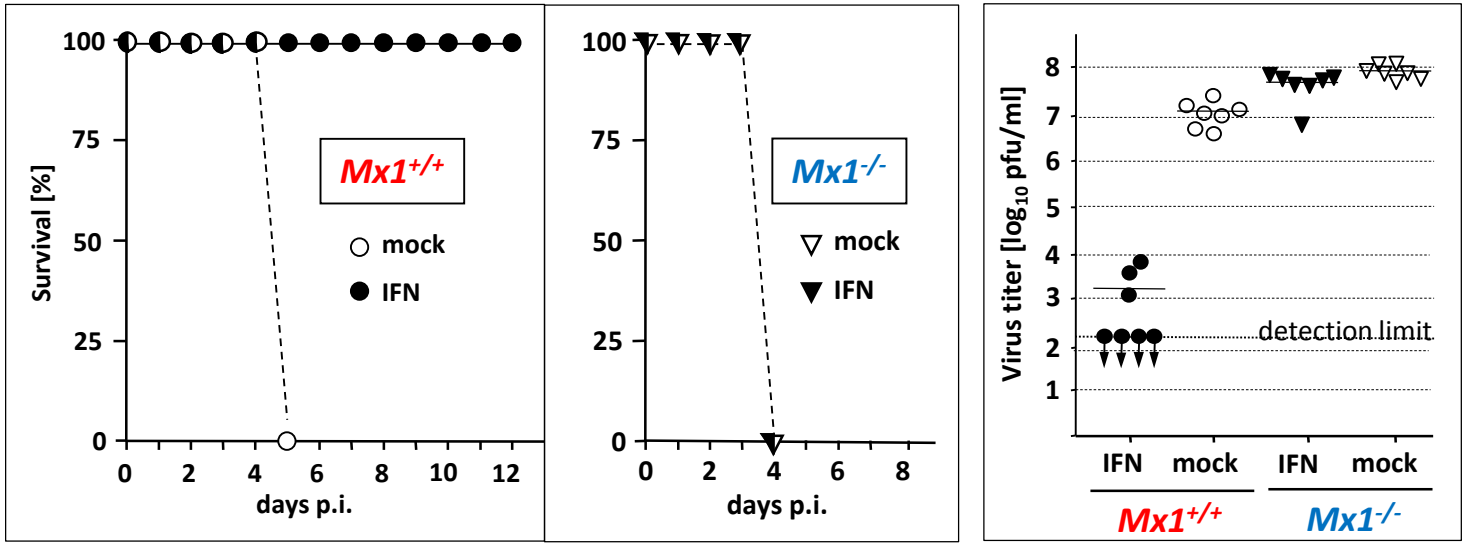


Antiviral effect of IFN, other candidates ?



C57BL/6 mice were treated i.n. with IFN-α,
before intranasal infection with 100 LD50 of hvPR8.
(Grimm et al., (2007) PNAS 104, 6806-6811)

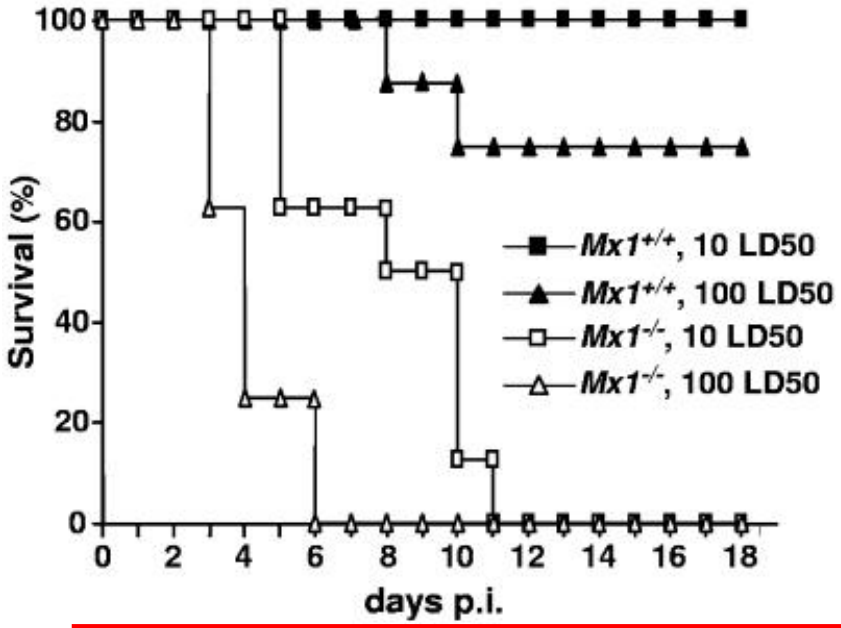
Antiviral action of Mx Proteins !



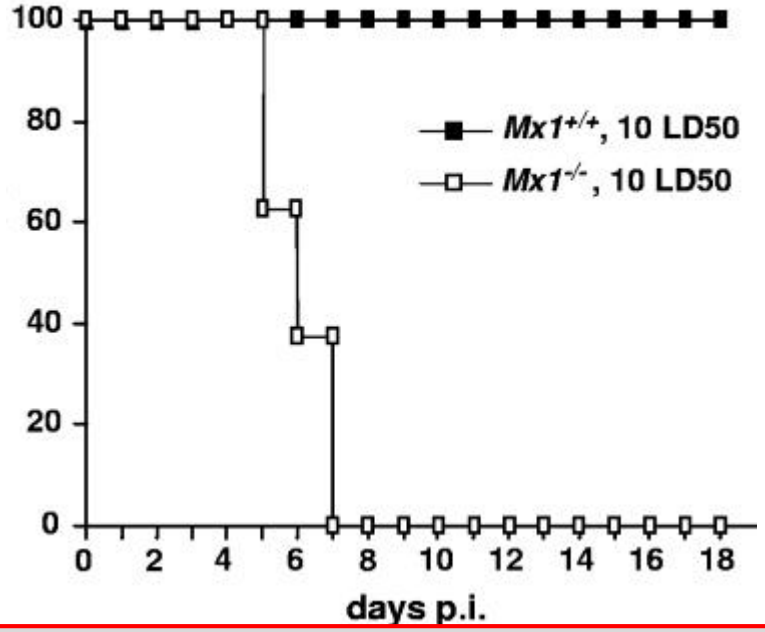
C57BL/6 and B6.A2G-Mx1 mice were treated i.n. with IFN-α, before intranasal infection with 100 LD50 of hvPR8.
(Grimm et al., (2007) PNAS 104, 6806-6811)

Antiviral action of Mx Proteins !

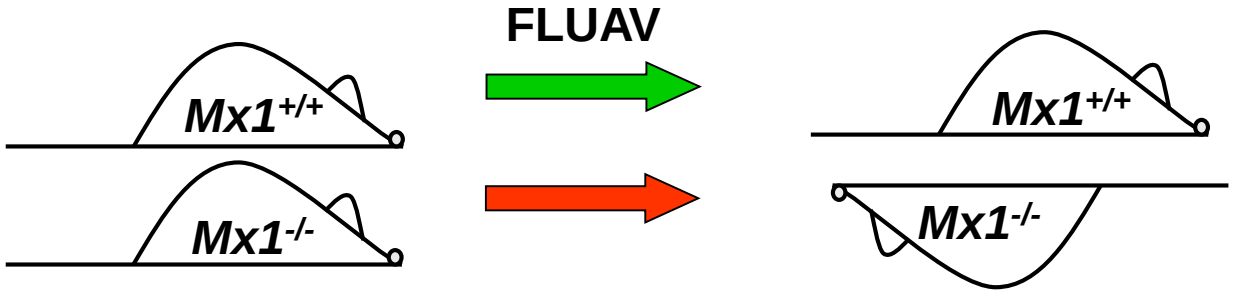
pH1N1-1918



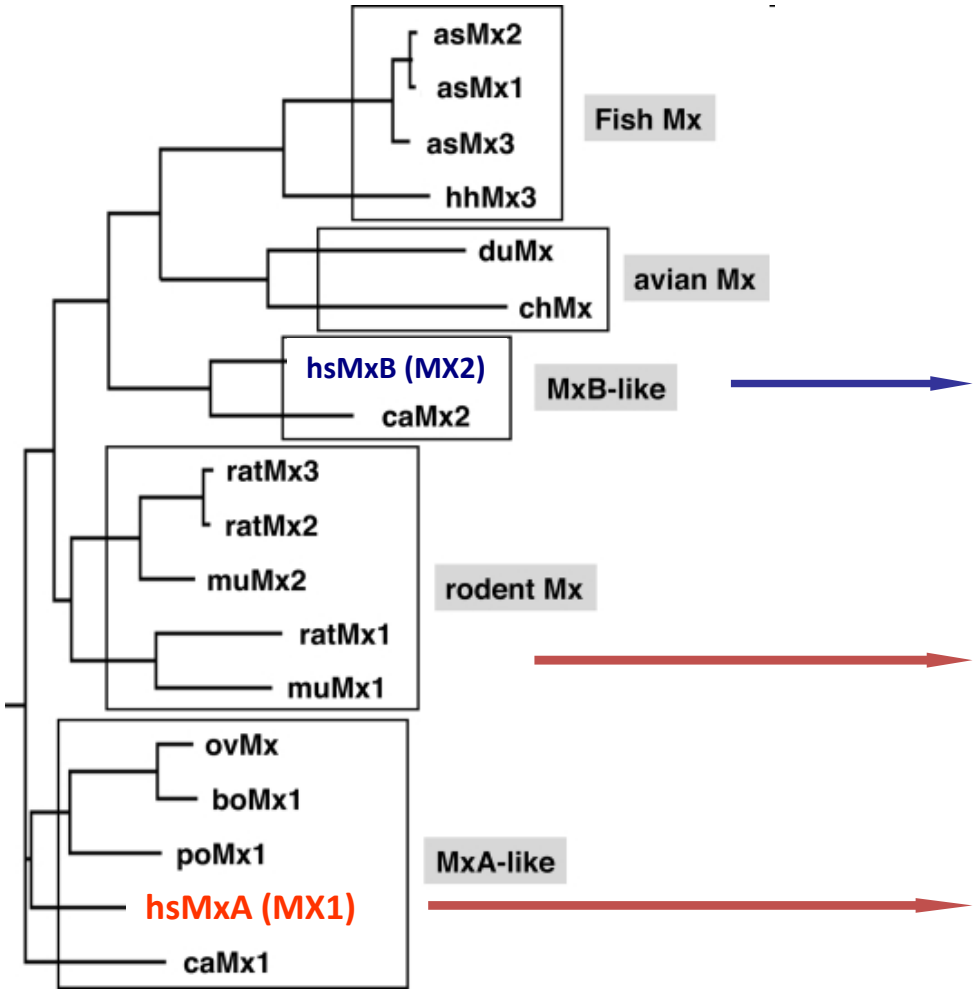
H5N1 – A/Vietnam/1203/04



Mx1 is a dominant antiviral restriction factor for influenza in the mouse.



Mx family members



anti- HIV-1 activity

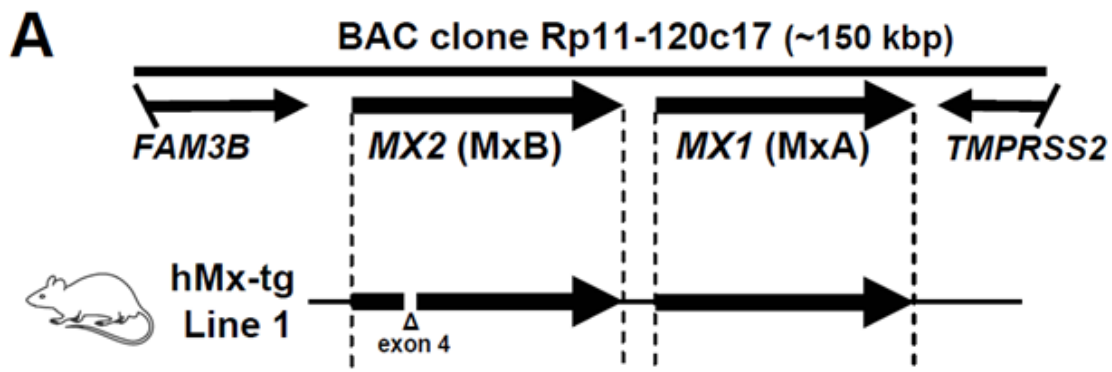
orthomyxoviruses

Influenza-, paramyxo- bunya-, rhabdo-, picorna- hepadna-, African swine fever viruses

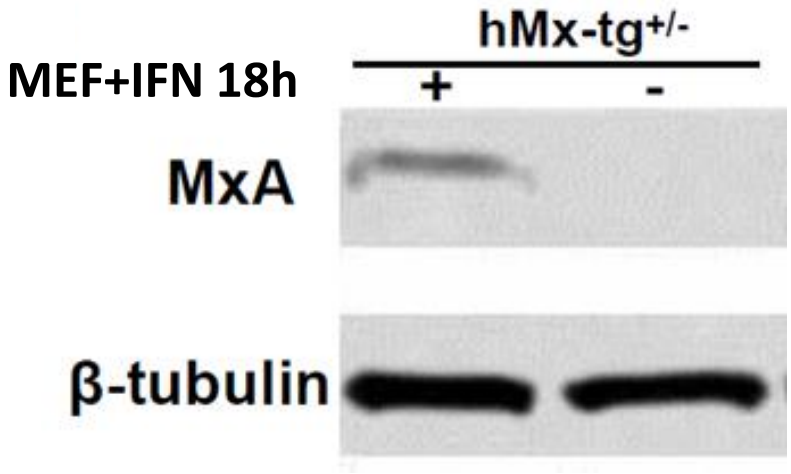
hsMxA active in vivo?

[*adapted from: Haller O. et al: Microbes and Infection, 2007*]

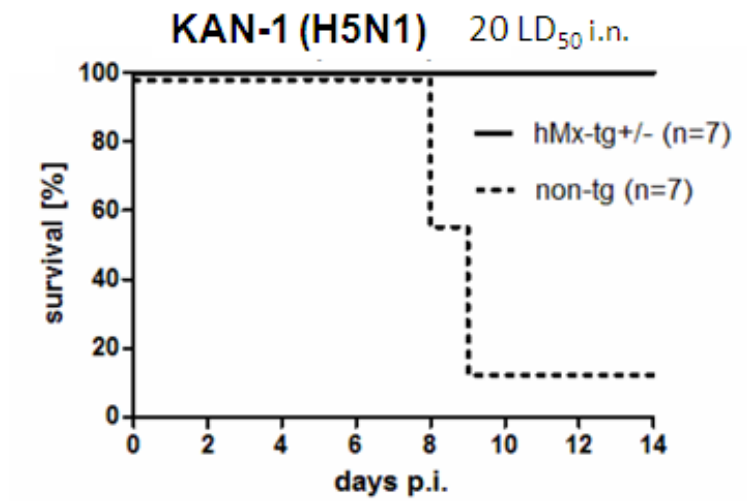
human MxA transgenic mouse

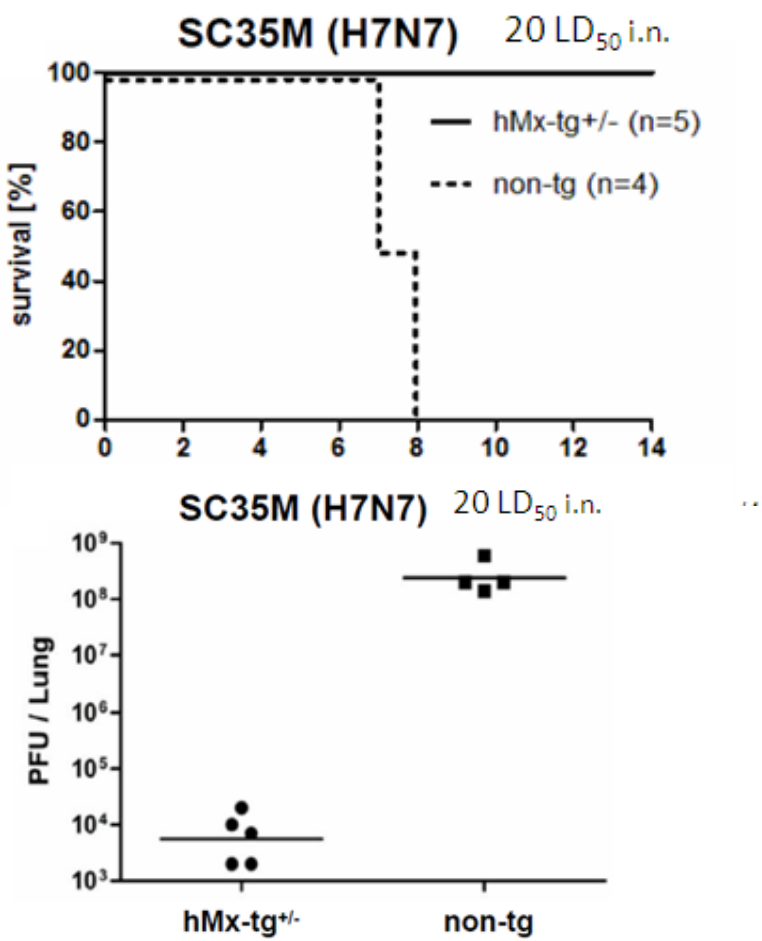


Peter Staeheli
Virology-Freiburg



Antiviral activity of human MxA ?



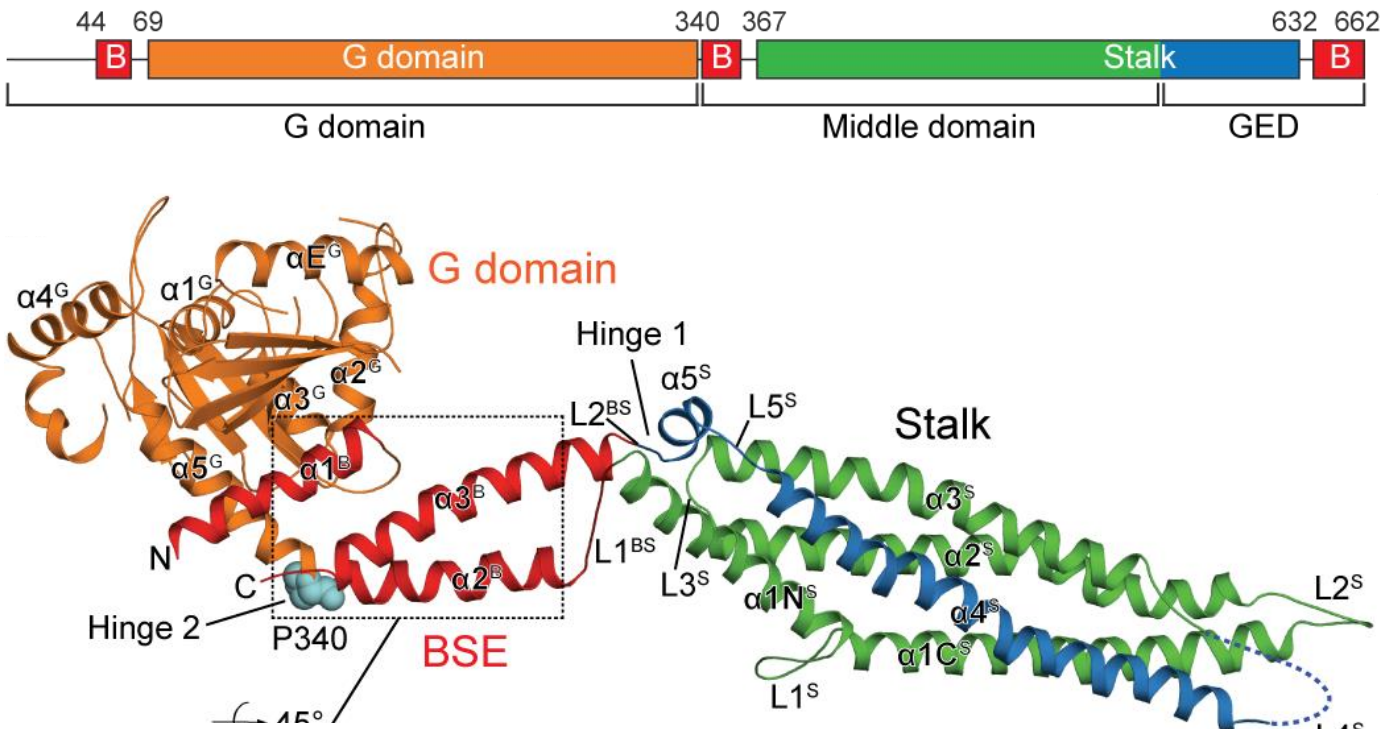


mice intranasal infected with lethal dose of flu (titers 5 d.p.i).
(Deeg&Staeheli (2014) in preparation)

Human MxA protects against lethal influenza A virus infection in the transgenic mouse model.

What are Mx proteins ?

- **Mx = <u>M</u>y<u>x</u>**ovirus resistance (1962 by Jean Lindenmann)
- **induced by IFN-α/β and λ** (used as a marker for IFN activity in vivo)
- **large dynamin-like GTPase (75 kDa)**
- **antivirally active against influenza viruses and other RNA viruses**



Oliver Daumke
MDC, Berlin

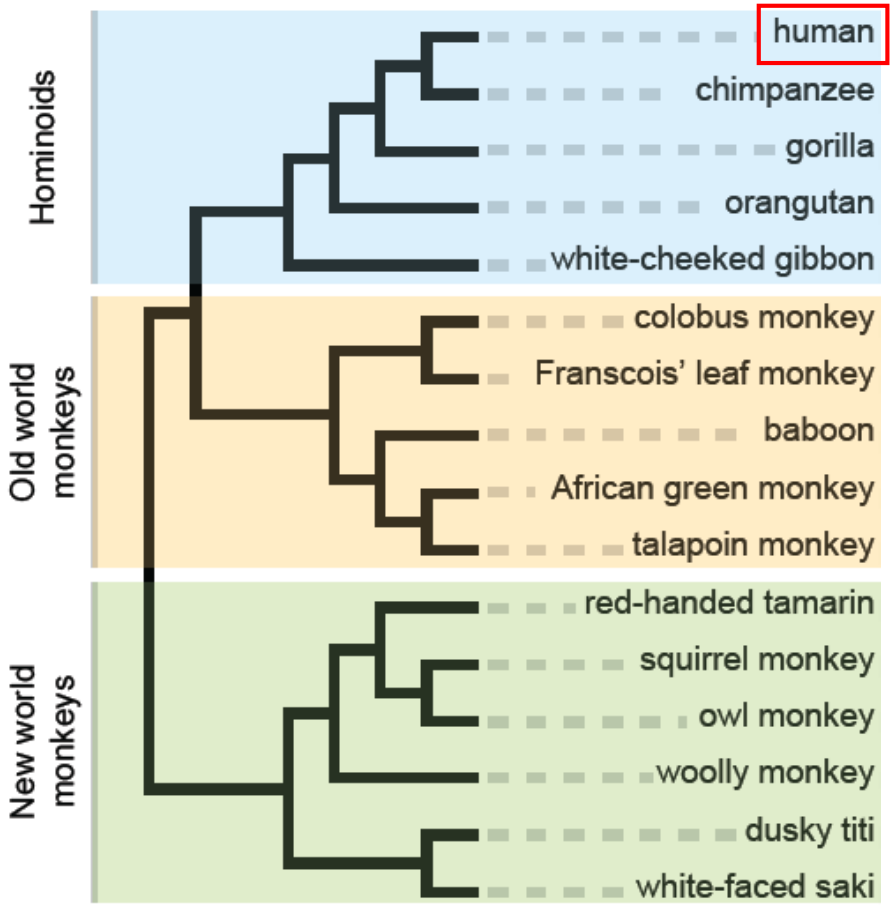
Gao S., von der Malsburg A. et al.; Immunity 2011

unstructured loop 4

Evolution guided analysis of primate MxA

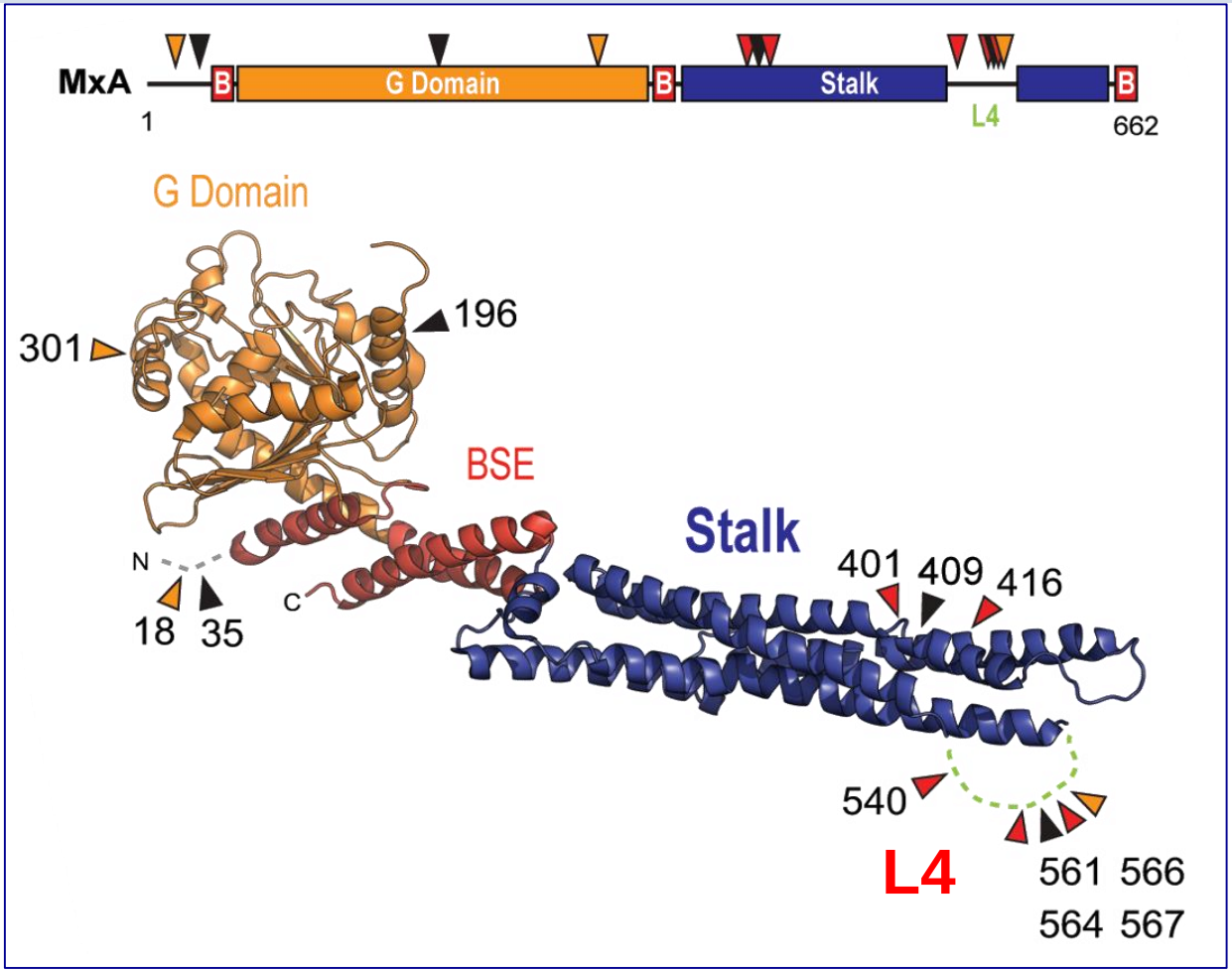
Dr. Harmit Malik
Cancer Center, Seattle

Dr. Corinna Patzina
Virology, Freiburg



→ Comparative analysis for positive selection as marker of host-target interfaces

MxA proteins from primates



- **Structure of MxA is highly conserved.**
- **L4 is a hotspot for positive selection.**

(Mitchell&Patzina et al., (2012) CHM 12, 598-604)

MxA proteins from primates

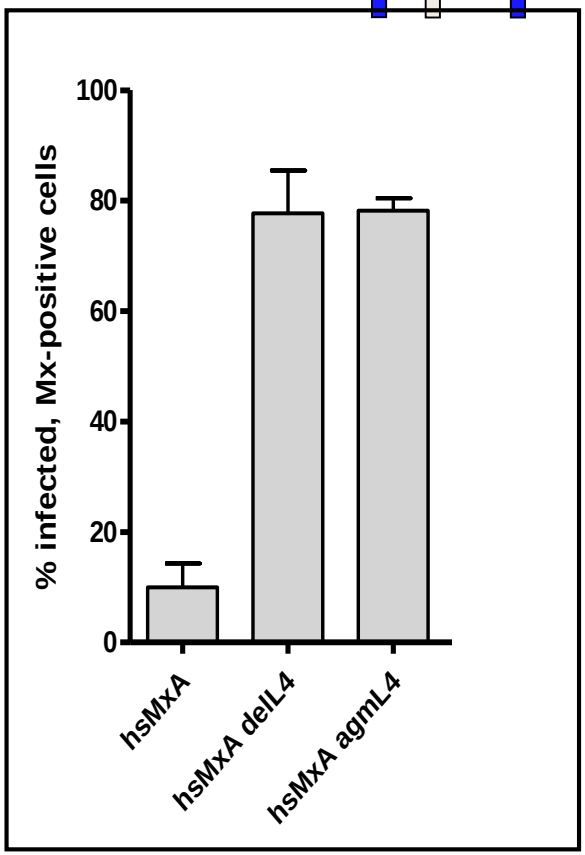
558 571

hsMxA ~CQDQVYRGALQKVREKELEEEKKKK~SWDFGAFQSSSATD~SSMEEIF
agmMxA ~CQDQVYRGALQKVREKELEEEKKKK~SWDVGTFQPSS~TD~SSM

→ Chimeric proteins:

hsMxA agmL4

agmMxA hsL4



old world human

African green monkey

MxA proteins from primates

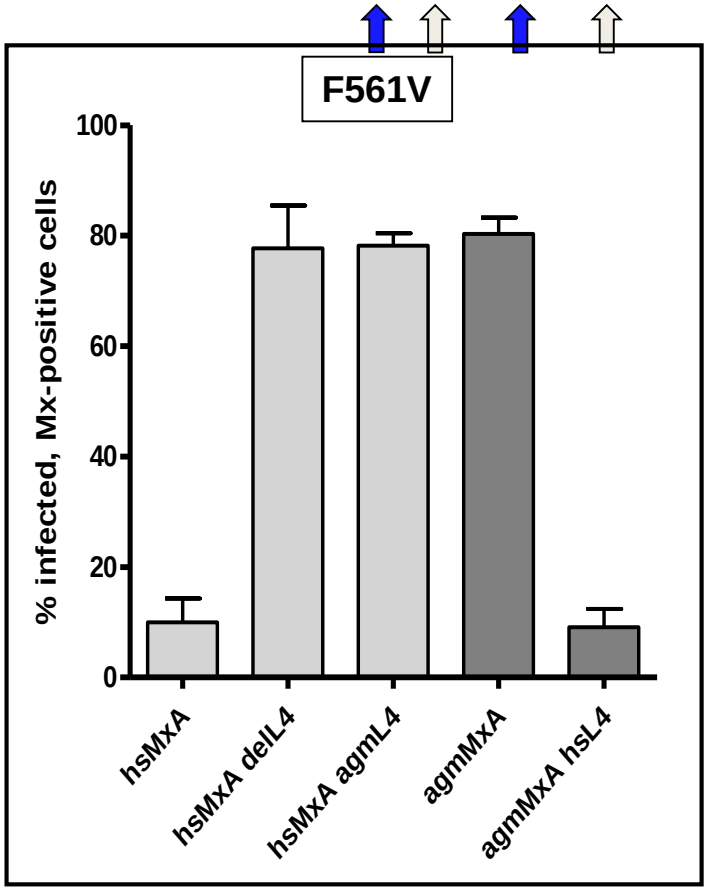
558 571

hsMxA ~CQDQVYRGALQKVREKELEEEKKKK~SWDFGAFQSSSATD~SSMEEIF
agmMxA ~CQDQVYRGALQKVREKELEEEKKKK~SWDVGTFQPSS~TD~SSMEEIF

→ Chimeric proteins:

hsMxA agmL4

agmMxA hsL4



MxA proteins from primates

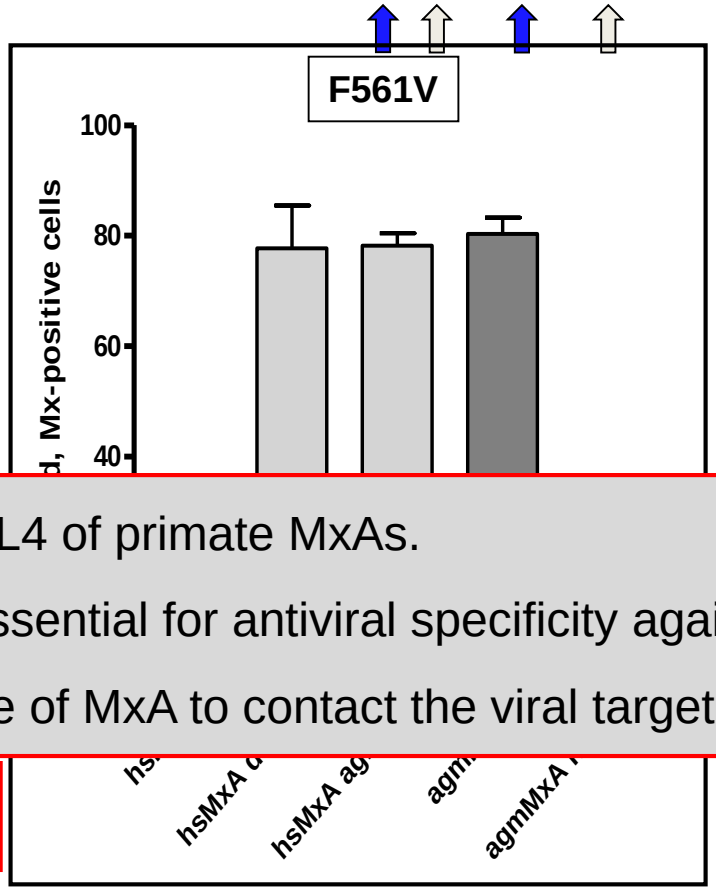
558 571

hsMxA ~CQDQVYRGALQKVREKELEEEKKKK~SWDFGAFQSSSATD~SSMEEIF
agmMxA ~CQDQVYRGALQKVREKELEEEKKKK~SWDVGTFQPSS~TD~SSMEEIF

→ Chimeric proteins:

hsMxA agmL4

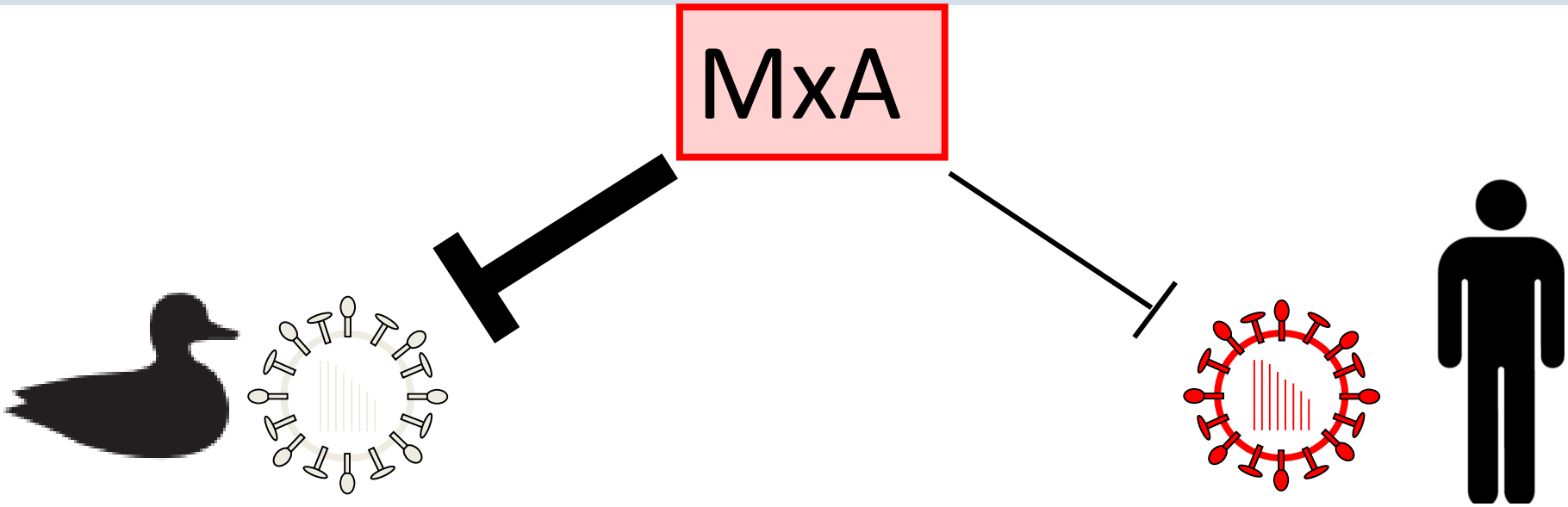
agmMxA hsL4



- positive selection in Loop L4 of primate MxAs.
- L4 and position 561 are essential for antiviral specificity against influenza A virus.
- L4 represents the interface of MxA to contact the viral target.

What is the viral target ?

Viral target of Mx Proteins ?

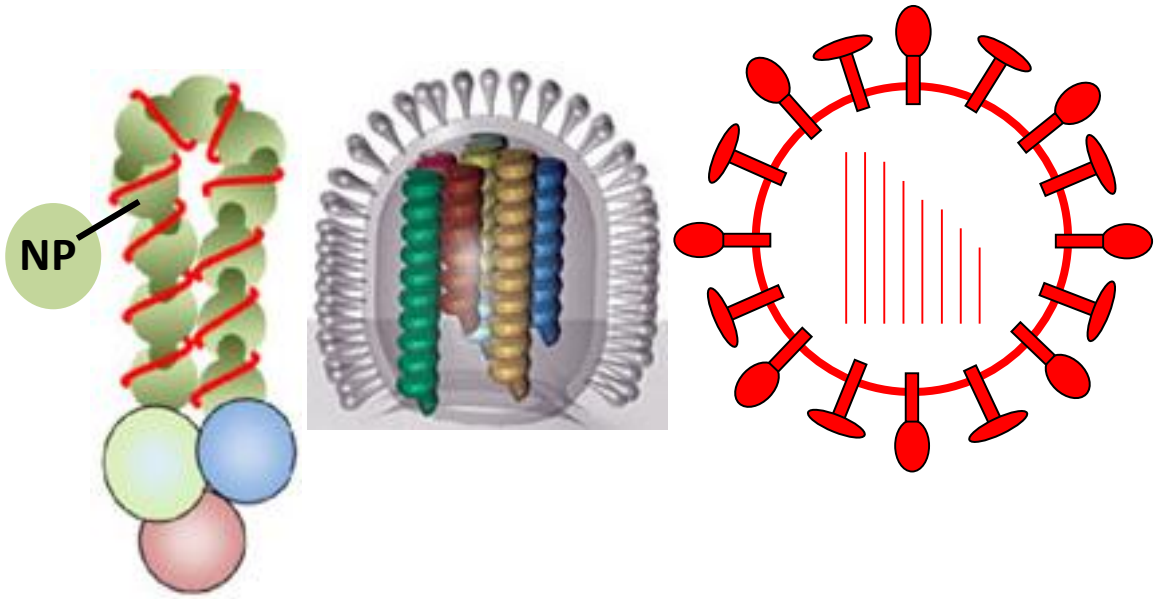


avian influenza viruses
A/Thailand/1/04 (H5N1)

Human flu isolates
A/Hamburg/4/09 (pH1N1)
A/BM/01/18

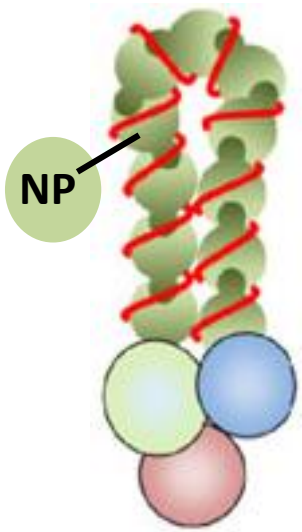
- Flu strains differ in their Mx sensitivity (Dittmann, 2008).
- The viral nucleoprotein (NP) determines Mx sensitivity (Zimmermann, 2011).

Viral target of Mx Proteins ?



? Why avian influenza viruses are so sensitive to MxA ?

Analysis of viral NP for Mx sensitivity



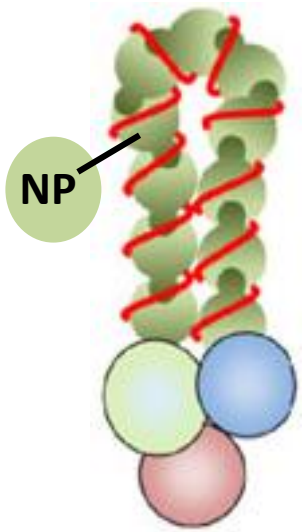
Martin Schwemmle,
Virology, Freiburg

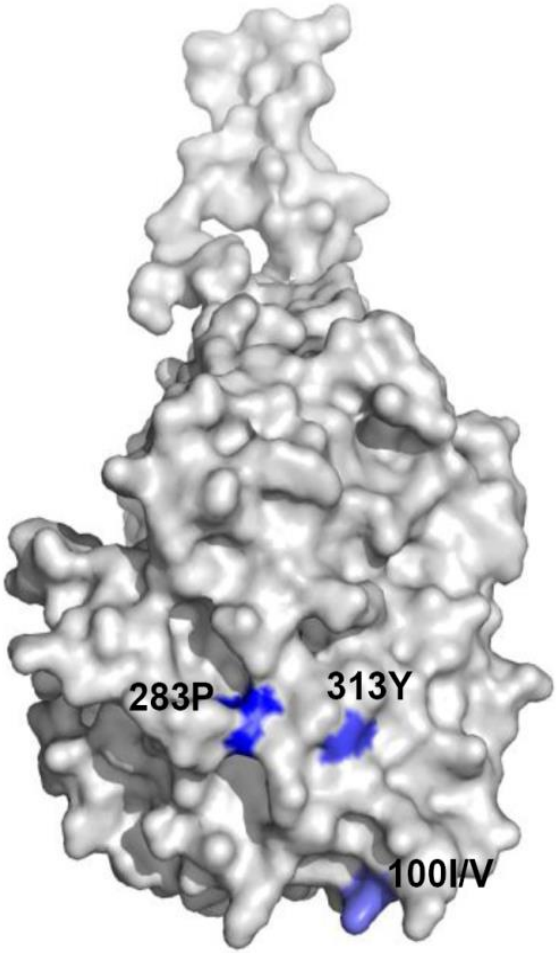
NP

Avian
H5N1

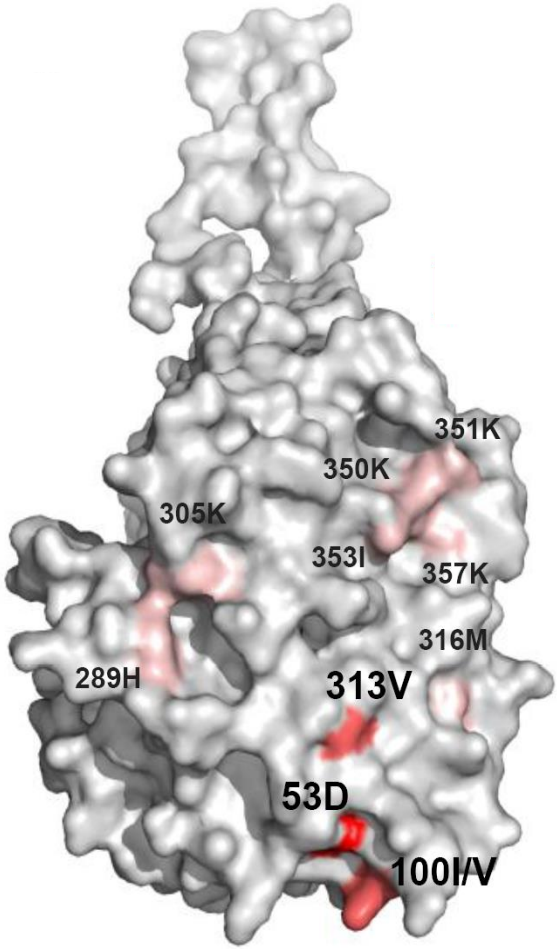
1918
14 aa
differences

2009
pH1N1
32 aa
differences





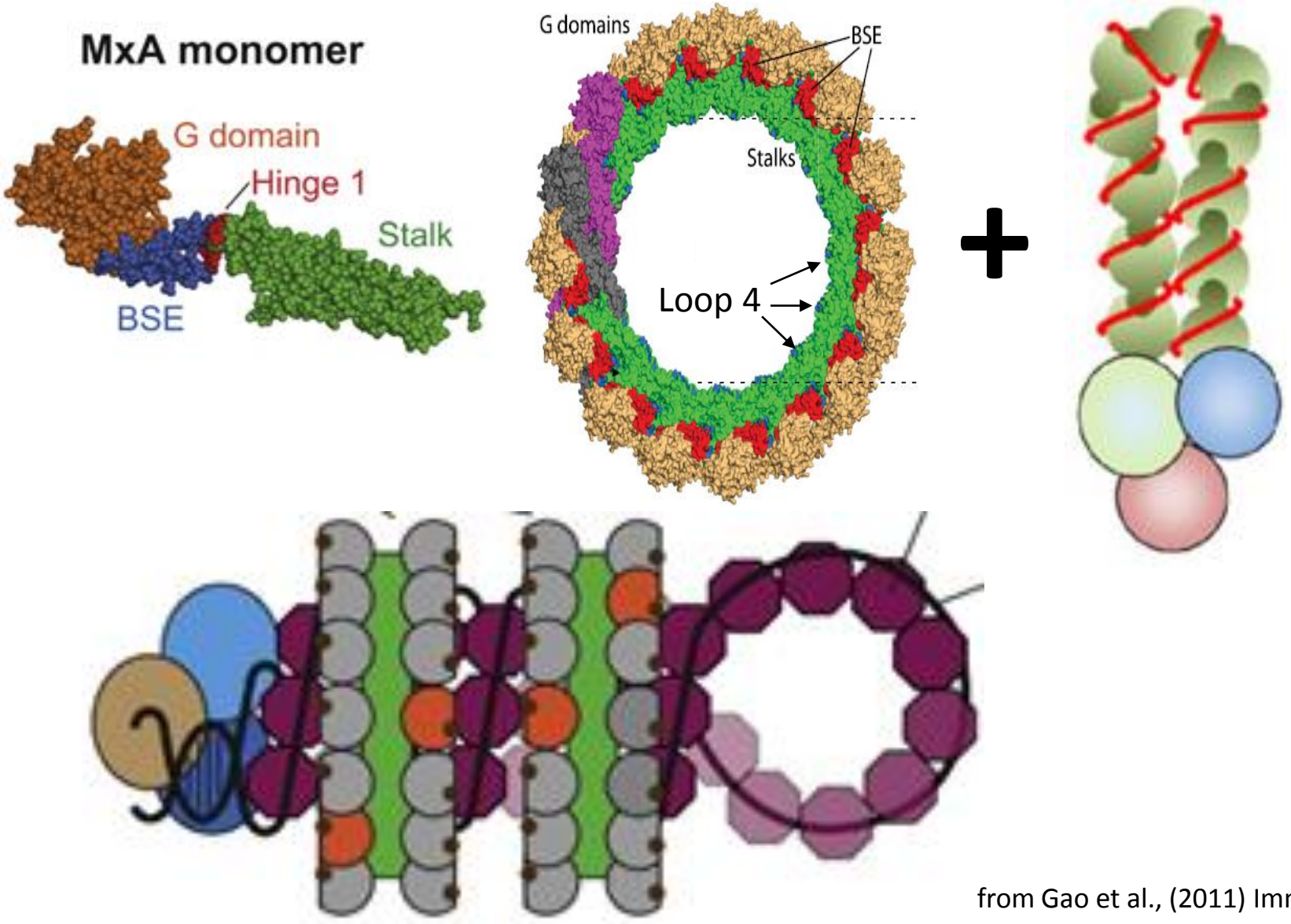
1918



pH1N1

- Specific positions in NP determine Mx sensitivity and might serve as interaction partners of MxA (Mänz, 2013)

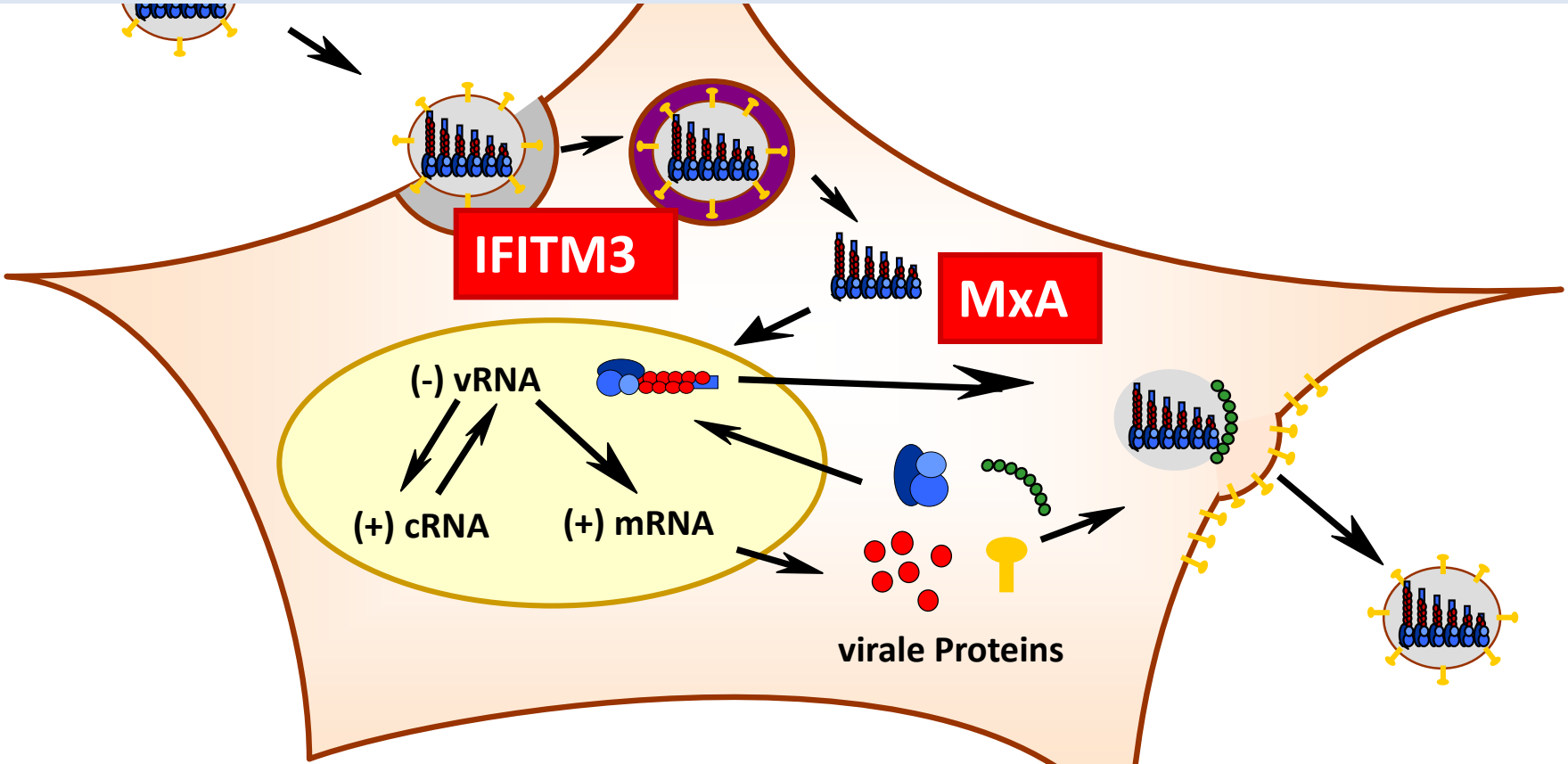
Model of MxA action



from Gao et al., (2011) Immunity 35,1-12

Summary:

- Analysis of candidates influencing susceptibility is difficult, polygenic effect.
- Two canidates, IFITM3 and MxA, block virus replication in vivo.
- IFITM3 polymorphism might determine susceptibility to severe influenza. helps to identify patients at higher risk and adequate treatment.
- MxA provides a barrier for zoonotic transmission of avian influenza viruses, might depict new strategies for antiviral treatment.



Otto Haller

Peter Stäheli

Martin Schwemmle

Oliver Daumke
MDC, Berlin

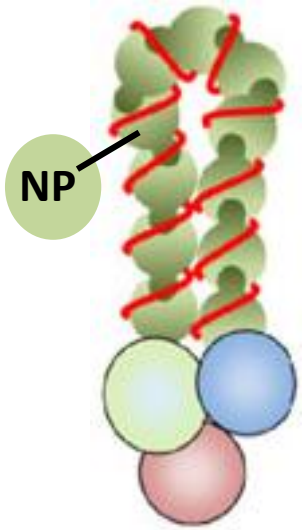
Harmit Malik
Fred Hutchinson Cancer Res. Center, Seattle

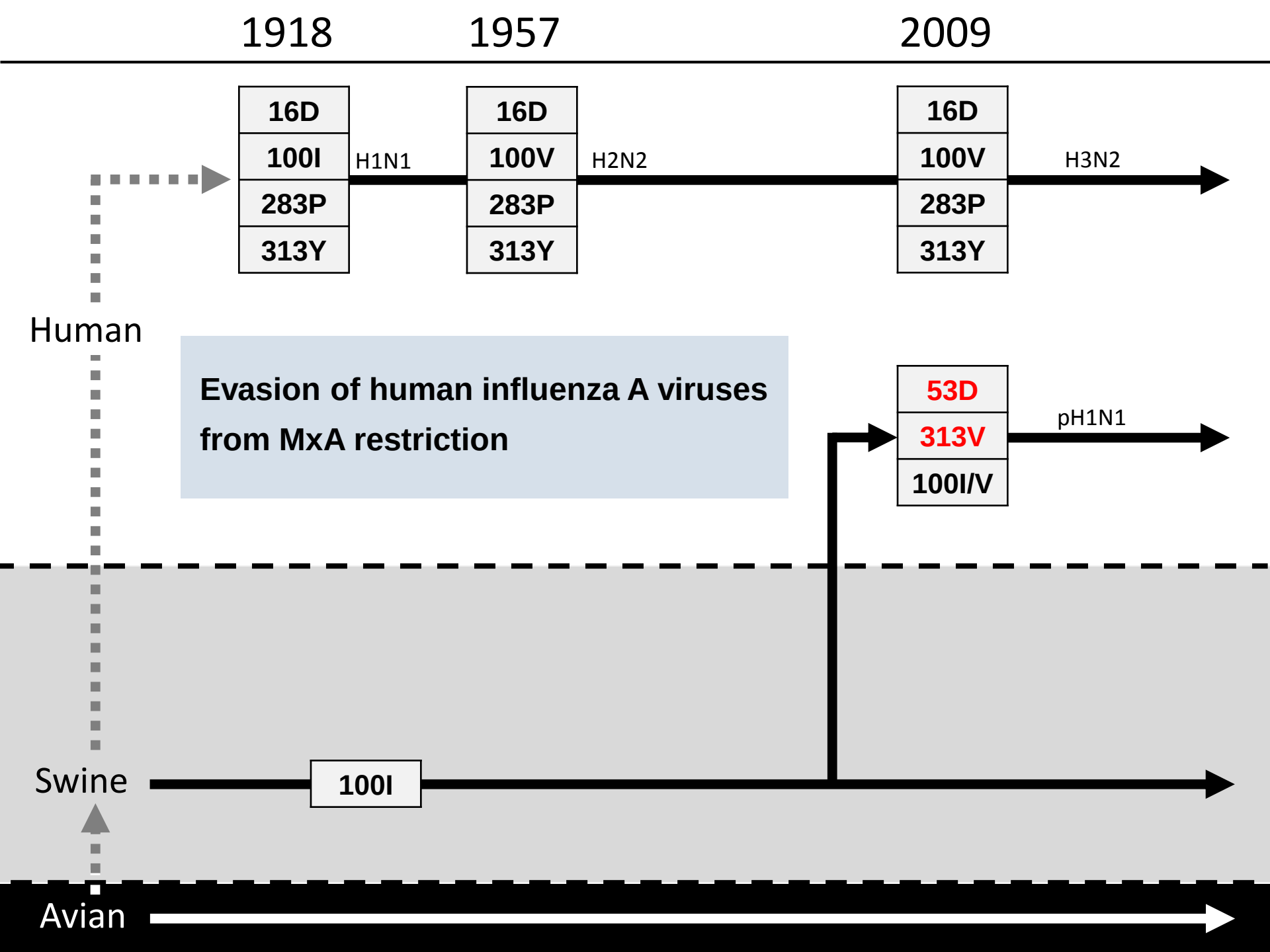
FREIBURG VIROLOGY

Fine

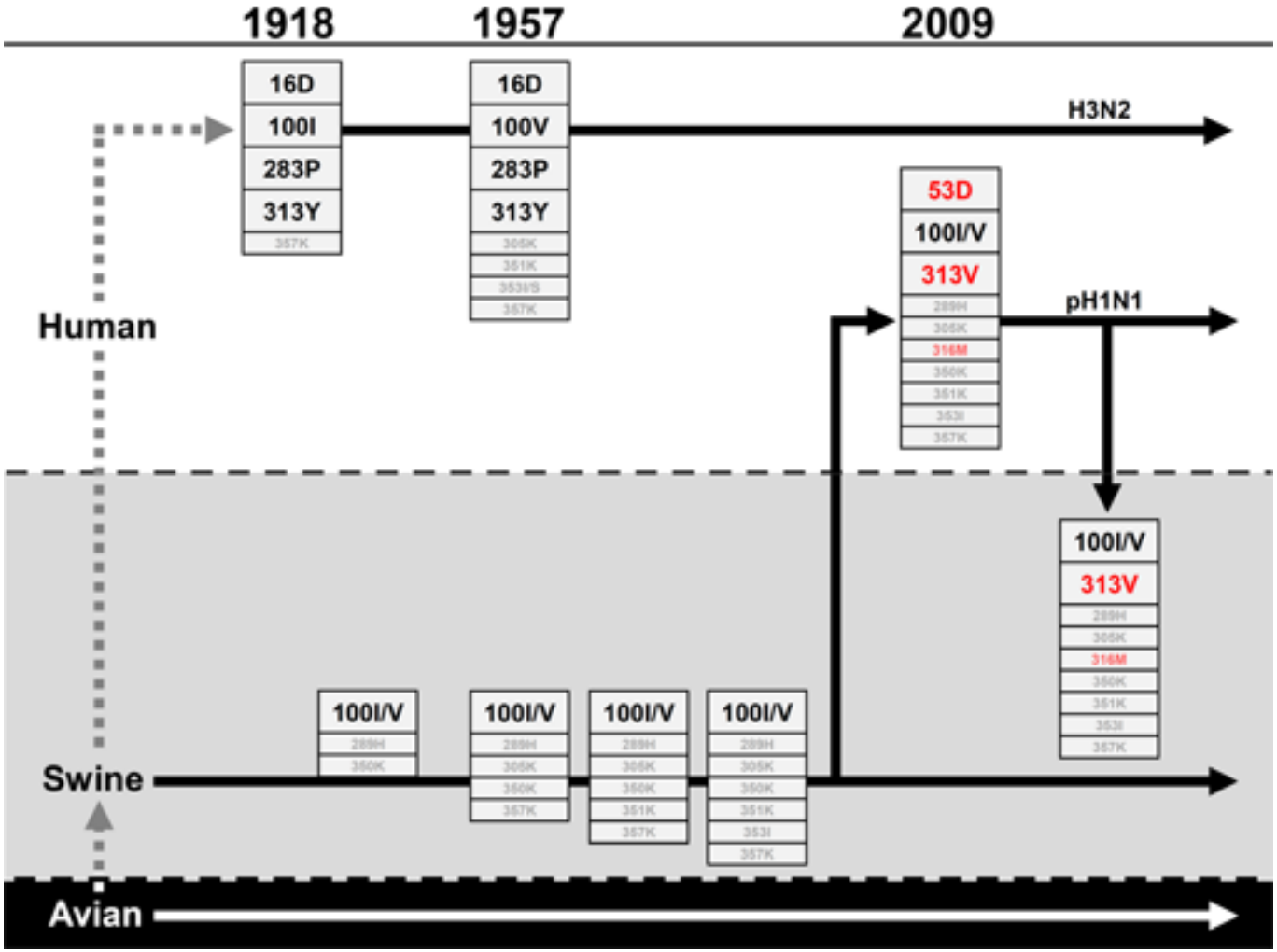
Analysis of viral NP for Mx sensitivity

	16	21	33	34	53	77	100	105	119	133	136	189	190	217	283	289	305	313	316	350	351	353	357	371	373	377	400	425	430	433	444	452	456	473	482	498	
pH1N1-NP	G	D	I	G	D	K	V	M	V	I	I	I	A	V	L	H	K	V	M	K	K	I	K	V	I	N	K	V	S	N	V	K	L	N	S	S	32 aa
H5N1-NP	G	N	V	S	E	R	R	V	I	L	L	M	V	I	L	Y	R	F	I	T	R	V	Q	M	A	N	R	I	T	T	I	R	V	N	N	N	14 aa
1918-NP	D	N	I	G	E	K	I	M	I	L	M	M	V	I	P	Y	R	Y	I	T	R	V	K	M	T	S	R	I	T	T	I	R	V	S	S	N	



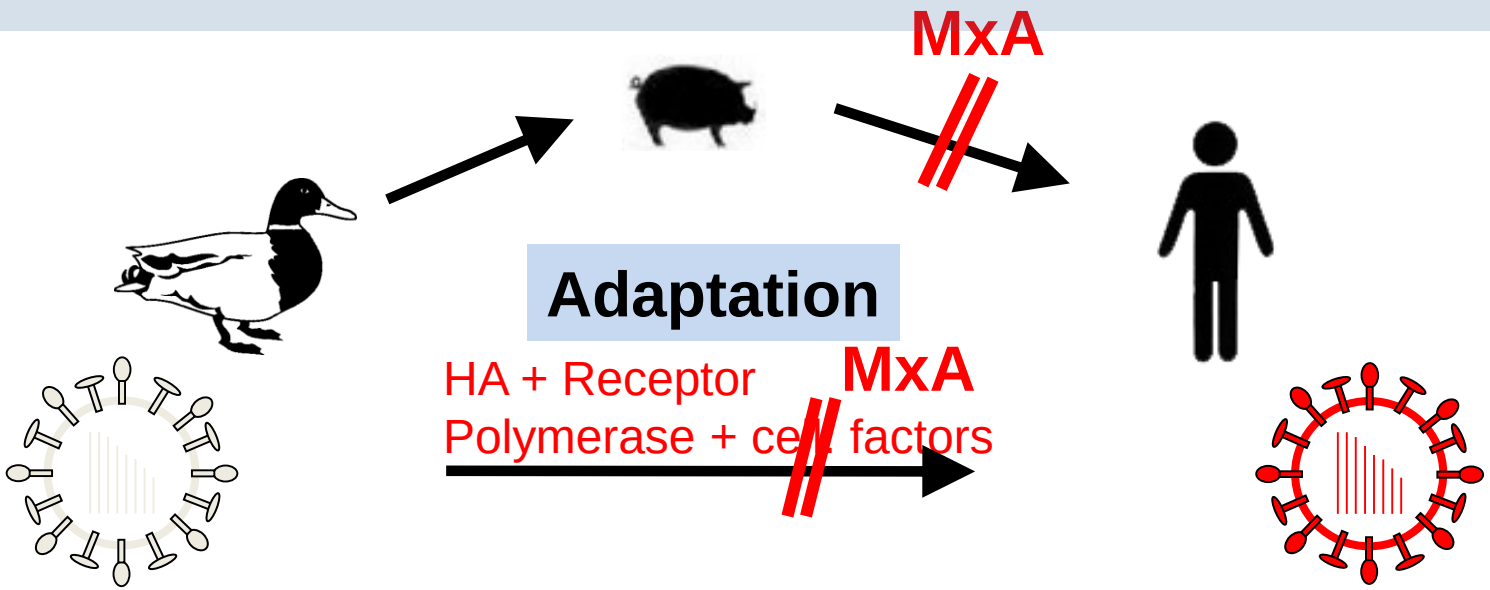

1918
1957
2009
16D
100I
283P
313Y
H1N1
16D
100V
283P
313Y
H2N2
16D
100V
283P
313Y
H3N2
Human
Evasion of human influenza A viruses from MxA restriction
53D
313V
100I/V
pH1N1
Swine
100I
Avian

Temporal appearance of Mx resistance enhancing mutations in influenza A viruses (Mänz et al., 2013)



Bold letters indicate amino acids shown to significantly increase Mx resistance, whereas amino acids highlighted in grey are minor contributors.
adaptive mutations that newly emerged with the appearance of the 2009 pandemic pH1N1 are depicted in red. 53D got partially lost after re-introduction into the swine host.

Influenza A virus & Mx proteins



Avian influenza

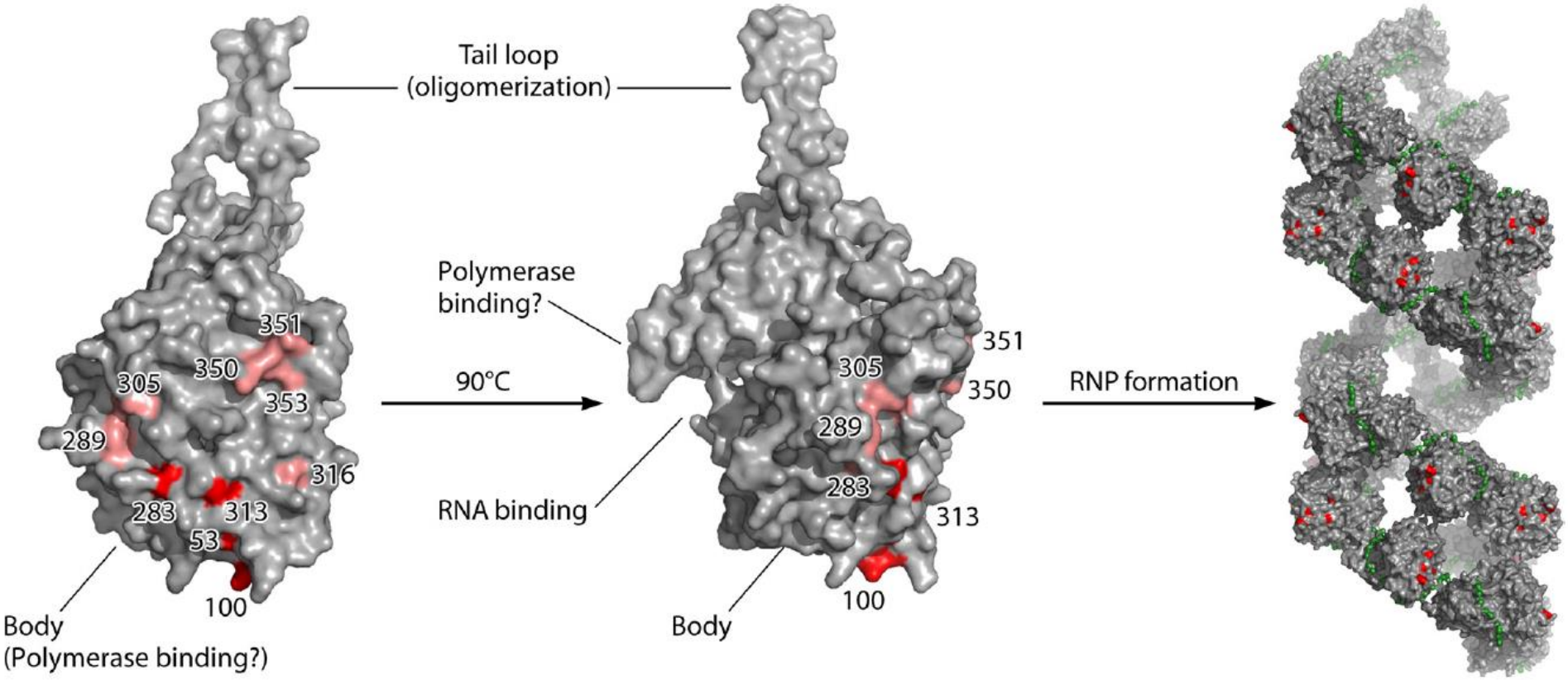
A/Thailand/1/04 (H5N1)-Kan1

Human adapted influenza

A/HH/01/09 (pH1N1)

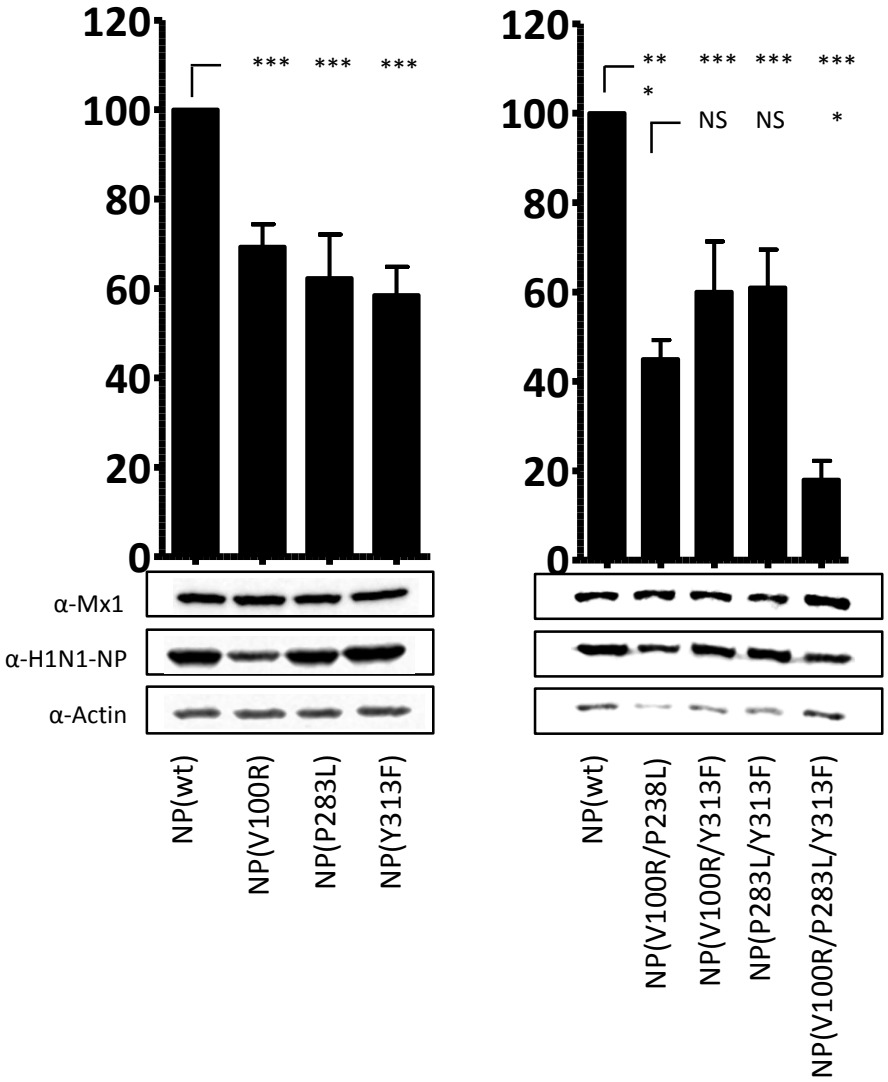
<u>Hypothesis:</u> Mx Proteins prevent Influenza A viruses from crossing the species barrier

INTRODUCTION: The Mx-Patch



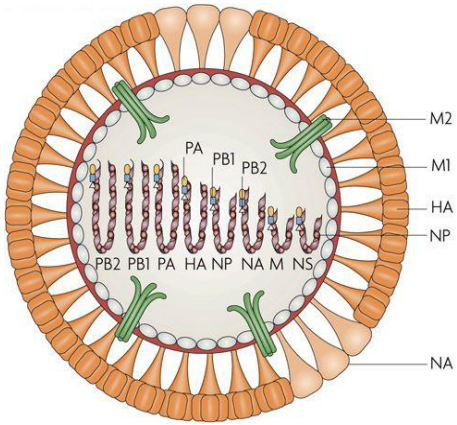
Verhelst, Hulpiau, Salens "Mx Proteins: Antiviral Gatekeepers That Restrain the Uninvited", MMBR, 2013

Mx-sensitivity of hvPR8-NP(V100R); (P283L); (Y313F) constructs in the minireplicon

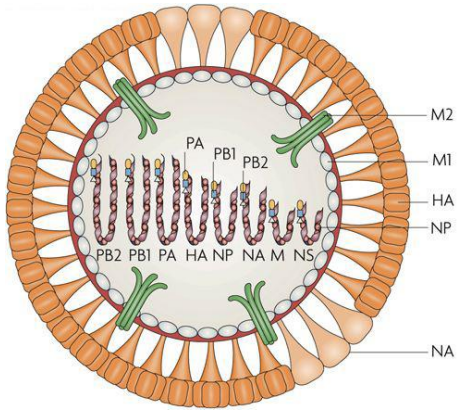


Creation of NP constructs

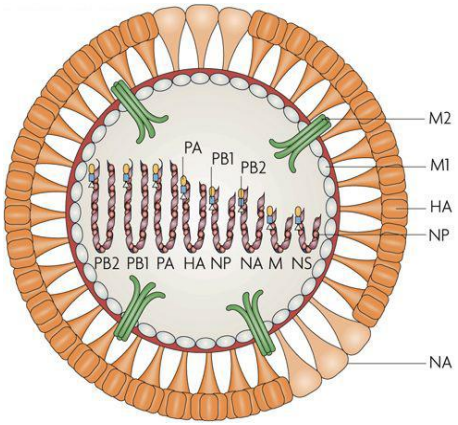
Rescue of PR8(mut) viruses



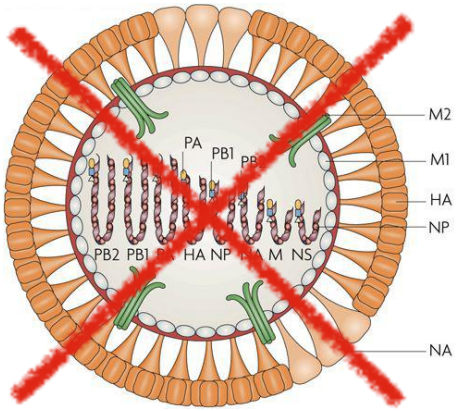
PR8-NP(V100R/P283L)



PR8-NP(V100R/Y313F)



PR8-NP(P283L/Y313F)

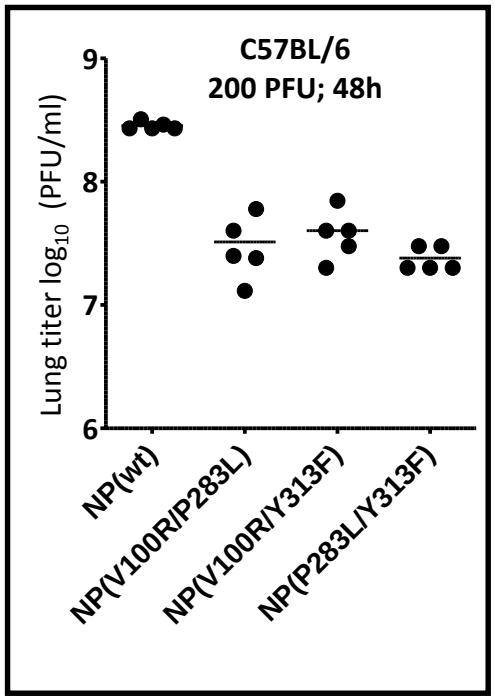


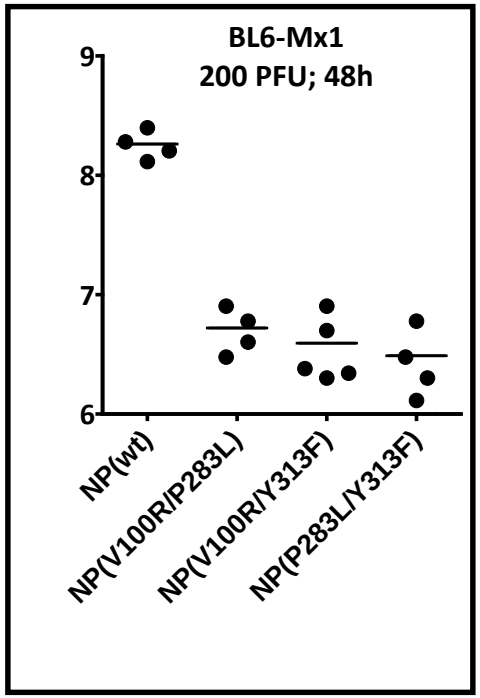
PR8-NP(V100R/P283L/Y313F)

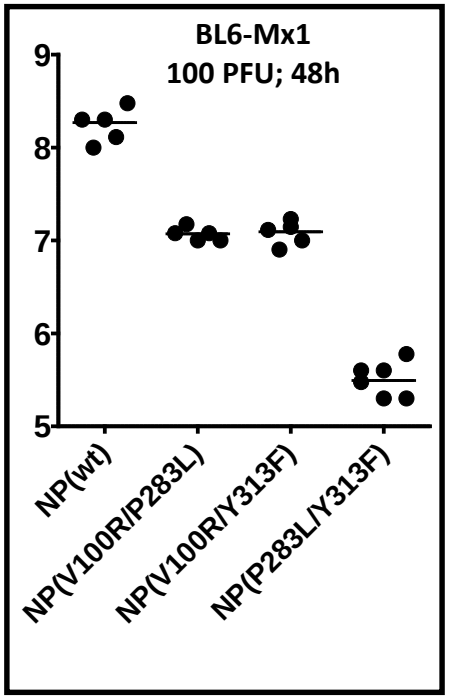
Rescue failed

Rescue of NP(mut) viruses

Fitness of hvPR8-NP(V100R); (P283L); (Y313F) viruses *in vivo*







LD_{50}

Virus	C57BL/6	BL6-Mx1
hvPR8-NP(wt)	<10	31,6
NP(V100R/P283L)	<10	100000
NP(V100R/Y313F)	<10	562
NP(P283L/Y313F)	<10	450000

Finding the right candidate for the passaging experiment

PASSAGING EXPERIMENT

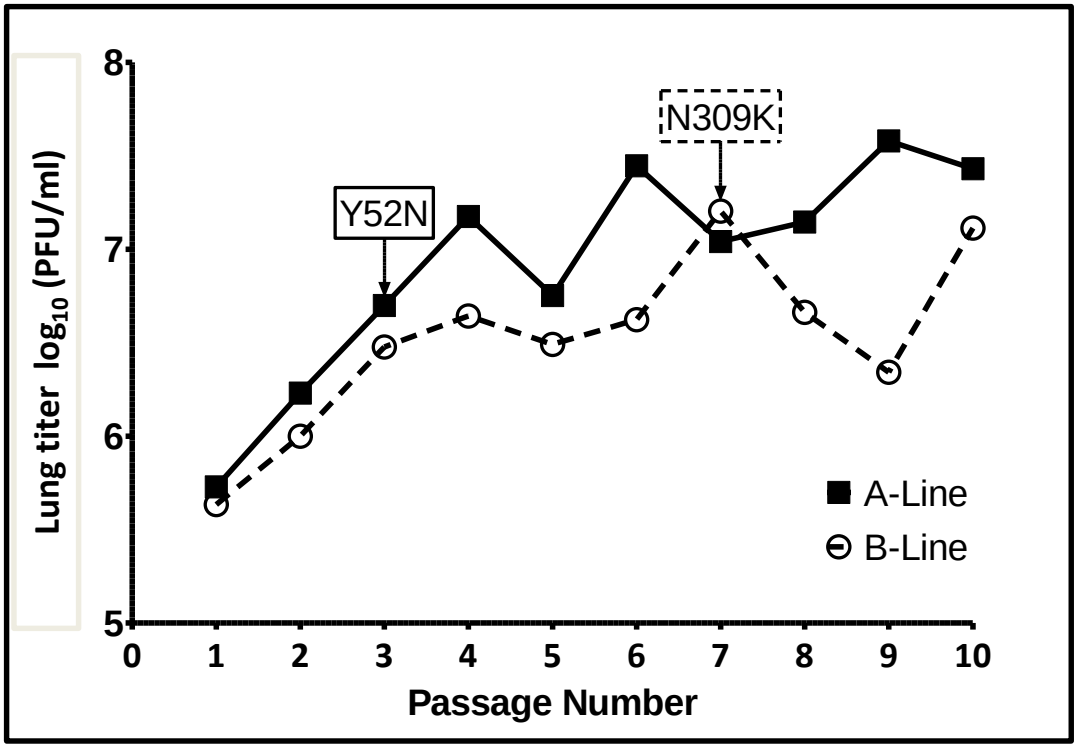
Start: Infection with hvPR/8-NP(P283L/Y313F)

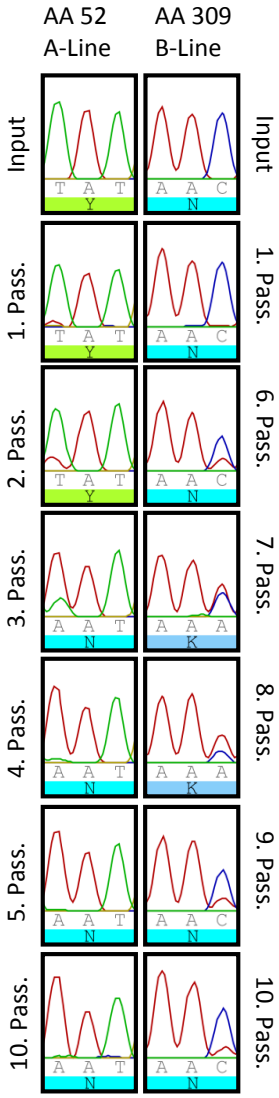
t=0: Infection with 100 PFU intra nasal

+48h: Preparation of lungs; homogenisation; virustitration

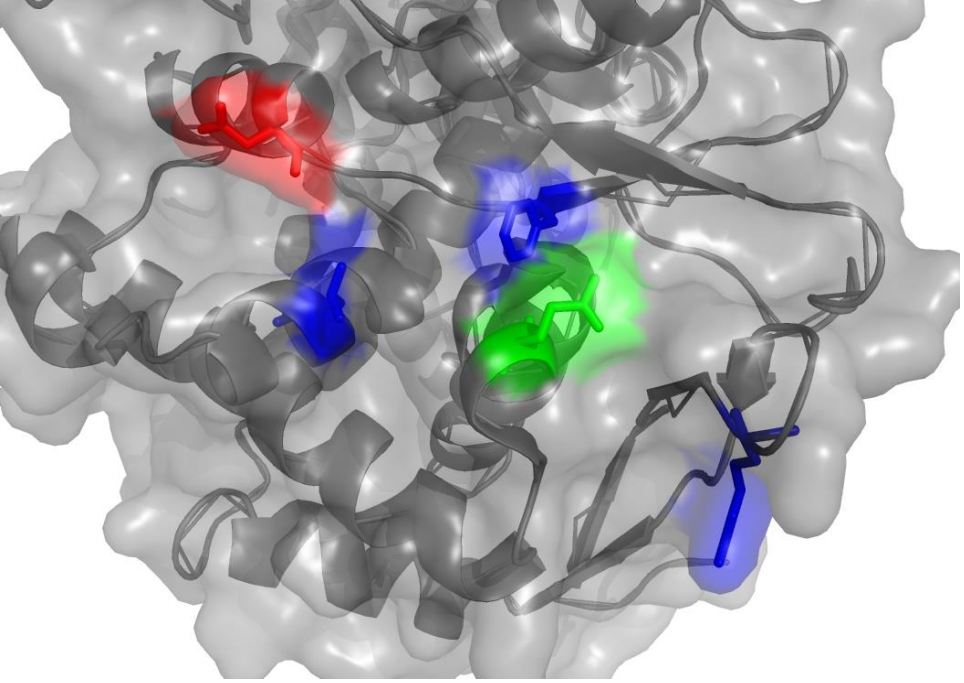
PASSAGING EXPERIMENT

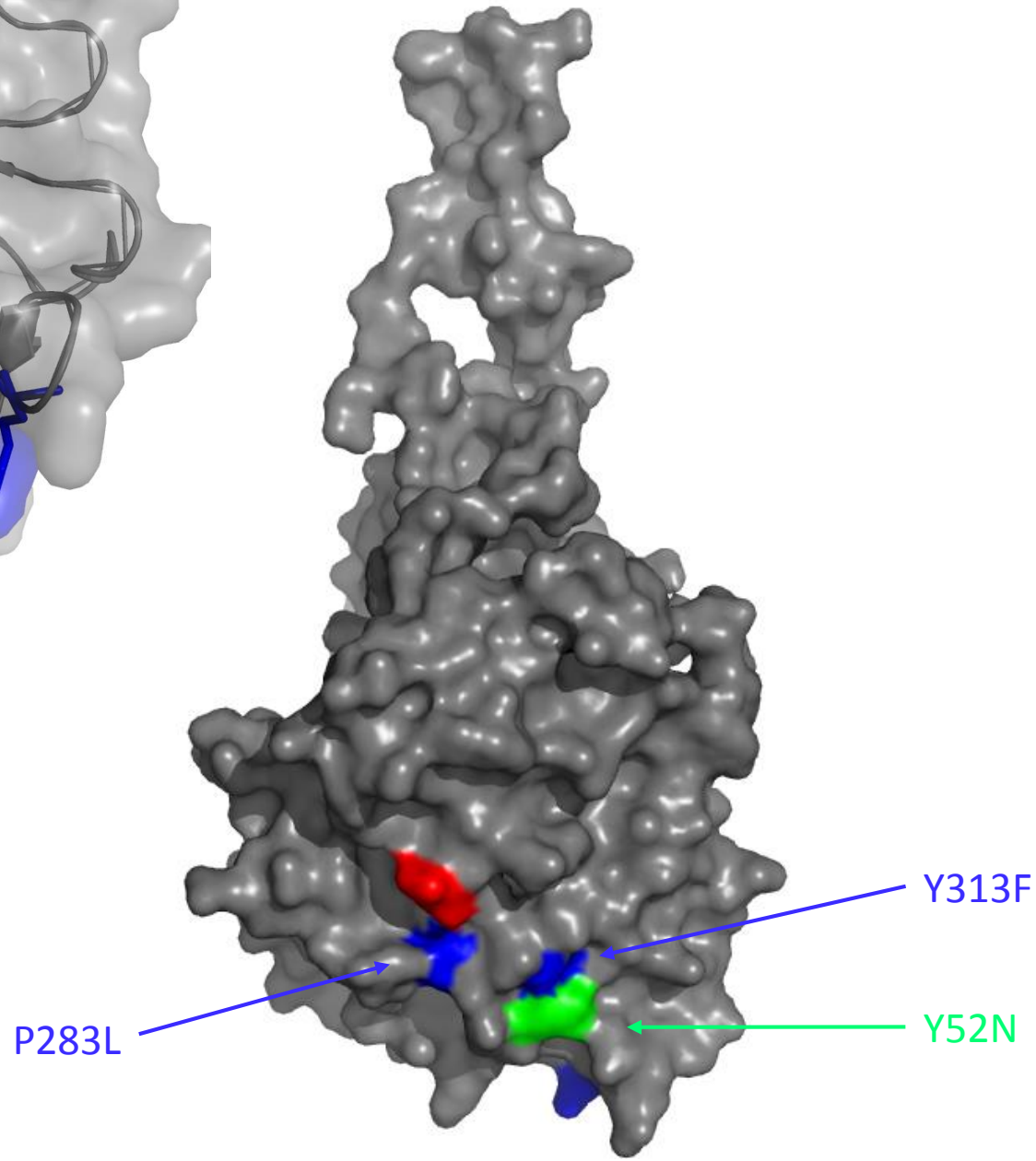
Development of lung titers during passaging and outcome of segment 5 sequencing





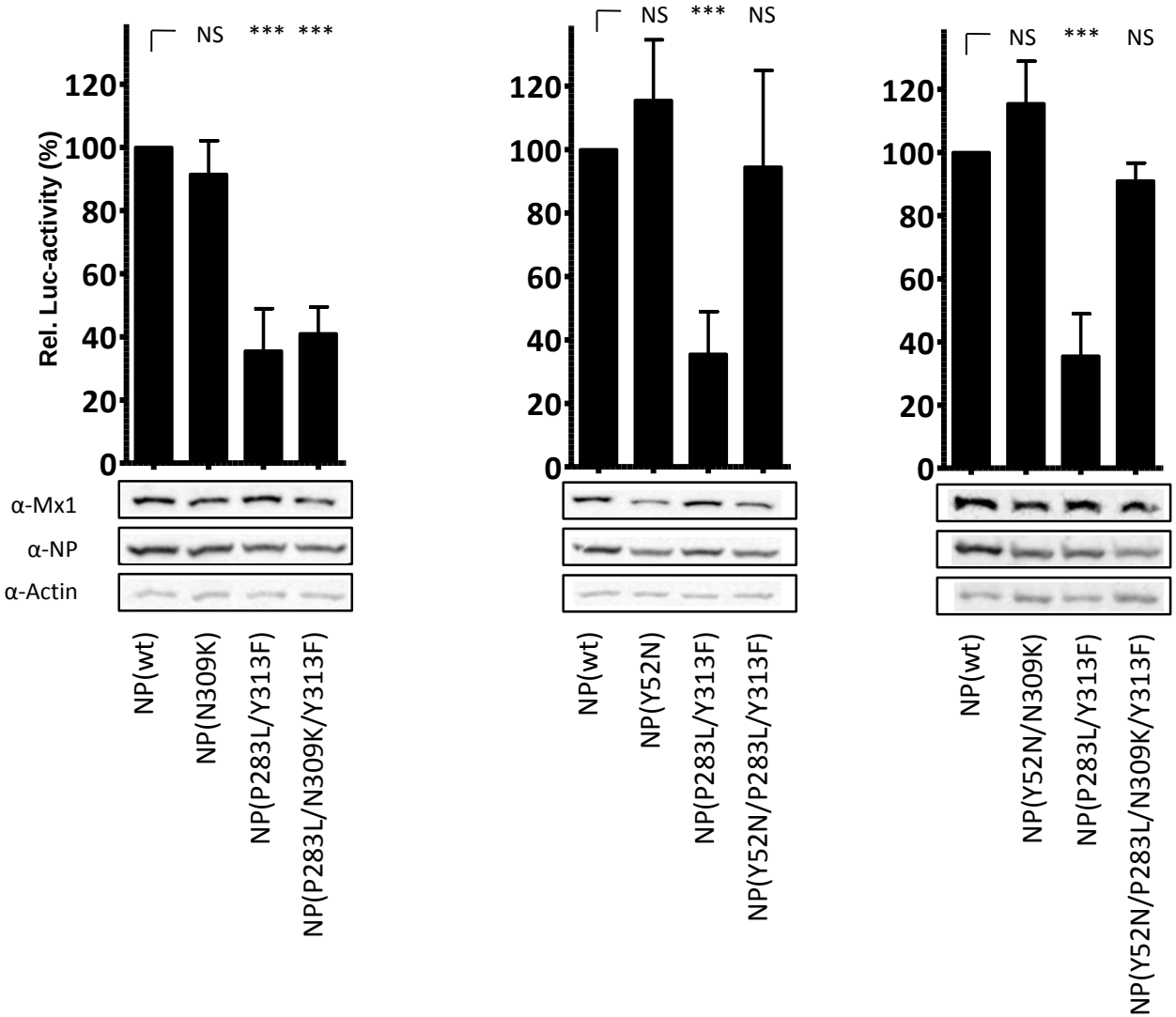
Two escape mutants were found





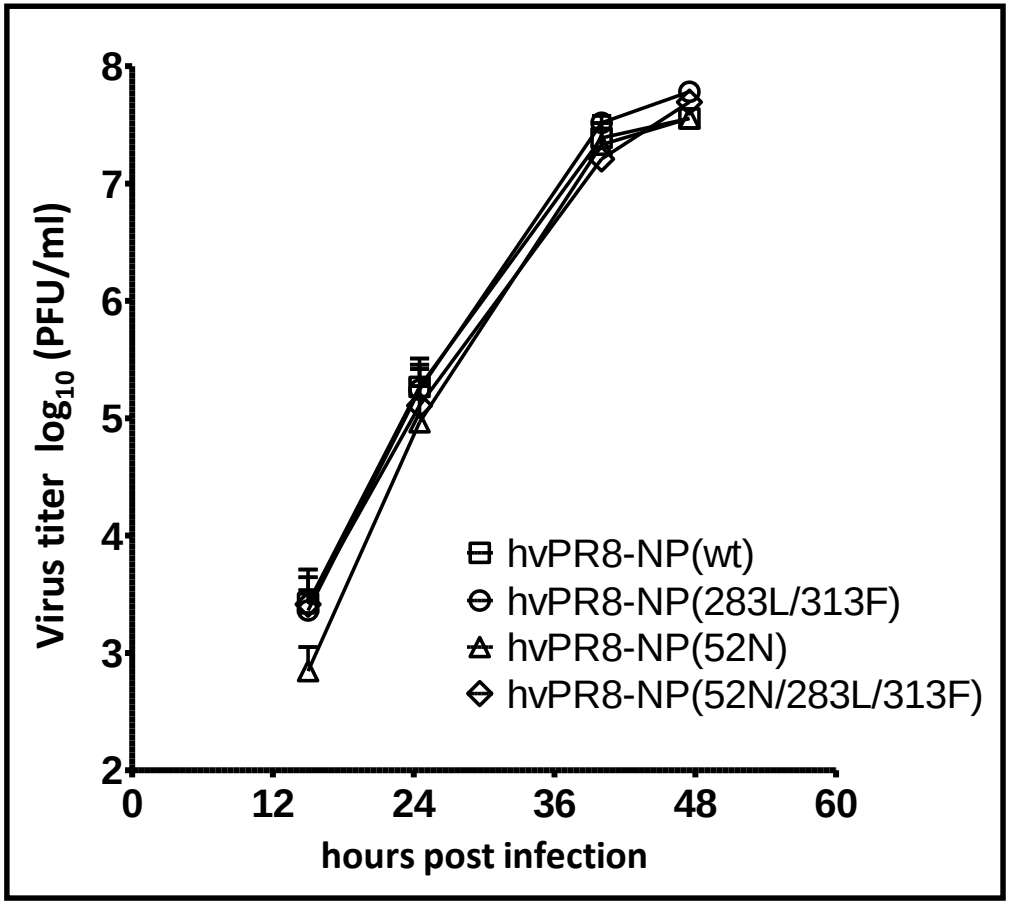
The new mutations are inside the Mx-patch next to the original knock-down mutations

Mx-sensitivity of hvPR8-NP(Y52N) & NP(N309K) constructs in the minireplicon



The Y52N mutation decreases Mx-sensitivity, N309K mutation has no effect

Fitness of hvPR8-NP(Y52N) & NP(N309K) viruses *in vitro*

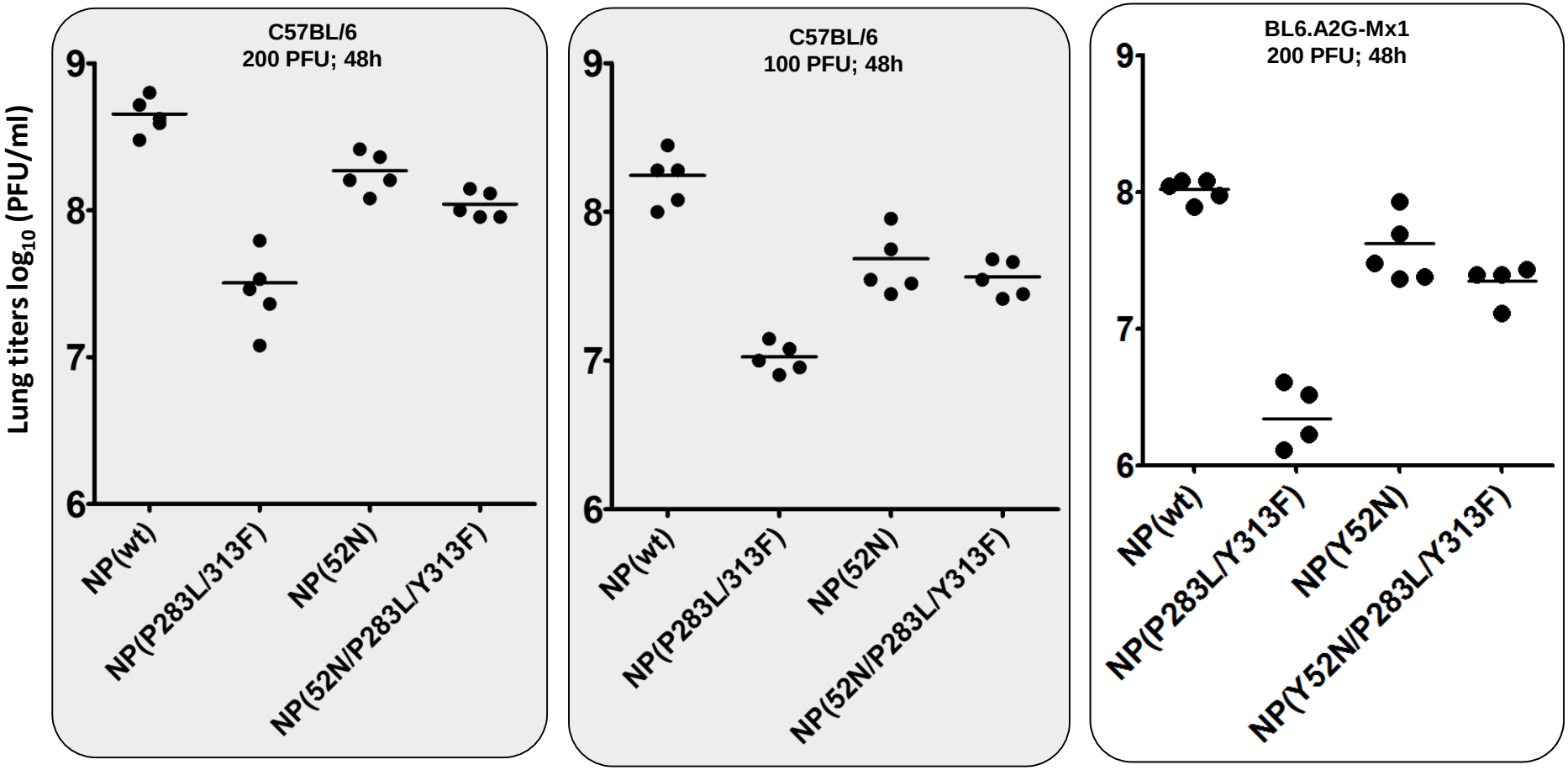


Calu-3 cells; MOI 0,001

Rescue and test of the interesting 52N mutant

Fitness of hvPR8-NP(Y52N); (P283L); (Y313F) viruses *in vivo*

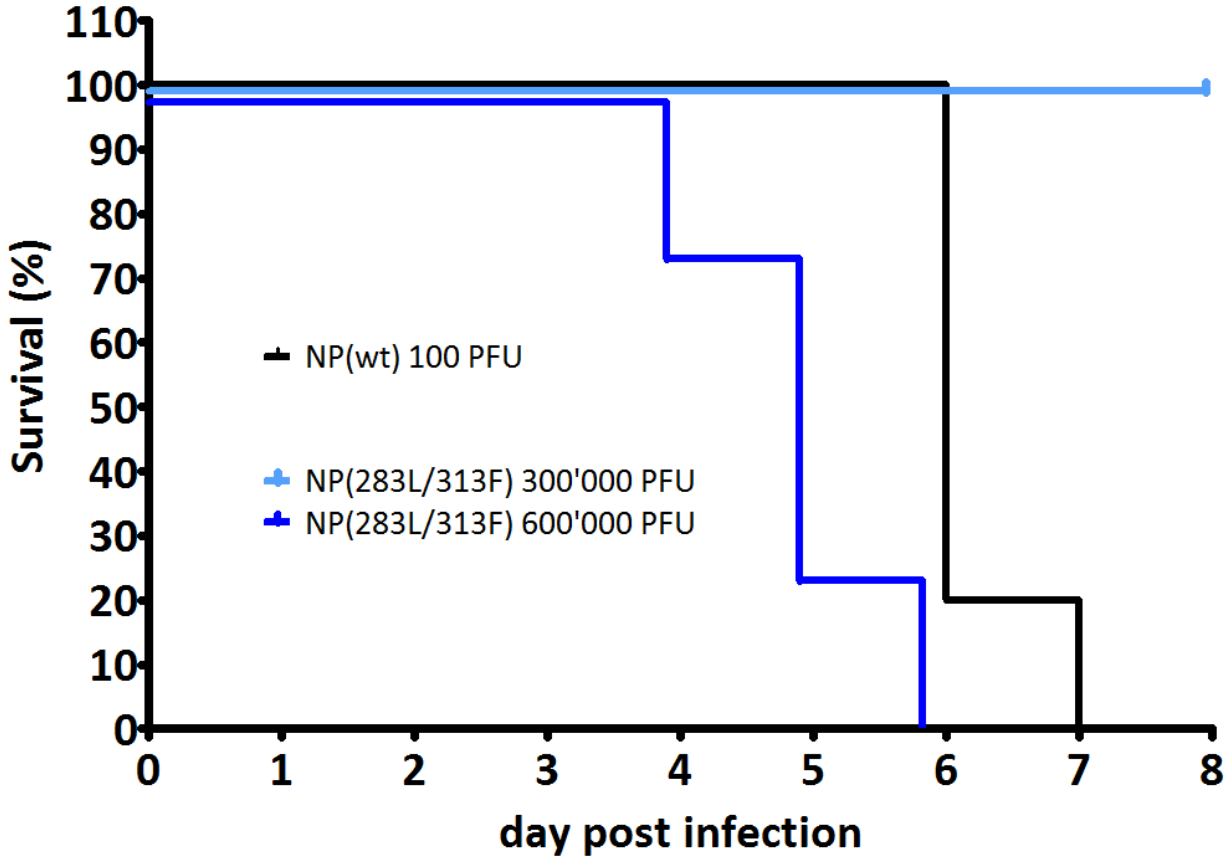
Lung titers in $Mx^{-/-}$ and $Mx^{+/+}$ mice



Rescue and test of the interesting 52N mutant

LETHALITY

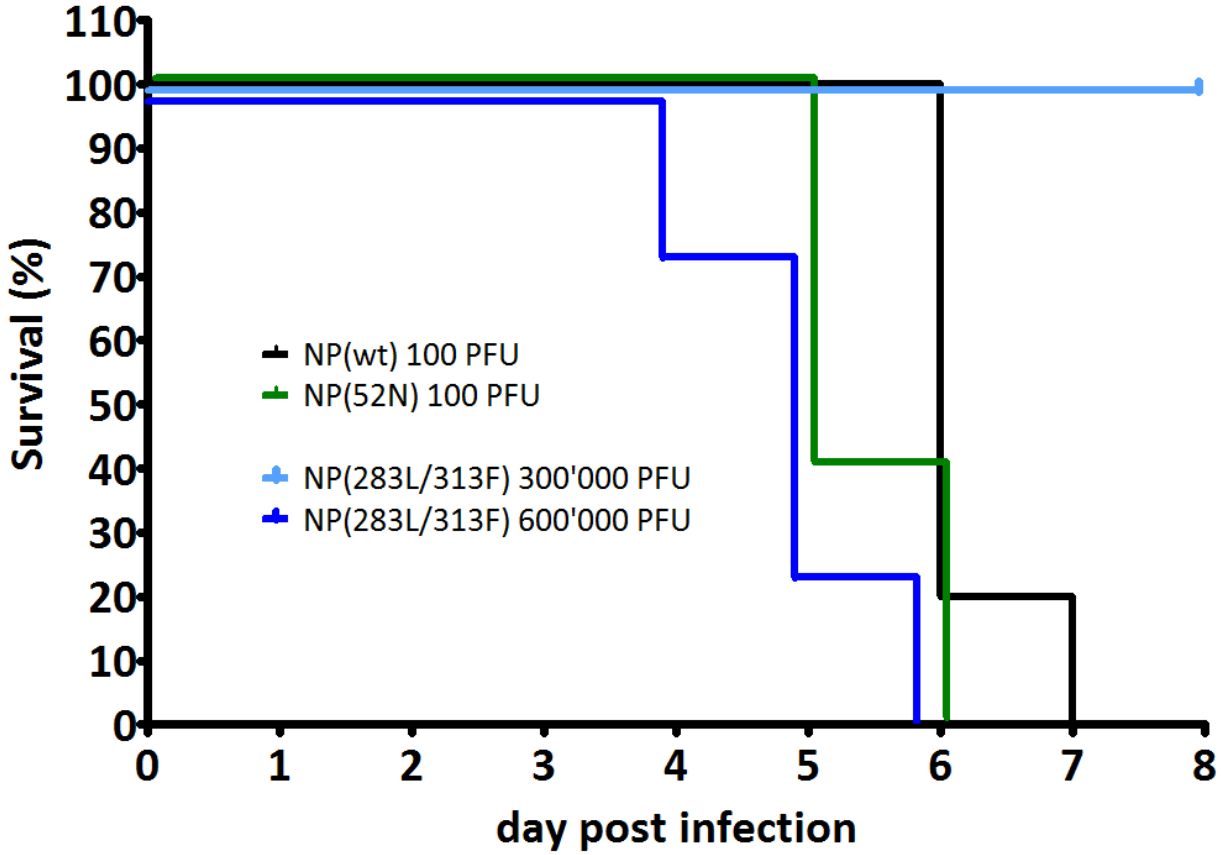
Survival of hvPR8-NP(Y52N/P283L/Y313F) virus in $Mx^{+/+}$ mice



NP(wt) is aggressive, NP(283L/313F) is attenuated in $Mx^{+/+}$ mice

LETHALITY

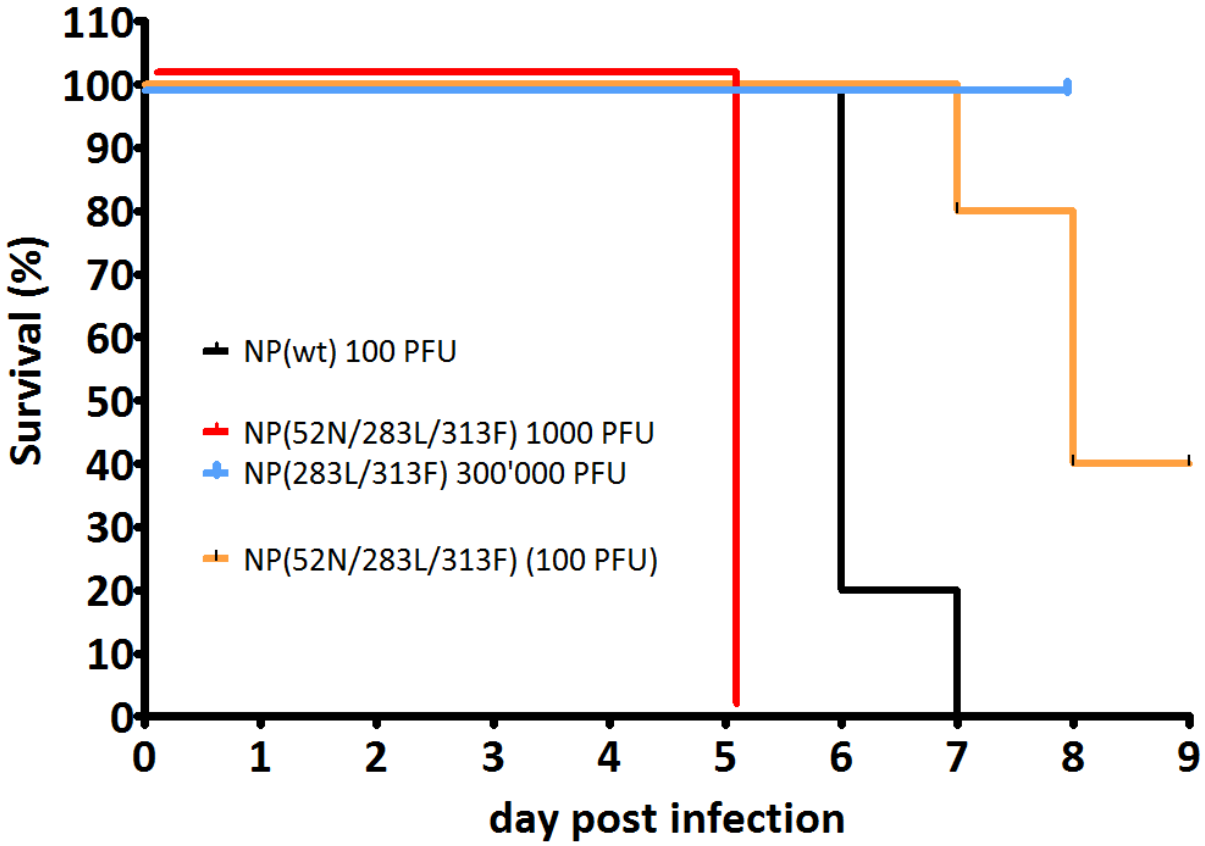
Survival of hvPR8-NP(Y52N/P283L/Y313F) virus in $Mx^{+/+}$ mice



NP(52N) is comparable to NP(wt)

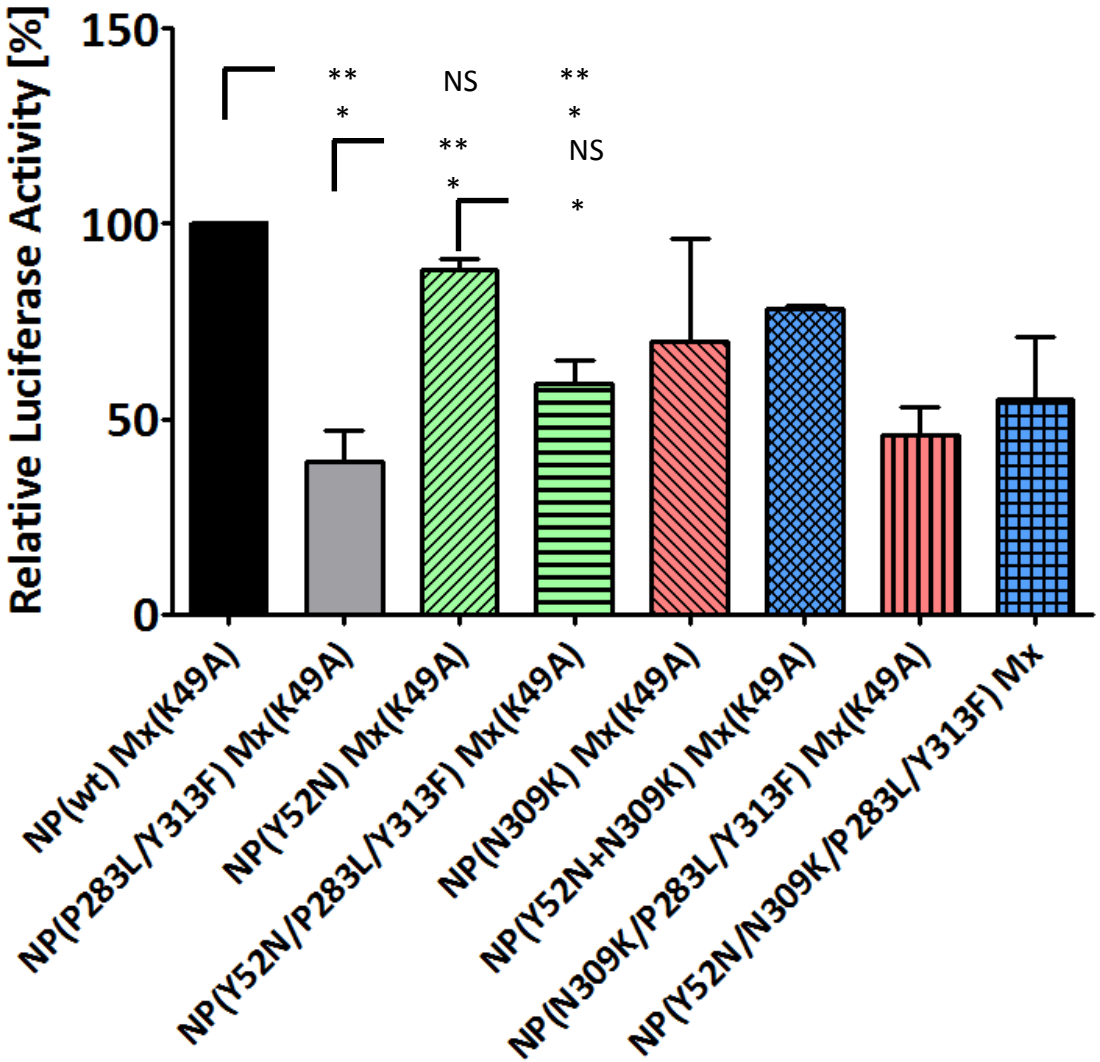
LETHALITY

Survival of hvPR8-NP(Y52N/P283L/Y313F) virus in $Mx^{+/+}$ mice



NP(52N/283L/313F) is nearly as aggressive as NP(wt)

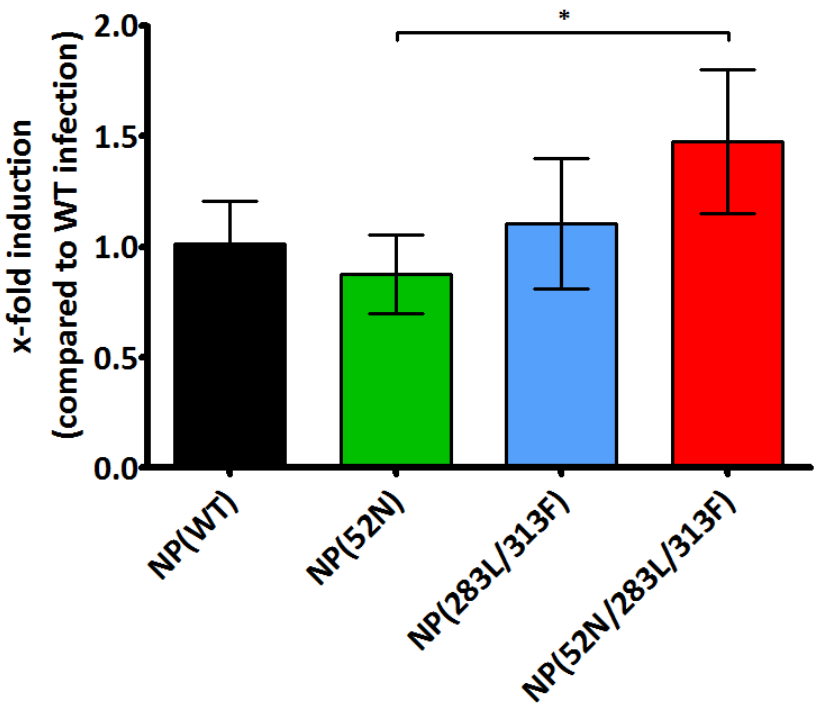
Relative polymerase activity
How do the mutations affect polymerase speed?



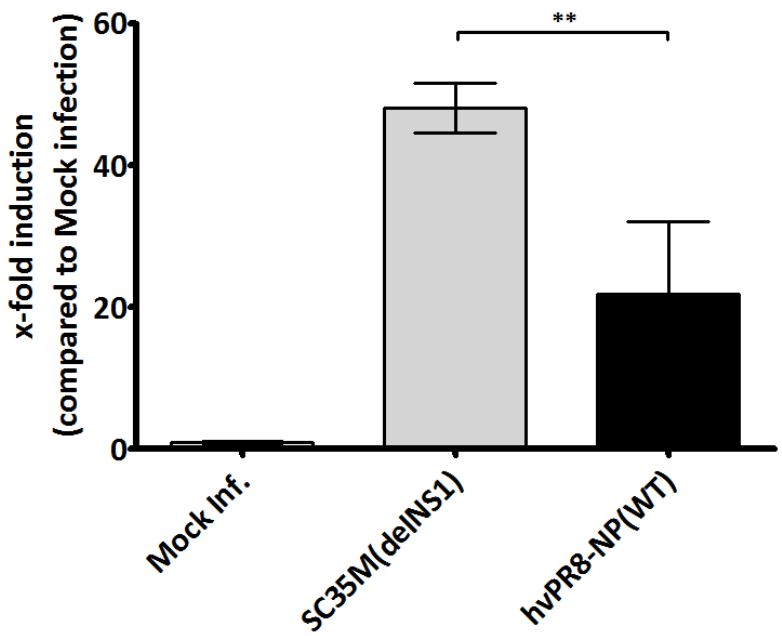
NP(283L/313F) is nearly on the same level as NP(52N/283L/313F)

qPCR:
Do the mutations affect Mx-induction??

Mx induction of hvPR8-NP(mut)



ISG15 induction of SC35M(ΔNS1)

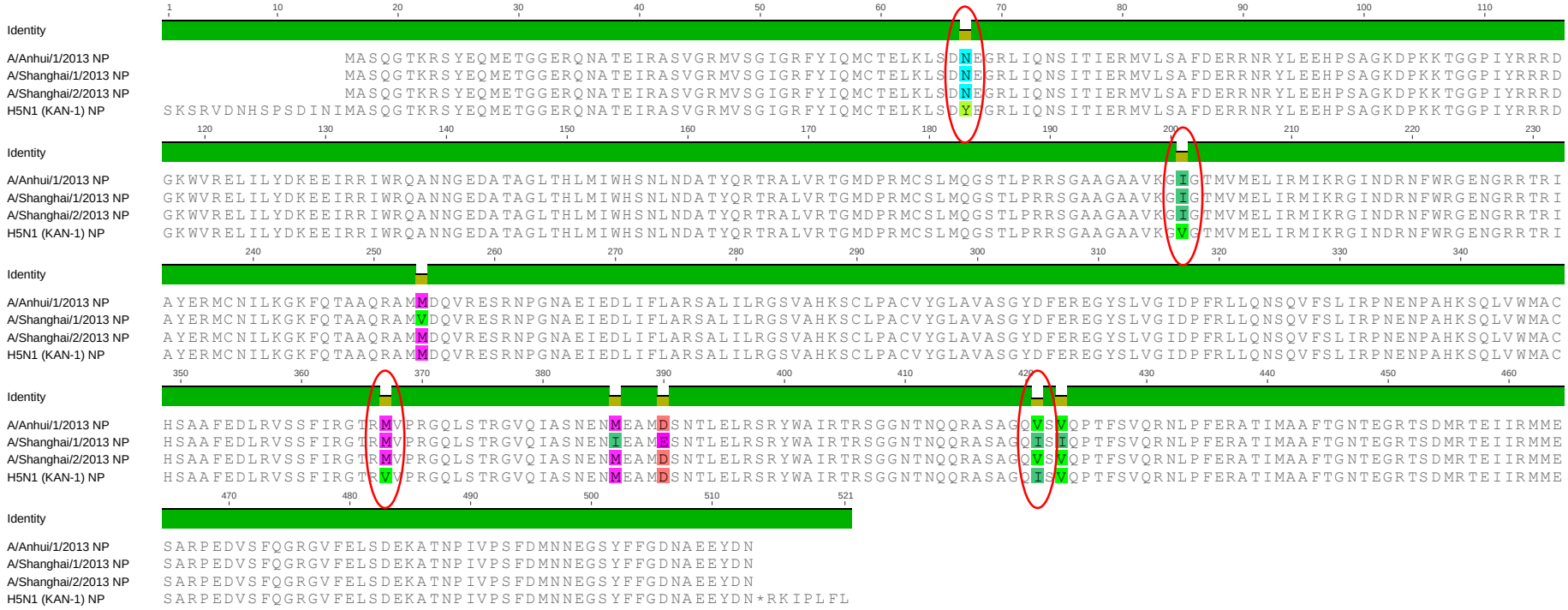


All mutations induce Mx1 to a comparable level


Project:
Doomsday

Outlook: H7N9

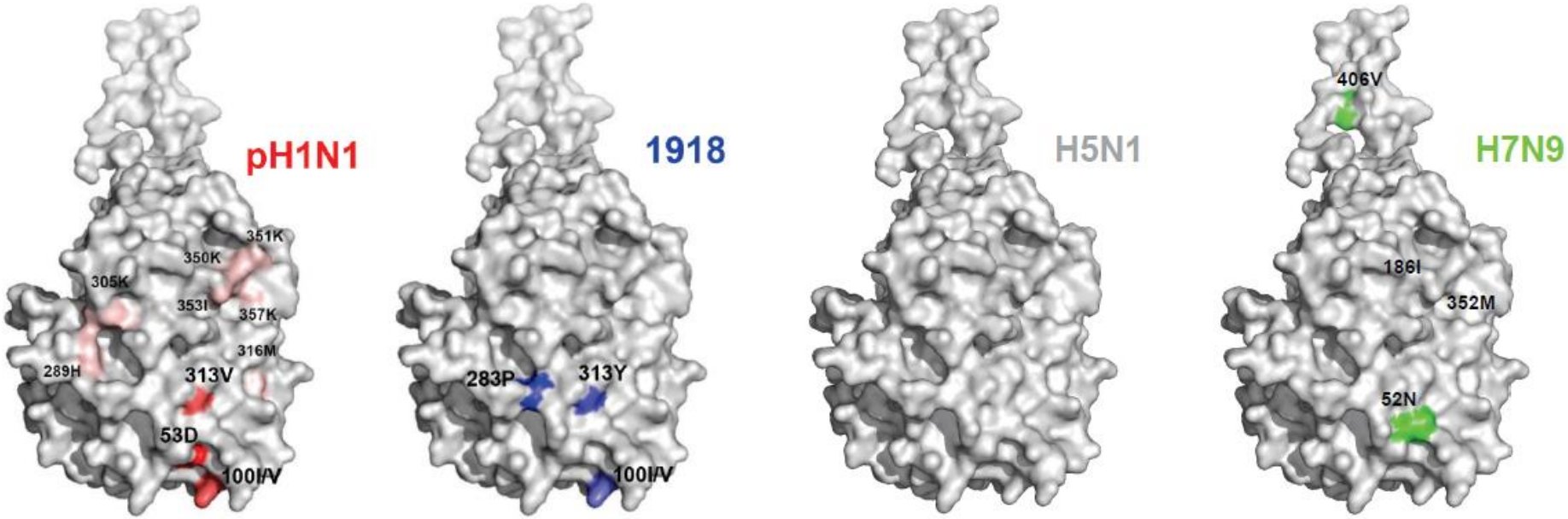
Analysis of H7N9-NP(wt), H7N9-NP(N52Y) and H7N9-NP(4X)



Together with Dominik Dornfeld (AG Schwemmle):
Checking H7N9 for Mx-sensitivity

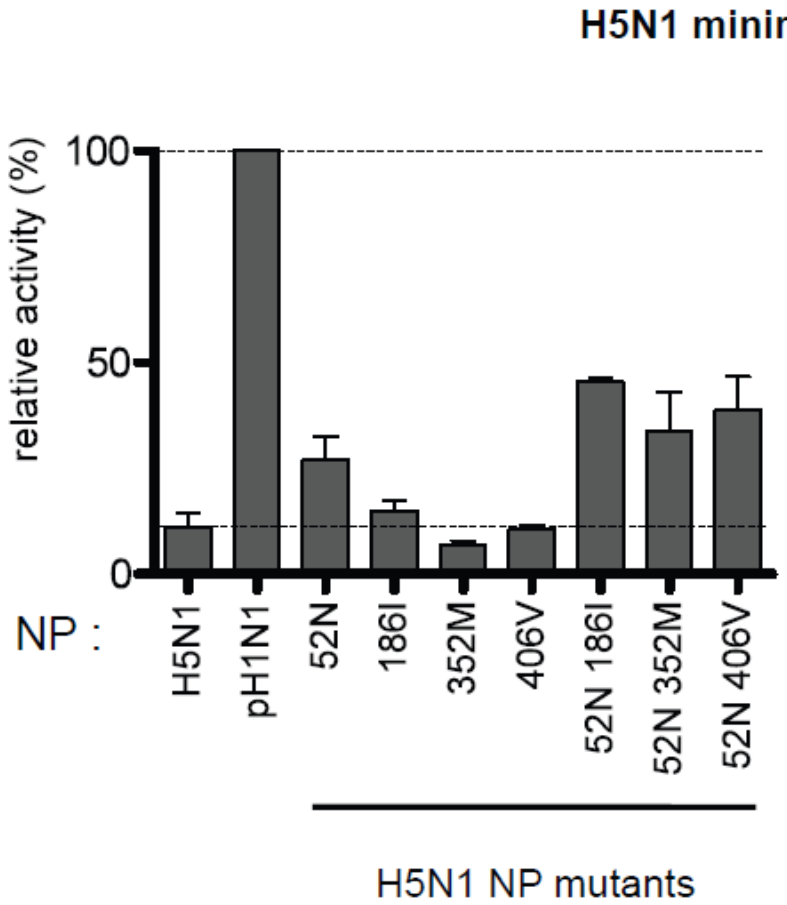
NP Comparison

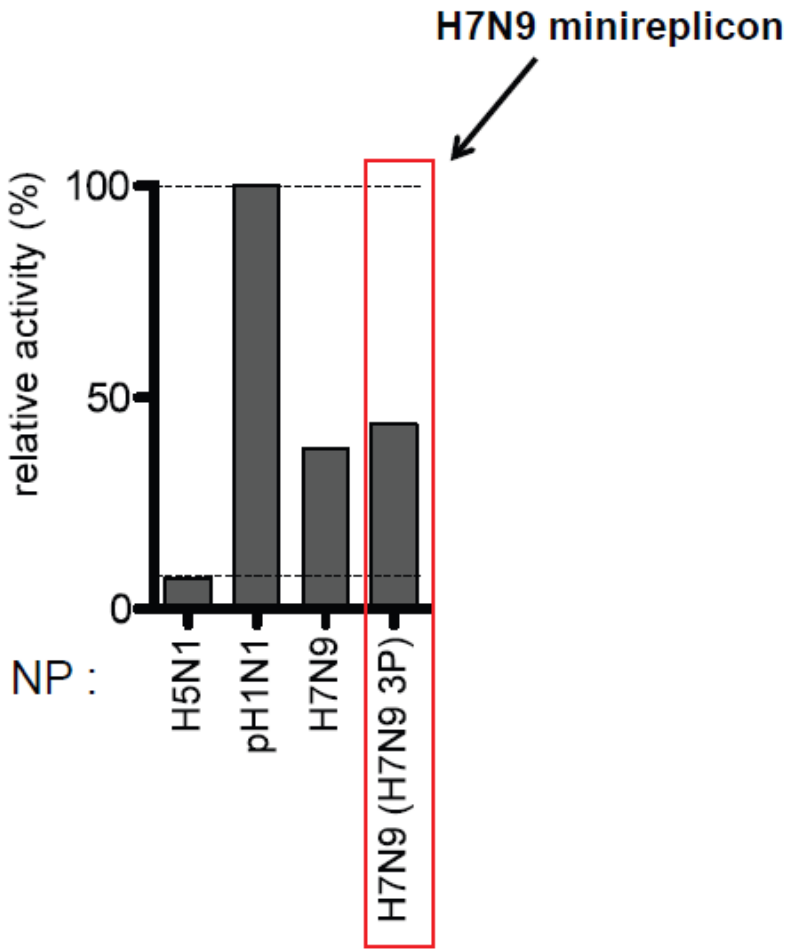
52N and 352M are close to Mx-patch positions



H7N9 Minireplicon

Intermediate Mx-sensitivity in the minireplicon

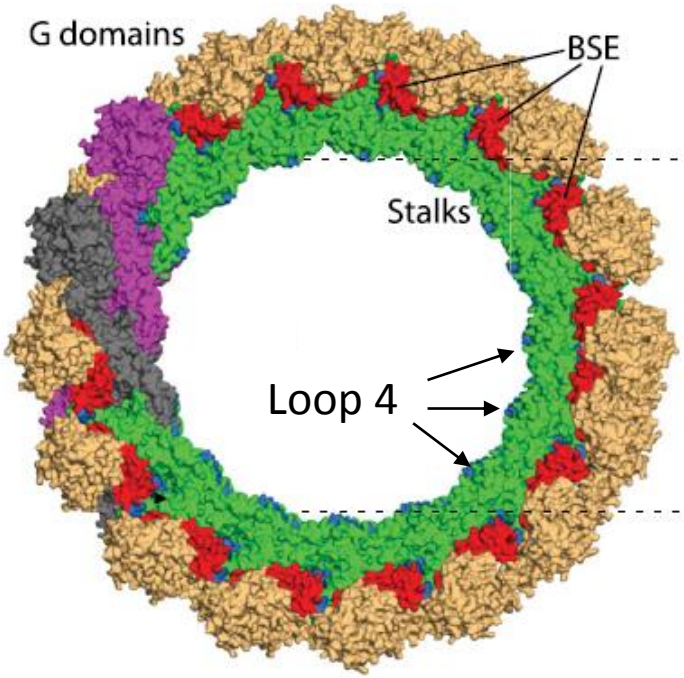


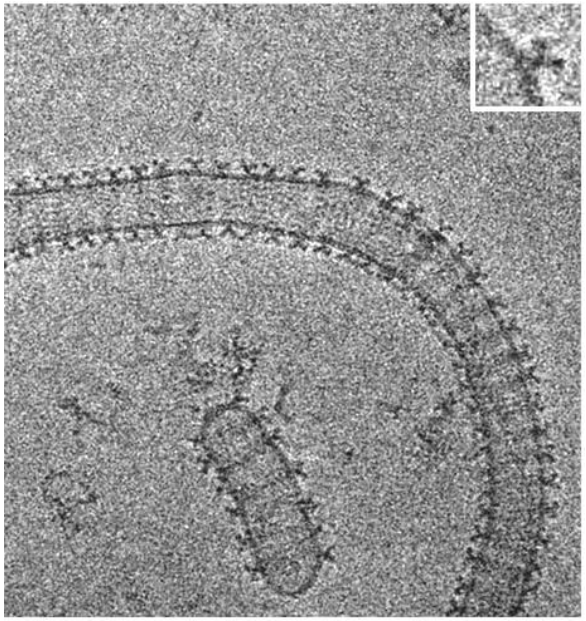


Minireplicon by Dominik

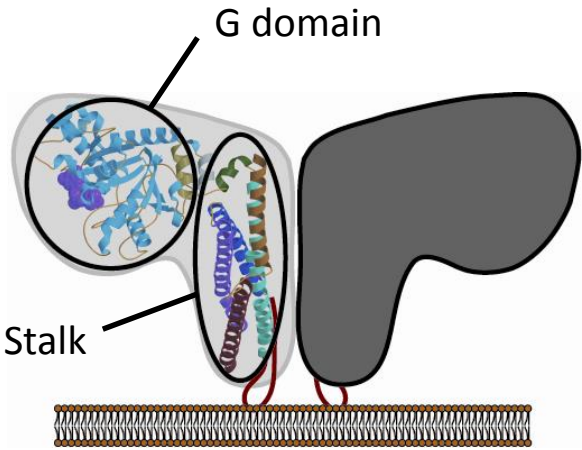
H7N9 has an intermediate Mx-sensitiviy between H5N1 and H1N1

Loop L4: binding site for lipid membranes





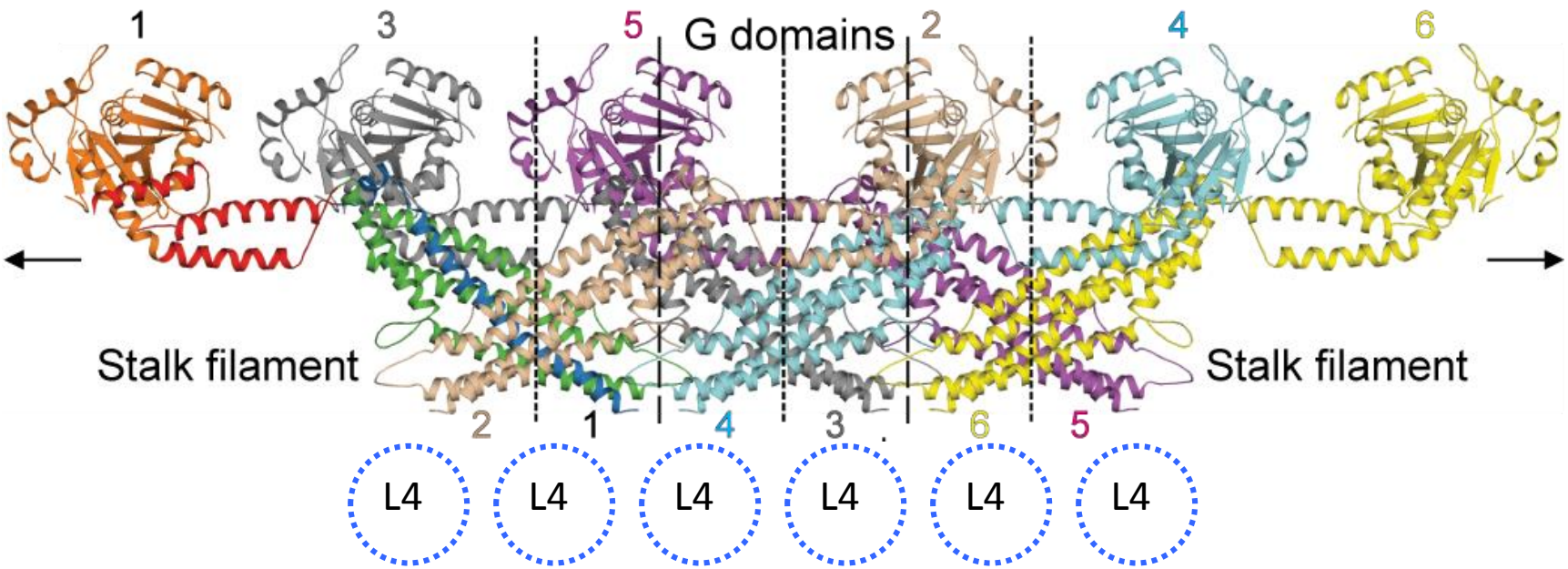
Cryo-TEM purified MxA on lipid membrane



T- bar shape of MxA

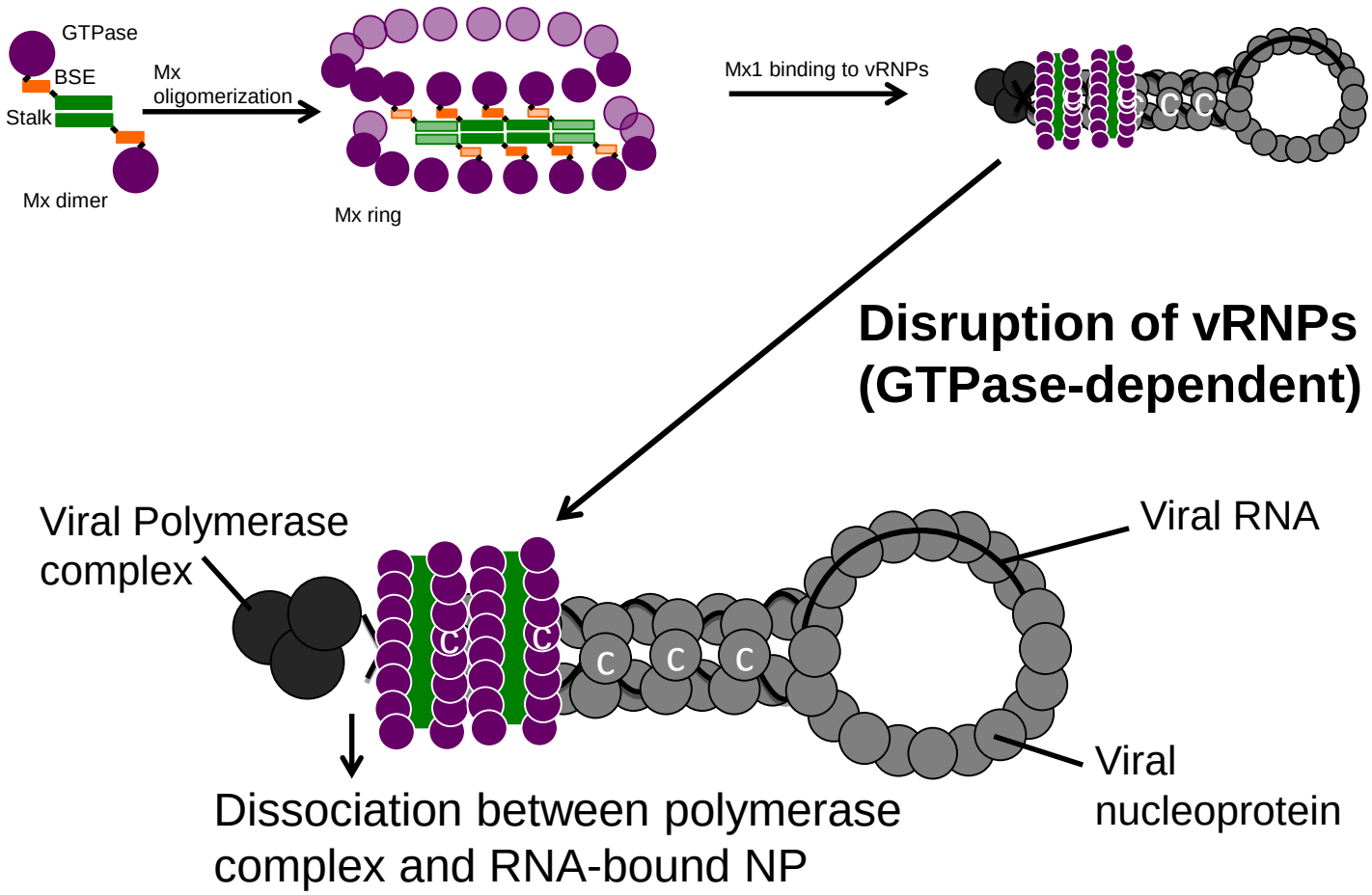
[*von der Malsburg A, et al., J. Biol. Chem, 286, 37858-37865 (2011)*]

Homo-oligomer of MxA



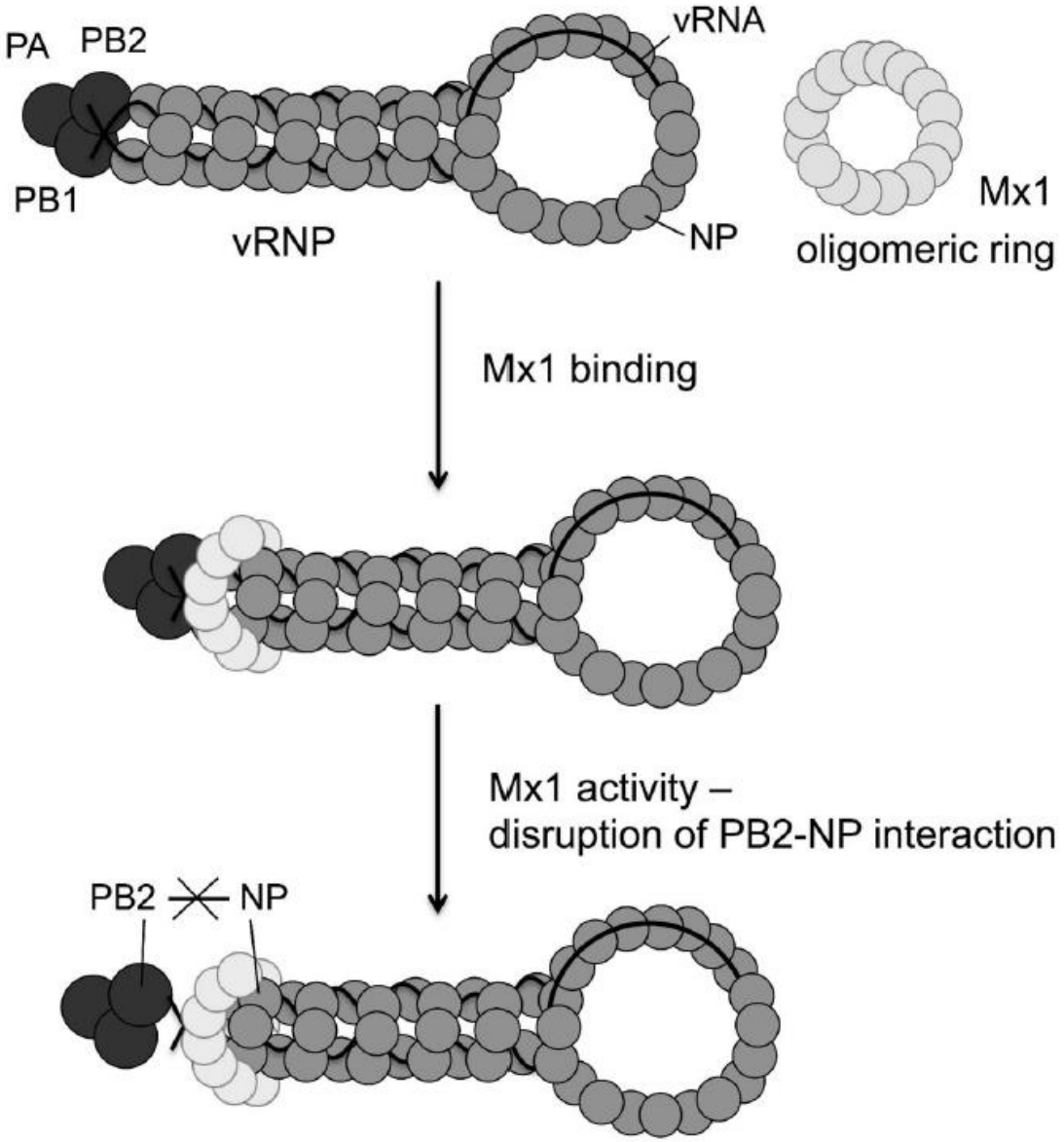
[*Gao S, von der Malsburg A, et al., Immunity, 35: 514-525 (2011)*]

Most recent model of mouse Mx1 action



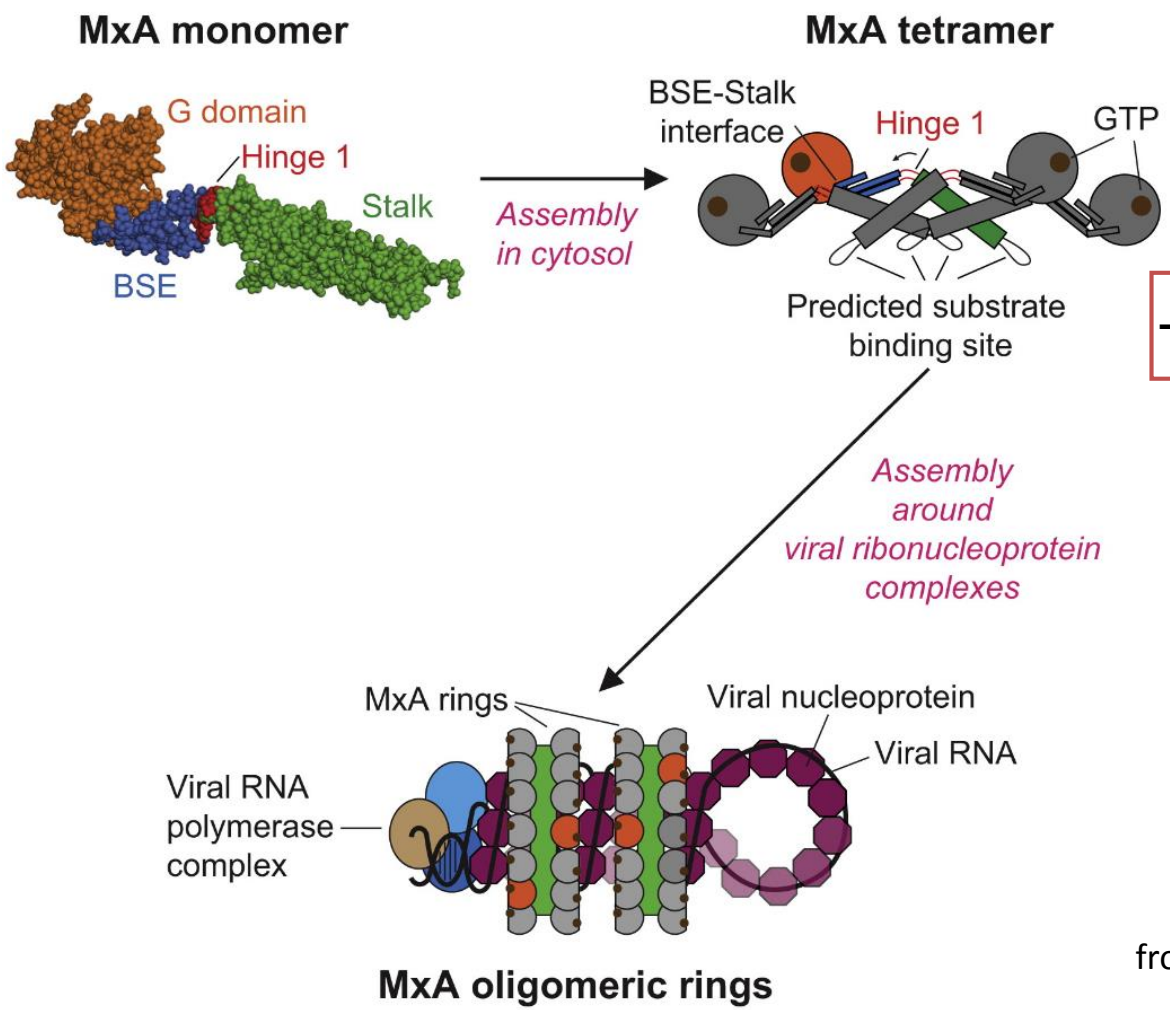
[*Verhelst … Fiers….Saelens, J Virol 86: 13445 - 13455 (2012)*]

Model of Mx action against influenza A viruses



Verhelst et al., (2012) JVI in press

Model of Mx antiviral action



→ Loop L4 of MxA

from Gao et al., (2011) Immunity 35,

Gao S., von der Malsburg A. et al.; Immunity 2011

CONCLUSIONS

1. Human MxA and MxB are IFN-induced antiviral restriction factors

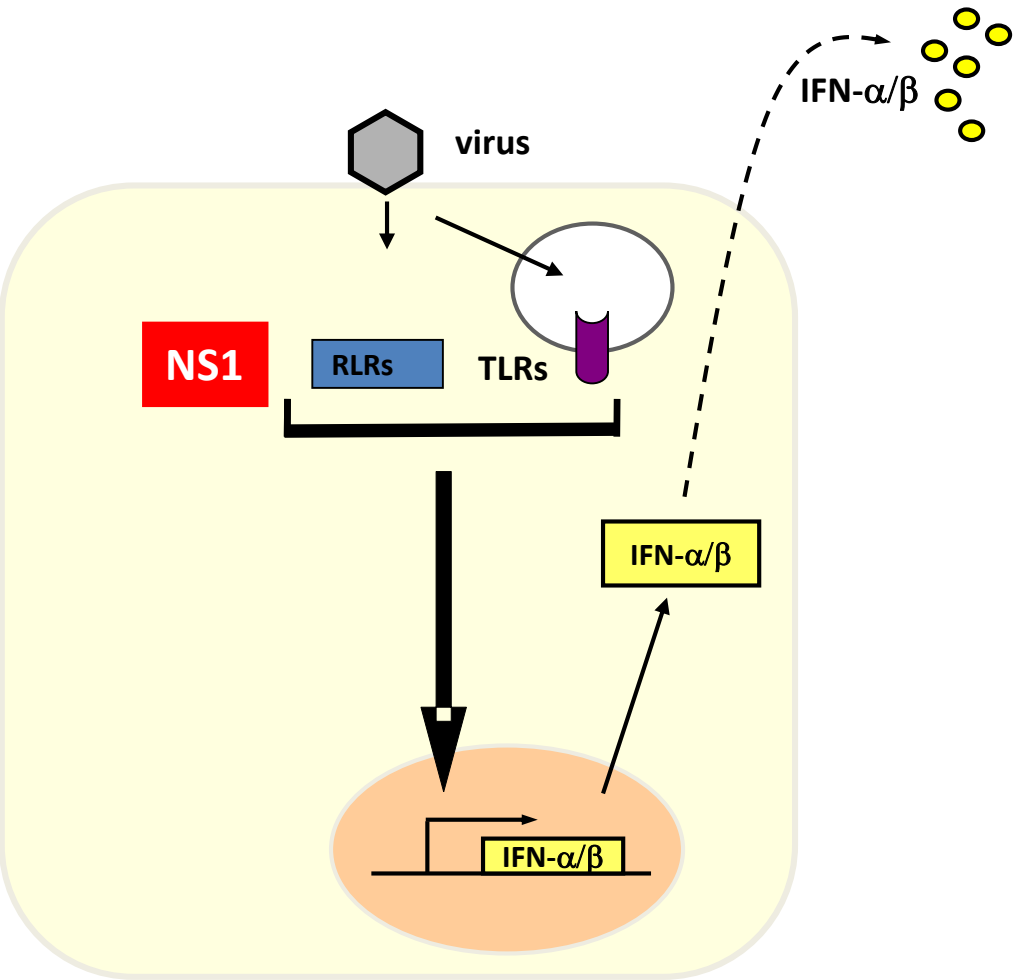
1. MxA provides a barrier against trans-species transmission of FLUAV

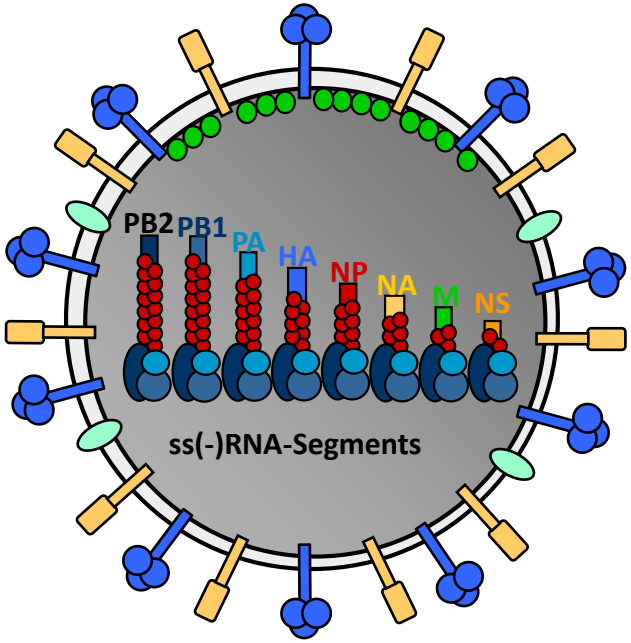
2. Disordered loop L4 is a target interface under positive selection

3. Adaptive mutations in viral NP allow a degree of MxA evasion

4. Host Mx proteins and viral components are „genes in conflict“
   in an evolutionary „arms - race“

Influenza A virus induces Interferons





AG - Kochs

Valentina, Barbara, David,
Mirjam, Basti, Corinna, Jonas



Otto Haller

Petra Zimmermann

Corinna Patzina

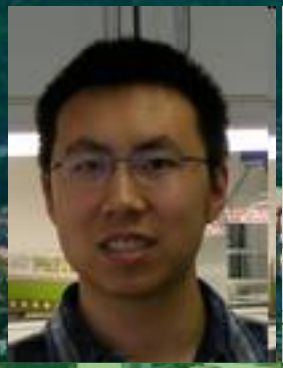


Martin Schwemmle

Benjamin Mänz

Dominik Dornfeld

Peter Stäheli




Oliver Daumke

Song Gao

MDC, Berlin

Harmit Malik

Patrick Mitchell

Fred Hutchinson Cancer Res. Center, Seattle

Deutsche Forschungsgemeinschaft

DFG