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Retroviral variants and their relationship to nonpermissive cells

Retrovirové varianty a jejich vztah k nepermisivním buňkám

PhD thesis

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Prague, 2020

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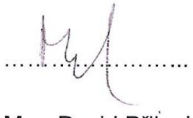

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November 2020, Prague

Preface

This thesis summarizes the results of two major projects that have been completed enough to be published (Lounková et al. 2017; Přikryl et al. 2019) and one ongoing project. All deal with variants of envelope glycoprotein, a protein vital for virus entry into the cell. As philosophers and biologists (one type of philosopher) agree, viruses are alive only inside living cells, and interactions of envelope glycoproteins with cell surface molecules, receptors, are a kind of kiss of the Prince to the Sleeping Beauty, as viruses attain “life” upon entering the cell. In contrast to the breaking of sleeping spell bestowed upon Sleeping Beauty by being kissed by the Prince (classified as a 410 tale type by the Aarne-Thompson-Uther classification system for folktales), some viruses may (or have to) be kissed by multiple Princes and some can even awake themselves, unkissed (which is usually coupled with rage in the form of pathology to the host).

All projects, as well as my entire work, strive to understand retroviral envelope glycoprotein interactions with respective receptors and non-receptor molecules and mechanisms of their function with a special interest in those exhibiting an extended host range. The work also includes unpublished results regarding the pathology of analyzed viruses. We believe that pathologies observed in our in vivo experiments highlight the importance of our work despite the fact that the symptoms and their underlying mechanisms remain mostly unexplained.

The Introduction to this thesis presents a brief review of the envelope glycoproteins of avian leukosis viruses and their receptors, mechanism of fusion, viral entry, and pathology. Materials and Methods are a mixture of methodology and solutions used in all experiments. In Results, the results of the two published papers and one unfinished project are supplemented with pathological analyses. In Discussion, our data are discussed with prospects for future work on our own or by others who found our papers interesting. Taken together, although our work is not about a new breakthrough methodology such as CRISPR, or paradigm-changing hypotheses such as the provirus hypothesis, I believe it presents new information and sheds more light on the life of viruses.

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Acknowledgment

I would like to thank a lot of people and I am sure that if I made a list, some people will be omitted regardless of their great help and support.

Here is the list:

First of all, greatest thanks go to Vláďa Pečenka and Vít Karafiát, who guided me to the world of retroviruses by knowledge learned from them, techniques inherited from them, enthusiasm caught from them, and support felt from them. I would never make it this far without Vláďas excellent experience with PCR, burning questions, and tasteful beer and without Víts skills as an avian pathologist, in vivo experiment executor, and philosophy discussant.

I would also like to thank Michal and Marta Dvořáks, who let me start my work in their laboratory despite the fact of the near end of the department. I would like to thank Jiří Hejnar, not just for taking me in for Master and Ph.D. studies, but also for the great colleagues he assembled in our lab and for creating an inspiring and friendly place, also for his time discussing all kind of stuff, work and science including, and for never turning his back on a pigheaded, solitary and carefree student such as me. I would also like to thank prof. Jan Svoboda, for his motivational speeches and his hard-working catchy attitude.

I would like to thank all my colleagues and collaborators for their work and help, namely Dana Kučerová, Lulu Pecnová, and Markéta Reinišová, who never go mad at me for questions such as “Where is this and that”? and “Could you order it, please?”, Jiří Plachý and Josef Geryk for their endless wisdom and knowledge of retrovirology and Dan Elleder, always sending me interesting papers labeled as “Did you know?”.

I would like to thank all the rest of the department, both past, and present, for all their questions at meetings polishing my answers and improvisation skills.

Abstract

In my doctoral thesis, I focused on studying the envelope glycoprotein of ALV (Avian Leukosis Viruses), a group of retroviruses infecting Galliformes and inducing a variety of diseases. Eradication of these viruses in the farming industry is in progress and information about the virus spread, namely corresponding receptors, is needed to successfully accomplish this feat. Furthermore, many variants of retroviruses infect cells regardless of the absence of the corresponding receptor, and understanding of this phenomenon is also crucial.

We analyzed a newly emerged subgroup of ALV, termed K for its sequence divergence from other subgroups, and determined its host range, interference, and receptor usage, to confirm whether this group deserves a new letter for its designation. We identified a receptor of ALV-K that proved to be Tva, the receptor also used by ALV-A. However, since the K subgroup differs from the A subgroup by its host range and inhibition by the soluble form of Tva, we expect the two subgroups use different epitopes of the Tva receptor.

We also analyzed a variant of ALV-C exhibiting an extended host range as it successfully infected hamster cells. We found that the extended host range correlates with the ability of the envelope glycoprotein to acquire activated prefusion state prematurely, without interaction with the receptor, which was demonstrated by the same sensitivity to temperature, pH, and selective inhibitors of fusion as other cases of envelope glycoproteins with a propensity for premature activation.

Finally, we tried to understand the cause and mechanism of virus-induced osteopetrosis, a disease characterized by bone enlargement caused by osteoblasts hyperproliferation. We compared the genome of a highly osteopetrotic strain, MAV-2.O, with non-osteopetrotic strains and verified the importance of the envelope glycoprotein gene in osteopetrosis induction. Furthermore, we analyzed its stability and ability to enter the cells lacking its receptor, Tvb.

Keywords: ALV, avian, glycoprotein, receptor, fusion, extended host range, osteopetrosis

Abstrakt

Ve své doktorské práci jsem se zaměřil na studium obalových glykoproteinů ALV (ptačích leukósových virů), skupiny retrovirů infikující hrabavé ptáky a vyvolávající řadu onemocnění. Informace o způsobech šíření viru, především o jejich receptorech, jsou nezbytné vzhledem k probíhající snaze o eradikaci těchto virů. Navíc, mnoho variant retrovirů infikuje buňky nezávisle na přítomnosti receptoru; pochopení tohoto jevu je rovněž nanejvýš důležité.

Analyzovali jsme nově objevenou podskupinu ALV, označenou K pro její sekvenční odlišnost od ostatních podskupin a určili její hostitelský rozsah a interferenci, abychom potvrdili, že si tato skupina zaslouží nové písmeno. Identifikovali jsme receptor pro ALV-K, kterým se ukázal být Tva, receptor využívaný také ALV-A. Vzhledem k rozdílům v hostitelském rozsahu a rozdílné inhibici rozpustnou formou Tva ovšem předpokládáme, že tyto dvě podskupiny využívají rozdílné epitopy Tva receptoru.

Také jsme analyzovali variantu ALV-C, vykazující rozšířený hostitelský rozsah, který se projevil její schopnosti infikovat křeččí buňky. Zjistili jsme, že rozšířený hostitelský rozsah koreluje se schopností obalového glykoproteinu přejít do aktivovaného předfúzního stavu předčasně, bez interakce s receptorem, což bylo demonstrováno stejnou citlivostí na teplotu, pH a selektivní inhibitory fúze jako v jiných případech obalových glykoproteinů s náchylností k předčasné aktivaci.

Nakonec jsme se pokusili porozumět příčině a mechanismu virové osteopetrózy, onemocnění charakterizovaného zvětšením kostí způsobeným hyperproliferací osteoblastů. Porovnali jsme genom vysoce osteopetrotického kmenu, MAV-2.O, s neosteopetrotickými kmeny a ověřili důležitost genu obalového glykoproteinu v indukci osteopetrózy. Dále jsme analyzovali jeho stabilitu a schopnost vstoupit do buněk postrádající jeho receptor, Tvb.

Klíčová slova: ALV, ptačí, glykoprotein, receptor, fúze, rozšířený hostitelský rozsah, osteopetróza

Contents

List of abbreviations

A	Alanine
AAV	Adeno-associated virus
AC	Chukar
AHV	Avian hemangioma virus
AIDS	Acquired Immune Deficiency Syndrome
Akt	AKR mice thymoma (protein kinase B)
ALV	Avian leukosis viruses
AMV	Avian myeloblastosis virus
AP	Domestic duck
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ASLV	Avian sarcoma leukosis viruses
AZT	Azidothymidine
BH	Bryan high-titer
BLV	Bovine leukemia virus
C	Cysteine
CA	Capsid
CC	California quail
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CEF	Chicken embryo fibroblasts
CG	Gambel's quail
CO^2	Carbon dioxide
CPE	Cytopathic effect
CPSF6	Cleavage and polyadenylation specificity factor subunit 6
CRD	Cysteine-rich domains
CRISPR	Clustered regularly interspaced short palindromic repeats
CV	Northern bobwhite
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
EAV	Endogenous retrovirus
ECL	Extracellular loop
EDTA	Ethylenediaminetetraacetic acid
ENA	Enzootic nasal adenocarcinoma
ENTV	Enzootic nasal tumor virus
ENV	Envelope glycoprotein
EpoR	Erythropoietin receptor
ER	Endoplasmic reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
ERV	Endogenous retrovirus
EtOH	Ethyl alcohol
F-MuLV	Friend murine leukemia virus
FACS	Fluorescent Activated Cell Sorting
FACT	facilitates chromatin transcription
FCS	Fetal chicken serum
FFU	Focus forming units
FGV	Fowl glioma virus
FP	Fusion peptide
GAG	Group of antigen
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GG	Red jungle fowl
GP	Glycoprotein
GPI	Glycosylphosphatidyl--inositol
GPV	Golden pheasant virus
GQV	Gambel's quail virus
gRNA	Genome RNA
GS	Gray jungle fowl
HEK	Human embryonic kidney
HIV	Human Immunodeficiency virus
HR	Heptad repeat
hr	Host range
hTERT	Human telomerase reverse

	transcriptase
HTLV	Human T-lymphotropic virus
Hsp90	Heat shock protein 90
IFITM	Interferon-induced transmembrane protein
IN	Integrase
ITAM	Immunoreceptor tyrosine-based activation motif
IU	Infection unit
Jak	Janus kinase
JNK	c-Jun NH2-terminal kinase
JQ	Japanese quail
JSRV	Jaagsiekte sheep retrovirus
L	Leucine
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LL	White-crested kalij pheasant
LMP	Low melting point
LPS	Lipopolysaccharides
LN	Silver pheasant
LTR	Long terminal repeat
M	Turkey
MA	Matrix protein
MAPK	Mitogen-activated protein kinase
MAV	Myeloblastosis associated virus
MCF	mink cell focus-inducing virus
MEK	Mitogen-activated protein kinase kinase
MMTV	Mouse mammary tumor virus
MOI	Multiplicity of infection
mRNA	messenger RNA
MSD	Membrane spanning domain
mTOR	Mammalian target of rapamycin
MuLV	Murine leukemia virus
NC	Nucleocapsid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHE	Sodium/hydrogen exchanger
NM	Guinea fowl
NP	Nucleoprotein
NRS	Negative regulator of splicing
NS	Not significant
OPA	Ovine pulmonary carcinomas
ORF	Open reading frame
PBS	Phosphate-buffered saline
PC	Ringed-neck pheasant
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PERT	Product-enhanced reverse transcriptase
PI3k	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMB	Polymyxin B
PP	Gray partridge
PR	Protease
PR-RSV-C	Prague strain RSV C
qPCR	Real-time PCR
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RAV	Rous associated virus
RNA	Ribonucleic acid
RON	Recepteur d'Origine Nantais
RPE1-hTERT	hTERT-immortalized retinal pigment epithelial cells
RPV	Ring-necked pheasant virus
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
S	Serin
SA	Splicing acceptor
SAMHD1	SAM domain and HD domain-containing protein 1
SD	Splicing donor
SDS	Sodium Dodecyl Sulfate

SERINC	Serine incorporator
SFFV	Friend spleen focus-forming virus
SH	Src homology
SH	Mrs. Hume's pheasant
SR	Reeve's pheasant
SR-A	Schmidt-Ruppin A
Src	Sarcoma
Stat5	Signal Transducer and Activator of Transcription
STK	Serine/Threonine protein Kinase
SU	Surface
T	Threonine
TLR-4	Toll-like receptor 4
TM	Transmembrane
TMD	Transmembrane domain
TNFR	Tumor necrosis factor receptor
TRIM5α	Tripartite motif-containing protein 5
Tva	Tumour virus A
Tvb	Tumour virus B
Tvc	Tumour virus C
Tvj	Tumour virus J
VLP	Virus-like particles
vr	Variable region
wt	Wild type
XC	90
Y	Tyrosin
ZAP	Zinc-finger Antiviral Protein

Introduction

Retroviruses

Retroviruses are infectious agents that can be found in most vertebrates and cause a variety of diseases. They have been known for more than a century and also became recognized by the general public because of the HIV (Human Immunodeficiency virus) outbreak in the 1980s resulting in AIDS (Acquired Immune Deficiency Syndrome) worldwide pandemic. In addition to immunodeficiency, retrovirus-related pathologies include tumorigenesis, wasting syndrome, anemia, and several others. Some are causing huge health problems in humans (AIDS and leukemia) or economical losses in farming (leukosis of hens).

Retroviruses piqued the interest of researchers on two major occasions. First, after their discovery at the beginning of the 20th century when their ability to induce tumors was observed (Rous 1910; Ellermann and Bang 1909; Rous 1911). Second, after the discovery of their replication protein, reverse transcriptase, an enzyme converting RNA to DNA, challenged the central dogma of molecular biology in the 1970s (Baltimore 1970; Temin and Mizutani 1970).

The Retrovirus family comprises viruses sharing the following essential features: they are enveloped viruses (i.e. wrapped into a membrane of cellular origin) that carry a pseudodiploid genome with two single-stranded RNAs of (+) polarity encoding three major polyproteins: gag, pol, and env. During the retrovirus life cycle, the RNA undergoes transcription into DNA which then integrates into the host genome and forms a provirus flanked by two identical LTRs (long terminal repeats). Retroviruses are further divided into several genera. Alpharetroviruses and gammaretroviruses are called simple retroviruses since they do not code for any accessory protein, while betaretroviruses deltaretroviruses, epsilonretroviruses, and lentiviruses are known as complex retroviruses for encoding many accessory proteins regulating their life cycle.

The Retrovirus family also contains a subfamily of spumaviruses with no disease association, thus being out of the mainstream of interest.

ALVs studied in this thesis constitute the major group of Alpharetroviruses and bear only basic genetic equipment.

Alpharetrovirus life cycle

The interaction of env (envelope glycoprotein) spikes on the virion surface with receptors on the surface of the target cell starts a series of events ending by the release of the viral core into the cytoplasm (for more details see next chapter). This is followed by uncoating (viral core disassembly), reverse transcription of the single-stranded RNA genome into the double-stranded DNA form and its integration into the host genome thereby becoming a provirus. As ALVs encode no accessory proteins and their proteins harbor no nuclear import signals, there is no way for them to enter the nucleus via an active process thus, they have to wait for the disintegration of the nuclear envelope during mitosis to reach the host genome. Integration of viral DNA is promoted by association of the integrasome with the FACT (facilitates chromatin transcription) complex (Winans et al. 2017). The selection of integration sites is close to random.

ALV transcription driven by the promoter and enhancer contained within LTR leads to the production of unspliced RNA and a single form of spliced mRNA. Unspliced mRNA codes for both gag (group-specific antigen) and pol (polymerase) proteins, initially in the form of a polyprotein which, inside the virion and simultaneously with the process of virus budding, is cleaved by viral protease into a series of subunits. Gag-derived subunits are MA (matrix), CA (capsid), and NC (nucleocapsid) proteins that together form the virion core, pol-derived are replicative proteins PR (protease), RT (reverse transcriptase), and IN (integrase). Spliced mRNA is translated into the env precursor protein, cleaved, and subsequently glycosylated in the Golgi apparatus into the SU (surface) subunit responsible for receptor recognition and the TM (transmembrane) subunit responsible for virion anchoring and fusion of membranes; the subunits remain linked together via disulfide bonds.
Unspliced mRNA also serves as gRNA (genome RNA), which after dimerization is encapsulated by assembling gag-pol polyproteins beneath the surface of the host membrane enriched by env spikes, followed by virus budding and so-called maturation - cleavage of proteins by PR inside the virion.

Permissive cells and restriction factors

Retroviruses penetrated nearly all branches of life. This wide distribution of retroviruses is accompanied by diversification as retroviruses have to adapt to the specific environment in certain hosts. Retroviruses strongly depend on cellular factors (virus dependence factors) at all steps of their replication cycle and many of these factors are species-specific. Thus, retroviruses can enter only susceptible cells and replicate only in permissive cells. Any change in the host range of a given retrovirus must be accompanied by the evolution of the viral proteins, particularly the envelope glycoproteins. Co-evolution of retroviruses and hosts is continual and host cells cope with selection pressure imposed by retroviruses by changes or loss of virus dependence factors (e.g. receptors for retroviruses).
Another anti-retroviral strategy of the hosts is the expression of restriction factors, which interfere with the virus life cycle. Restriction factors typically obstruct virus entry (SERINC family - Rosa et al. 2015, IFITM family - Bailey et al. 2014) reverse transcription and replication (APOBEC family - Mangeat et al. 2003, Samhd1 - Laguette et al. 2011, CEM15 - Sheehy et al. 2002), uncoating (TRIM5α - Gao, Guo, and Goff 2002), nuclear entry (CPSF6 - Lee et al. 2010) viral mRNA production (ZAP - Gao, Guo, and Goff 2002), and virus release (tetherin - Neil, Zang, and Bieniasz 2008, also called BST-2 - Van Damme et al. 2008). Most restriction factors have been identified in HIV-1- and MLV-resistant cells whereas restriction factors blocking the replication cycle of e.g. avian leukosis virus remain to be systematically explored (Krchlíková et al. 2020).
An excellent example of cell non-permissiveness is the mammalian cell lines transformed with avian Rous sarcoma virus (RSV), which do not express viral mRNAs and produced no viral progeny (Svoboda et al. 1963) until cell fusion with permissive chicken fibroblasts complements the lacking virus dependence factors (Svoboda and Dourmashkin 1969; Machala, Donner, and Svoboda 1970). A similar phenomenon was observed in rodent cells non productively infected by HIV. Virus replication was forced upon cell fusion with permissive human cells (Bieniasz and Cullen 2000; Mariani et al. 2000). The nature of permissiveness-inducing factors remains to be solved, while the first evidence of mRNA

aberrant splicing and impaired nuclear export in RSV-infected mammal cells is being collected (Lounková et al. 2014).

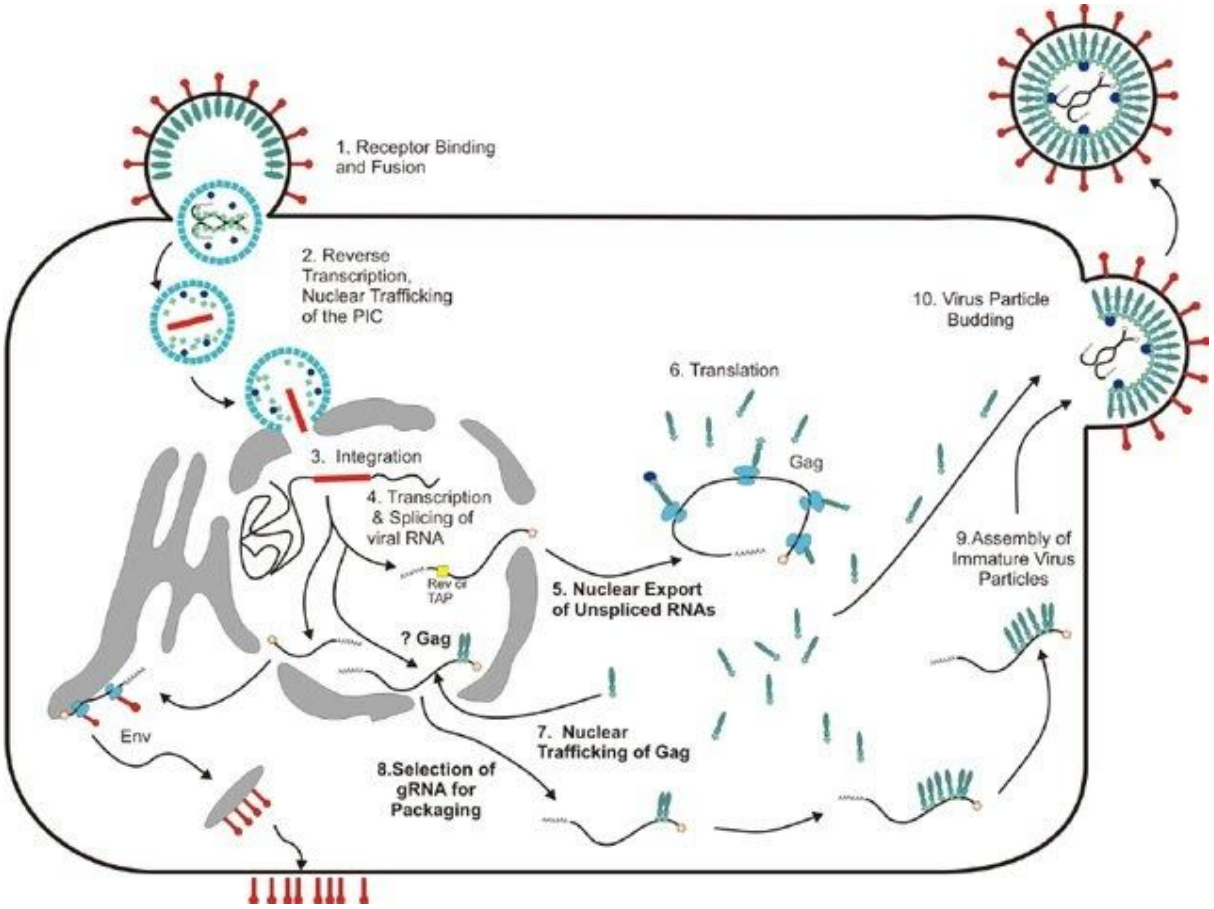

Figure 1.: Scheme of retrovirus life cycle

The first step of the retrovirus life cycle consists of interaction with specific receptors (and in some cases co-receptors) and fusion of viral and host membranes (1.). The fusion may occur immediately, on the plasma membrane (HIV) or later, in an acidified endosome (ALV). Virus entry is followed by the import of capsid core into the cell nucleus, reverse transcription (2.), uncoating, and integration of proviral DNA into the host genome occurs (3.). LTR-driven transcription generates spliced and unspliced RNAs (4.), which are exported from the nucleus (5.) to serve as a template for translation (6.) or to associate with Gag (7.,8.). As the immature virus particles are assembling beneath the surface of cells (9.), they interact with newly synthesized envelope glycoproteins and leave the cells through budding of the host membrane and undergo maturation (10.). (Stake et al. 2013)

Cell entry mediated by envelope glycoprotein

The first step in the retrovirus life cycle, attachment of the virion to the cell membrane followed by virus entry, is mediated by the interaction between virus env (ligand) and corresponding cell surface protein (receptor).

Although env sequences and properties differ among retroviral genera, they share basic hallmarks. Env is translated as a polyprotein consisting of a signal peptide, SU, and TM subunits. As the nascent env is being translated, the signal peptide binds to the signal recognition particle and becomes associated with the membrane of the ER (endoplasmic reticulum). Restored translation extrudes most of the polyprotein into the lumen of the ER and becomes anchored via a hydrophobic sequence (called transmembrane or membrane-spanning domain) near the C-terminus. As soon as the translation is finished, the polyprotein forms trimers via a homo-binding domain localized at the TM subunit. After cleavage of the signal peptide, the polyprotein is transported to the Golgi apparatus where N-glycosylation and proteolytic cleavage by furin or furin-like proprotein convertase takes place, producing SU and TM subunits linked through disulfide bonds at the C-terminus and N-terminus, respectively (Wills, Srinivas, and Hunter 1984; Perez and Hunter 1987).

The signal peptide was shown to play an important role in the life cycle of some retroviruses upon entering the nucleus; however, this was not proved in the case of ALVs (Yolitz et al. 2018; Hofacre, Nitta, and Fan 2009).

The SU subunit is responsible for receptor recognition. Five regions of ALVs SU were suggested to be the most important for this feat and designated vr1-3 (variable regions 1-3) for shorter regions and hr1-2 (host range regions 1-2) for longer regions. This hypothesis was formulated based on the fact that a large proportion (20-40 %) of sequence differences between envs of different subgroups are concentrated in these relatively short regions. Analysis of chimeras between selected envs identified hr1, hr2, and v3 as the major players since their switching shifted receptor usage and host range towards receptor usage and host range of the donor molecule while switching vr1 and vr2 had no effect (Bova, Olsen, and Swanstrom 1988; Bova, Manfredi, and Swanstrom 1986).

While the SU subunit is highly variable, the TM subunit is the most conserved part of the envelope glycoprotein. This is not surprising since the TM subunit is not involved in receptor recognition and thus is not subject to selective pressure during the evolutionary arms race between the virus and its host where receptor usage changes are typical events. On the contrary, the TM subunit represents a device for the fusion of viral and cellular membranes, a crucial and highly orchestrated event, and most changes in TM might interfere with it.

The TM subunit contains five regions worth mentioning. The fusion peptide at the N-terminus is a hydrophobic sequence that is hidden within the env trimeric structure until the virus-receptor interaction occurs. This interaction induces conformational changes of SU evoking the whole env trimer rearrangement leading to exposure of fusion peptides before they get inserted into the cell membrane so-called priming (Walther Mothes et al. 2000). The virion is thus anchored to the cell via TM subunit already hooked up in the virion by its transmembrane domain (another hydrophobic sequence near the C-terminus). While anchored, the virion is transferred inside the cell by endocytosis. As the endosome matures, its inner environment is acidified. Protonation of two TM regions, HR1 and HR2 (heptad repeats 1 and 2), increases their affinity to each other and promotes the formation of

six-helix bundles concurrently aligning viral and endosomal membranes along with each other. This is followed by spontaneous lipid bilayer fusion and pore formation for releasing the virion core into the cell cytoplasm. The last TM region worth mentioning is its intracellular C-terminus. A role in virion assembly, fusion, immunosuppression, and pathogenicity was observed for the C-terminus of env-J (T. Li et al. 2020), but its function in other subgroups is unclear.

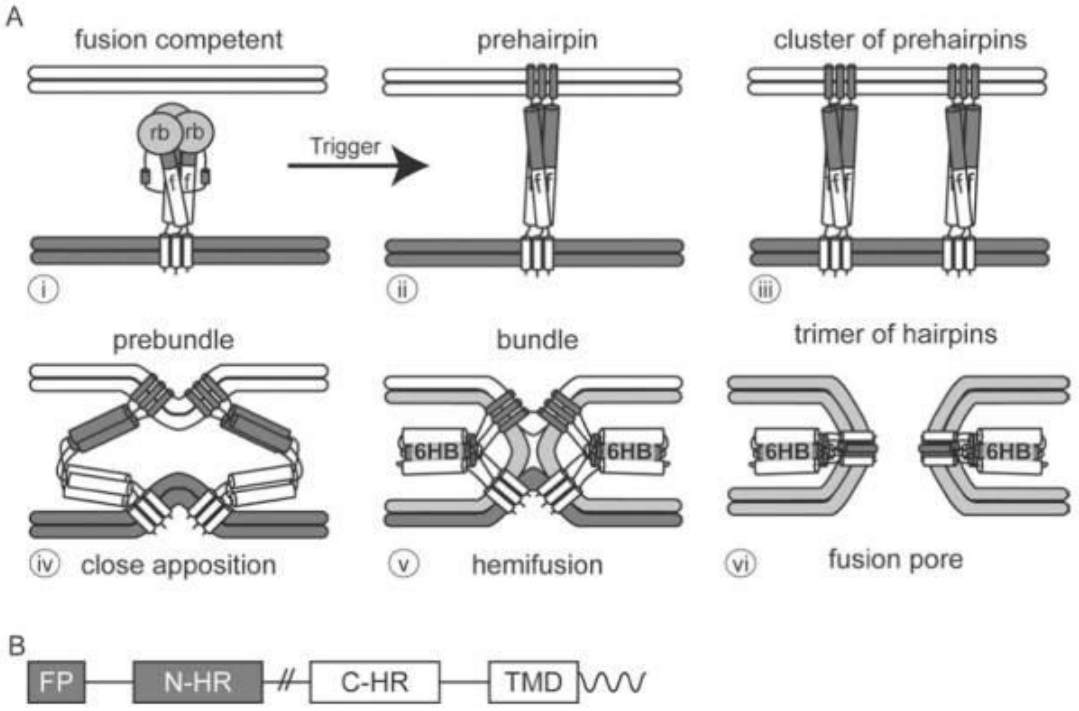

Figure 2.: Steps of env-driven membrane fusion

(A) The envelope glycoprotein anchored in the membrane of the virion in close proximity to the host cell membrane (i) undergoes structural changes upon interaction with the receptor, leading to exposure of the fusion peptides that insert into the host cell membrane (ii). A number of activated glycoproteins is necessary to cooperate (iii) in bending the membranes (iv), bringing them to close proximity until lipid monolayer mixing occurs (v) and a fusion pore is formed (vi).

(B) The TM subunit consists of four regions involved in membrane fusion, fusion peptide (FP) responsible for attaching to the host membrane, two heptad repeats (N-HR and C-HR) which are attracted to each other upon protonation, bringing both membranes to close proximity and the transmembrane domain (TMD) anchoring the protein into the viral membrane. (White et al. 2008)

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Classification and pathogenicity of Avian leukosis viruses

ALV subgroups

With the introduction of new techniques, such as agar overlay of cultures of chicken embryo cells at the end of the 1950s, a new era of retrovirology began. In the following years, in vitro experiments with RSV (the first member of alpharetrovirus family identified) revealed that some embryo-derived cell cultures contain transmissible resistance factors blocking RSV foci induction. Analysis of these factors leads to the discovery of a set of avian retroviruses persisting in chicken flocks due to vertical transmission from hens to the offspring. These retroviruses were similar to RSV in basic features except the ability to induce foci in cultures and sarcomas in chicken. They were termed ALV for their connection with avian leukosis.

The above phenomenon, later named receptor interference (or superinfection interference), proved common across different viral genera, starting from bacteriophages and ending with retroviruses. Upon virus entry and envelope glycoprotein expression further entry of virions using the same receptor is blocked via either receptor downregulation caused by env binding or via receptor occupancy. Receptor downregulation was demonstrated in beta-, gamma- and delta- retroviruses. In the case of alpharetroviruses, however, some subgroups use the same receptor yet they poorly interfere with each other which implies two conclusions: first, these subgroups contact different epitopes on the same receptor, and second, the receptor downregulation by these subgroups, if any, is far from complete.

Based on the interference of individual ALV strains with individual RSV strains, the first five subgroups were defined, termed A to E (Vogt and Ishizaki 1965). Later, F to I subgroups were established based on interference patterns of viruses released from non-gallus embryonic cells (Hanafusa et al. 1976). In 1987, a worldwide outbreak of myelocytomatosis in meat-type broilers was observed and linked to the spread of ALV of a novel subgroup, termed J (Payne, Gillespie, and Howes 1992). Lastly, in 2012, in an attempt to test a population of Luhua chickens for the presence of either J or A subgroup, viruses with env sequences distant from any other previously reported were found, possibly forming a new subgroup, termed K (Wang X., Zhao, and Cui 2012).

Thus, 11 ALV subgroups have been identified so far, distinction and nomenclature of which is based on the host range and interference pattern which is in turn determined by the receptor they use and is reflected in the sequences of env.

Subgroups of high risk to poultry: A, B, J, and K

Subgroups A, B, J, and possibly K comprise naturally occurring exogenous viruses causing economic losses in poultry farming worldwide. In the past, the prevalence of ALV in commercial flocks was very high and the associated mortality rate was about 2 %. With improved hygienic standards and the selection of more viable lines, the mortality rate and virus prevalence dropped significantly. However, ALV-incurred depression of production (egg numbers, fertility, hatchability) is still significant and remains to be solved (Stedman and Brown 1999); (Lin et al. 2016).

Different strains of ALV have a tendency to induce different diseases. The disease specificity generally reflects the virus tropism - the tissue in which the virus preferentially replicates. Tropism is primarily determined both by tissue specificity of expression of the receptor used

to enter the cell (i.e. by a subgroup) and by tissue specificity of the viral promoter and enhancer contained within LTR. Other parts of the retroviral genome, however, may influence disease specificity as well.

Subgroup A viruses have occurred frequently and still remain one of the most frequent types of avian retroviral infection (Fenton, Reddy, and Bagust 2005; Burstein et al. 1984; Zhang et al. 2010). Together with subgroup B viruses, which are rarer (D. Li et al. 2013), they induce a variety of diseases, including a range of neoplasias (leukosis, erythroblastosis, myelocytomatosis, hemangiomas, gliomas, nephroblastomas, osteopetrosis, etc. (Hatai et al. 2008; Nakamura et al. 2011)). Most neoplasias result from alteration of cancer gene expression or structure by provirus integration, a phenomenon called oncogenesis by insertional mutagenesis (see below). The penetrance of neoplasias is especially high when individuals are infected congenitally which evokes immunological tolerance to the virus. Under such conditions, there is no or very low immune reaction against the virus and high provirus load builds up in the blood and other tissues. If an individual is infected post-hatching, when the immune system is fully developed, viremia is only transient and is usually followed by anemia.

An example of subgroup-specific disease is osteopetrosis, characterized by osteoblasts hyperproliferation and enlarged bones, sometimes coupled with stunting (Powers et al. 1988; Ralph E. Smith, Davids, and Neiman 1976; R. E. Smith and Morgan 1984; Fritzsche and Bahnemann 1969; Schmidt and Smith 1981). Osteopetrosis is typical of B subgroup ALVs, though some A subgroup viruses were also connected with this disease (Barbosa et al. 2010). Another unique feature of osteopetrosis is that, contrary to other analyzed ALV-induced tumors, it is not caused by insertional mutagenesis (Robinson et al. 1983).

A further example of subgroup-specific disease is subgroup J-induced myelocytomatosis which had a huge impact on poultry farming just recently (D. Zhou et al. 2019; Venugopal et al. 2000). The prototype strain, HPRS-103 (Payne 1998), emerged after a series of recombinant events between exogenous ALVs and ev/J loci of the EAV family of endogenous retroviruses (Melanie A. Sacco et al. 2004; M. A. Sacco et al. 2000; Bai, Payne, and Skinner 1995). Such recombinations seem to occur frequently, as new isolates are being found regularly and show no kinship with the previously reported ones (Su et al. 2020; Sun and Cui 2007; Melanie A. Sacco et al. 2004).

Subgroup K was established upon isolation of three strains (JS11C1-3) of Luhua chicken-based solely on the sequence of the envelope glycoprotein (Cui et al. 2014; Wang X., Zhao, and Cui 2012). The virus emerged as a result of multiple recombinations between exogenous and endogenous viruses and, so far, it exhibits poor replication ability (Su, Li, Cui, et al. 2018). No data are available about its receptor usage or host range and only little is known about its pathogenicity (Liang et al. 2019; Zhao et al. 2018b).

Subgroup of low risk to poultry: E

Subgroup E represents the principal type of chicken endogenous proviruses, named ev family (Astrin et al. 1980). Although most of the endogenous proviruses of ev type are defective, some are not and have the potential to produce exogenous virus. However, the produced viruses have only quite a low pathogenicity as their activity is hampered by a weak enhancer and promoter in the LTR (Crittenden et al. 1980; Cullen, Skalka, and Ju 1983; Jenkins and Cooper 1980). Nevertheless, the presence of ev locus encoding functional virus

was shown to result in reduced production of eggs (Gavora et al. 1991). Even the presence of defective endogenous proviruses has adverse effects. Through evoking tolerance to antigens shared between ALVs, endogenous proviruses mitigate the immune response to exogenous ALVs which makes the animal more sensitive to ALV infection and aggravates the outcome (Crittenden, Smith, and Fadly 1984; Gavora et al. 1995; Crittenden, Fadly, and Smith 1982). Importantly, subgroup E viruses were detected in vaccines produced in chicken embryos (Barbosa, Zavala, and Cheng 2008; Fadly et al. 2006) which brought up concerns about their possible pathogenicity.

Subgroups of no risk to poultry: C, D, and F to I

ALVs of subgroups C and D have not been found in the poultry flocks on farms, and for the present, they thus do not inflict any damage. They are represented by two laboratory strains of RSV: Prague C and Schmidt-Ruppin D (Sandelin and Estola 1975) which provided valuable data concerning viral evolution, replication mechanisms, and pathogenicity. Subgroup C induces stunting in infected chickens (Carter and Smith 1984), while subgroup D was found to infect and transform some mammalian cell lines and induce tumors in newborn hamsters (Diglio, Wolfe, and Meyers 1983).

Subgroup F, represented by RPV (ring-necked pheasant virus), originally emerged as a recombinant of chicken exogenous virus and pheasant endogenous loci (Fujita et al. 1974; Hanafusa and Hanafusa 1973). Neoplastic diseases such as lung angiosarcomas are induced by this subgroup and cytotoxicity was observed *in vitro* (Simon, Smith, and Hayward 1984; Simon et al. 1987; Weller, Joy, and Temin 1980). However, much like C and D subgroups, subgroup F has not been detected in the poultry on farms.

Subgroups G, H, and I are a group of endogenous viruses isolated from non-junglefowl members of genus *Galliformes*. Prototype viruses are GPV from Golden pheasant - subgroup G (Hanafusa et al. 1976), (Tal et al. 1977; Fujita et al. 1974), RAV-62 from grey partridge - subgroup H (Hanafusa et al. 1976), and GQV from Gambel's quail - subgroup I (Troesch and Vogt 1985). They were isolated as recombinants with exogenous Bryan high-titer strain of Rous sarcoma virus (BH-RSV), a virus lacking the env gene, which makes it dependent on a helper envelope glycoprotein for spreading. After BH-RSV infection of non-junglefowl embryonic fibroblasts followed by irradiation, PEG treatment, or spontaneously, recombinant viruses were arising that carried the newly acquired env. Partition into G, H and I subgroups was based solely on the host range and interference with other subgroups. Basically, no data concerning their sequences or pathogenicity are available (Purchase et al. 1977). Subgroup G was also shown to be non-ALV later on (Chen and Vogt 1977).

Viral receptors

In the case of enveloped viruses, the term viral receptor is reserved for the host surface molecule triggering, through physical interaction, irreversible changes in the envelope glycoprotein resulting in virus anchorage to the host membrane and virus entry into the cell (Walther Mothes et al. 2000). Some retroviruses need the simultaneous binding of multiple different molecules; an example is HIV which requires one major receptor (CD4) and one of two alternative coreceptors (CCR5 or CXCR4) differentially expressed on CD4+ T-lymphocytes and some nonlymphoid cell lineages (Deng et al. 1996; Broder and Dimitrov 1996). In most cases, however, including all studied ALV receptors, a single molecule performs the task.

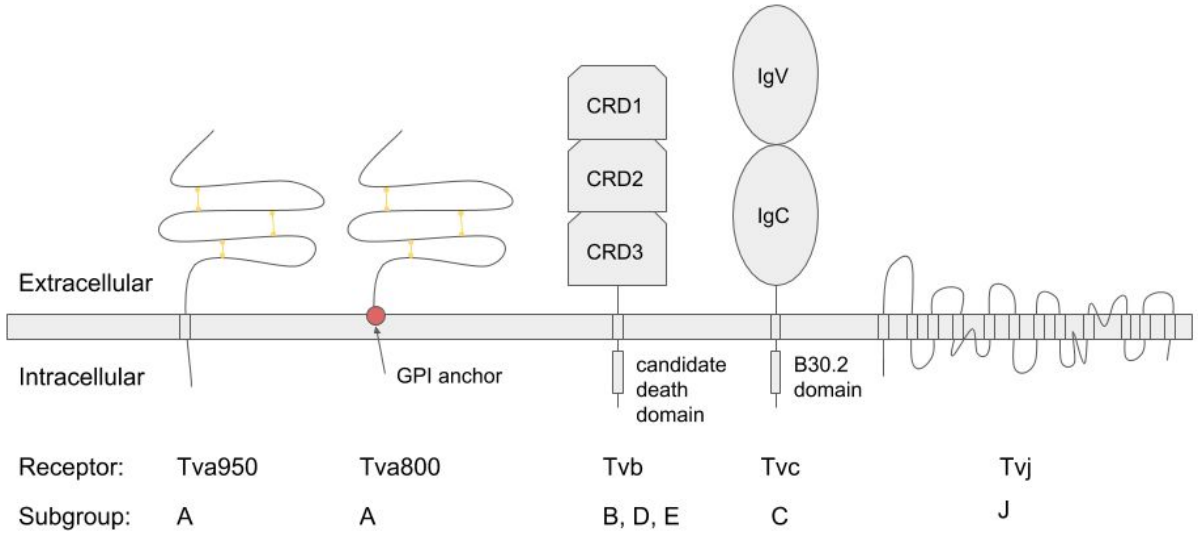


Figure 3.: ALV receptors
Depicted are receptors used by ALV to enter the host cells: Tva950 and Tva800, transmembrane and GPI-anchored forms of Tva receptor used by A subgroup, Tvb receptor used by B, D and E subgroups, Tvc receptor used by C subgroup and Tvj receptor used by J subgroup.

Tva receptor

Tva, the ALV-A receptor, was recently shown to be a cellular receptor for the transcobalamin II/B12 complex (Krchlíková, unpublished data). Its LDL-like structure defined by multiple cysteine bridges across the binding domain is crucial for virus entry as some variants or receptors lacking cysteine bridges render host cells resistant to infection (Rong and Bates 1995; Bélanger, Zingler, and Young 1995). The receptor is expressed in two forms, longer membrane-spanning form TVA950 and shorter GPI-anchored form TVA800 (Bates, Young, and Varmus 1993). The virus can enter the cell via both forms, though some studies have shown differences in virus-receptor interaction and in the following pathways used for internalization (Narayan, Barnard, and Young 2003). Both forms are present in all tissues (Merkin et al. 2012; Barbosa-Morais et al. 2012).

As mentioned before, the binding site for the virus is dispersed through the cysteine-rich region, with the crucial role of W48 of the DEW motive (Zingler and Young 1996). As tryptophan is frequently associated with virus entry, W33 was also investigated, but its mutations showed no effect on virus entry (Contreras-Alcantara, Godby, and Delos 2006). W48 is certainly not the sole determinant of virus entry since some species are resistant to ALV-A while W48 in Tva is untouched. Experiments (Melder et al. 2015)) based on swapping parts of quail and chicken Tva suggest that residues E14, F16, R22, L31, and L34 are also important and possibly participate in forming a pocket-like binding site.

Tvb receptor

Based on sequence homology, the Tvb receptor was categorized as a member of the tumor necrosis factor superfamily. Although homology does not necessarily imply analogical physiological function, the fact that subgroup B has a tendency to trigger apoptotic pathways suggests that the death domain at the cytoplasmic terminus of Tvb is functional and may be activated by binding of the virus (Melder et al. 2015; J. Brojatsch et al. 1996). The extracellular terminus contains three cysteine-rich domains (CRD1-3) crucial for virus entry (Heather B. Adkins, Brojatsch, and Young 2000). Receptors are ubiquitous in all tissues.
Tvb is exceptional among ALV receptors by being used by three different subgroups: B, D, and E. The interaction shows a non-reciprocal interference pattern - while infection by B or D subgroups prevents superinfection by all three subgroups, infection with the E subgroup interferes only with superinfection by the E subgroup (Melder et al. 2015; J. Brojatsch et al. 1996; E. J. Smith et al. 1998; H. B. Adkins et al. 1997). To explain this phenomenon, the existence of two different isoforms of Tvb was postulated and confirmed. The isoforms, called receptor type 1 and type 2, are produced from the same gene (Heather B. Adkins, Blacklow, and Young 2001). Type 1 serves as a receptor for all three subgroups while type 2 is specific for B and D. Thus, env B and D appear to downregulate both forms of the receptor whereas env E leaves form 2 available for ALV-B and -D entry.
It was later demonstrated that the epitope used by subgroup B is a 15-amino acid peptide contained within CRD1, positions 32-46, which, when expressed alone, is sufficient to promote virus binding and fusion (Knauss and Young 2002). Amino acid residues L36, Q37, L41, and Y42 were shown to be crucial for virus binding and entry. On the other hand, amino-acid residues necessary for subgroup E entry are Y67, N72 and D73 contained within CRD2 (Knauss and Young 2002; Klucking and Young 2004). CRD1 and CRD2 are present on both forms of the receptor; the nature of the differences between the isoforms was not elucidated, having the same apparent size and the possibility of different glycosylation was not ruled out (Knauss and Young 2002).

Tvc receptor

According to sequence homology, the Tvc receptor belongs to the butyrophilin family, a subclass of the immunoglobulin protein superfamily; its physiological role in chicken is still unclear (Elleder et al. 2005). The receptor is present in cells of the immune system. Tvc contains two extracellular immunoglobulin-like domains, IgV and IgC, and one cytoplasmic, likely protein-binding domain, denominated B30.2/SPRY domain. The domain responsible for interacting with the env C glycoprotein is IgV, with at least two aromatic amino acid

residues, W48 and Y105, being critical for efficient ALV-C infection (Munguia and Federspiel 2008).

Tvj receptor

The Tvj receptor was identified as sodium/hydrogen exchanger 1 (NHE1), an ubiquitously expressed integral membrane protein controlling cytosolic pH and cell volume (Chai and Bates 2006). Unlike other known ALV receptors, Tvj is multiple membrane-spanning proteins.

The region responsible for env J binding and ALV-J entry into the cell is the extracellular loop 1 (ECL1) while an ECL1-derived peptide containing amino acid residues 28 to 39, when expressed alone, is sufficient to promote virus entry (Guan et al. 2017). It was demonstrated that residues A30, V33, W38, and E39 are crucial for virus binding. The importance of W8 was highlighted by the fact that multiple species substituted this residue to gain resistance to the subgroup J virus while preserving the physiological function of NHE1 (Reinišová et al. 2016).

Unknown receptors (F-I)

To this day, receptors used by F, G, H, and I subgroups remain unknown. As those subgroups cause no economic losses, no attempts to identify their respective receptors have been made so far.

Nonreceptor molecules interacting with env

Not all cell surface molecules interacting with env fulfill all attributes of the receptor definition. Regularly, the virus makes multiple weak and dynamic contacts with molecules present in high numbers on the cell surface (e.g., acidic proteoglycans such as heparan sulfate or molecules carrying sialic acid). These molecules (sometimes called adhesion receptors) do not trigger env rearrangement and cell entry but facilitate infection by effectively increasing the concentration of virions in the proximity of receptors as they limit the virion movement to rolling or sliding on the cell surface until they contact the genuine receptor (Lehmann et al. 2005). The same mechanism may also retain virions in the tissue where the appropriate target cells occur.

Special and interesting types of molecules interacting with env are so-called soluble receptors. These are truncated versions of the receptor containing the env-binding domain but missing the transmembrane domain. Some of them retain the ability to induce priming (rearrangement leading to activated prefusion state with exposed fusion peptide) after binding to env. Primed env then can, without the need of the receptor, start the process of fusion and virus entry whenever the virion contacts the cell surface (Damico and Bates 2000). However, since primed env is unstable and tends to undergo further rearrangements, it has a limited time to fortuitously encounter the cell surface; docking virions on the cell surface by adhesion receptors mentioned above is thus very instrumental. Hence, soluble receptors can fulfill the function of full-length receptors though they do not fully comply with the definition of the receptor by not being a cell surface molecule. On the other side, soluble receptors that bind env but lost the ability to induce priming can, at sufficient concentration,

block the interaction between env and the genuine receptor thus imitating neutralizing antibodies (S. L. Holmen and Federspiel 2000; Sheri L. Holmen et al. 1999).

Resistant forms of receptors

Many allelic variants of Tva, Tvb, and Tvj receptors, incapable of mediating virus entry into the cells were found both in individual domestic chicken lines and in non-chicken birds (Elleder et al. 2004; Klucking, Adkins, and Young 2002; Elleder et al. 2005; Reinišová et al. 2016). Fixation of these variants is an obvious result of the virus-host arms race. Pathogenic effects of ALV infection had created selection pressure resulting in segregation of receptor alleles with substitutions or indel mutations that had changed the receptor structure or expression so that the receptor-virus interaction was abrogated or impaired.

Ideally, from the point of view of the host, the newly formed virus-resistant allele should preserve its cellular function. Indeed, no harm to both animals and cell lines seems to be inflicted by mutations that created virus-resistant receptor variants such as C40T substitution in the cysteine-rich region of Tva (Elleder et al. 2004), C125S substitution in the cysteine-rich region of Tvb (Reinisová et al. 2008) or either deletion or substitution of W38 amino acid residue in Tvj (Kucerova et al. 2013).

In some virus-resistant alleles, however, substitutions or indel mutations changed the reading frame and/or created a premature stop codon close to the beginning of the coding sequence, thus most likely totally abolishing all gene functions. Examples are the ALV-A-resistant 7^2 cell line, where the Tva gene is disrupted by 4-bp insertion in exon 1 (Elleder et al. 2004) or in ALV-B-resistant chickens with C172 codon of Tvb mutated to a premature stop codon (Klucking, Adkins, and Young 2002). Even animals with such a gene in a homozygous state are perfectly healthy suggesting that the functions of the protein are redundant and can be substituted by other proteins.

A special type of variant was observed for Tva: deletions in the intron region led to low levels of properly spliced mRNA and low levels of the receptor on the cell surface. Decreasing the levels of the protein was sufficient to render cells resistant to the virus; loss of the cellular functions was considered but not proven (Reinišová et al. 2012).

Evolution of ALV env glycoproteins and arms race between the virus and the cell

The sequence comparison of env A, B, C, and E indicates that they have a common predecessor. That, together with the existence of receptor variants resistant to ALV of the relevant subgroups, suggests that ALV diversification into several subgroups was a step in the continual virus-host arms race, a response of the virus to the effort of the host to escape virus infection by segregating resistant receptor alleles. To bypass the loss of a functional receptor, the virus was forced to change its receptor specificity by adapting to changes in the binding pocket on the receptor, by switching to another epitope on the same molecule, or even by switching to a new receptor. When Mark Federspiel´s group tested the proficiency of multiple ALV-A isolates with substitutions and indel mutations in env to recognize different variants of the Tva receptor, they found that some receptors, though nonpermissive for some env variants, are still used by other env variants (Melder et al. 2015) and suggested this is a manifestation of past arms races. Earlier, they even selected variants of ALV-A with mutations of the hr1 region, that enabled the virus to infect cells pre-infected with the same subgroup (S. L. Holmen and Federspiel 2000; Melder, Pankratz, and Federspiel 2003).

Since they observed a very low but detectable interference between these ALV-A-derived variants and ALV-B or ALV-C subgroups, they suggested that selected variants of ALV-A acquired the ability to interact with Tvb or Tvc (Mark J. Federspiel 2019). This phenomenon is not limited to ALV-A as a similar effect of mutations in env was observed for ALV-B (Yin et al. 2019). All the above facts point to the same notion: to bypass the loss of a functional receptor, the virus was forced to change its receptor specificity by adapting to changes in the binding pocket on the receptor, by switching to another epitope on the same molecule or by switching to a new receptor.

There is yet another (and maybe the most efficient) way how the virus can deal with the loss of functional receptors on cells. Some env mutations apparently decrease the energy barrier for priming thus destabilizing the envelope glycoprotein (naturally semi-stable already before acquiring the mutation) and capacitating it to switch into an activated prefusion state without the need of the receptor. Viruses mutated in this way are able not only to enter the cells that lost the functional receptor but also infect cells from other, previously nonpermissive species including mammals (Rainey et al. 2003; Taplitz and Coffin 1997; Kawai et al. 1989).

Receptor-independent entry, however, brings negative side effects, namely decreased virus viability as the destabilized env has a short lifespan and is temperature-sensitive (Bova-Hill, Olsen, and Swanstrom 1991). Thus, env destabilization might be only a transient solution that creates an opening for further adaptation after which the env may be stabilized again.

This group of viruses, often called viruses with extended host range, might be responsible for interspecies virus transmissions. Such enterprise is, however, much more complicated than just tailoring env to a new receptor the virus must make many more adjustments, e.g., readjust different regulatory sequences (enhancer and promoter within its LTR, sequences influencing splicing, transport, etc.) and take measures to evade different restriction factors and provirus silencing in non-avian species (Brown and Robinson 1988; Searle et al. 1984).

The extended host range provided by destabilization of envelope glycoprotein is not specific for ALV. It was also observed in other groups of retroviruses such as HIV (Keller et al. 2018) and in distant viruses such as the measles virus (Jurgens et al. 2015).

In the past, attempts to eradicate pathogenic ALV strains from poultry farms were done by killing off the whole flocks whenever ALV infection was detected and replacing them with a new cohort of ALV-negative hens and cocks (Spencer et al. 1977). Recently, the CRISPR/Cas9 system was successfully used to generate chickens with a mutation in the Tvj gene that prevents infection with ALV-J (Koslová et al. 2020). The same procedure should be easy to employ for any ALV subgroup. However, examples of arms races discussed above raise the question about the endurance of nonpermissiveness based only on a single amino acid change in the receptor. Due to a very high mutation and recombination rate of retroviruses, the virus adjustment may not take a long time. Indeed, comparative analysis of recently emerged subgroup J and postulated subgroup K showed a high mutation rate of env sequences (P. Wang et al. 2018; Pan et al. 2012; X. Li et al. 2016; Zhao et al. 2018a).

In the light of the above facts, understanding of retroviral envelope glycoprotein interactions with respective receptors including their evolution during the virus-host arms race is of utmost importance in the effort to eradicate pathogenic ALVs from poultry farms.

Mechanisms of retroviral tumorigenesis

Retroviruses can induce tumorigenesis by several mechanisms each having very distinct hallmarks. Only the two of them were observed with ALVs so far.

The first mode of action is carried out by retroviruses encoding a viral version of the cellular proto-oncogene, collectively called oncogene-transducing retroviruses. A great many genes were taken over this way such as src (Barnekow et al. 1981), myb (Radke et al. 1982), myc (Enrietto 1989) and erbA/B (Graf and Beug 1983) by alpharetroviruses, mos (Stoica 1994), ras (Rothstein et al. 1985) and fes (Groffen et al. 1983) by gammaretroviruses to name just a few of the most familiar ones. Tumors induced by these viruses arise shortly after infection and have a polyclonal origin as the tumor develops from many independently infected cells. It must be emphasized that, outside the laboratory, transducing retroviruses do not spread and do not survive (a consequence of their excessive pathogenicity and replication incompetence) and each isolate has arisen de novo.

The second mechanism (the most typical and frequent one) is the insertional mutagenesis, already mentioned in the chapter on pathogenesis. This process takes a longer period of time since it requires infection of a huge number of cells until a cancer gene is hit by provirus integration in one or a few cells. Resulting tumors are in principle clonal and are characterized by the presence of a provirus in the same locus in independent tumors (so-called common site of integration harboring a cancer gene (Kung and Vogt 2012)).

The third mechanism is connected to the side effects of viral accessory genes that regulate gene expression - transactivating proteins like Tax of HTLVs (human T-lymphotropic virus) (Millen et al. 2020) or non-coding RNAs, such as of AS1 locus of BLV (bovine leukemia virus) (Safari et al. 2020). Tumor induction by these proteins is characterized by low penetrance and long latency. The tumors are clonal, probably due to the requirement of the further infrequent event to happen.

Finally, the fourth mechanism of retroviral oncogenesis implicates one of the basic retroviral genes: the env. Solid evidence accumulated showing that the envelope protein of some retroviruses possesses the ability to transform infected cells. In the following chapter the respective viruses will be listed and molecular details of this process, where known, will be reviewed.

Envelope glycoproteins as tumorigenic agents

Ovine pulmonary carcinomas (OPA) that manifest themselves by dyspnea in herded sheep and goats were first observed in the nineteenth century in South Africa (Sharp et al. 2008). The origin of this condition, called jaagsiekte (compound of African words for driving, jaagt, and sickness, ziekte), was discovered much later and named Jaagsiekte sheep retrovirus (JSRV, (Palmarini et al. 1999). JSRV does not carry any transduced oncogene. Insertional activation was excluded due to low expression of the virus and the rapid onset of OPA in infected animals. The alternative open reading frame inside the pol gene (orf-x) with the unknown function was shown to be dispensable for infection and transformation both in vitro and in vivo (N. Maeda et al. 2001). Thus, attention turned to possible oncogenic properties of either gag, pol, or env genes. It was found that env, when expressed alone, was able to transform several rodents, human, and chicken cell lines, (N. Maeda et al. 2001). The dominant and crucial role of JSRV-env in tumorigenesis was then demonstrated in vivo: a

replication-defective vector expressing JSRV env gave rise to lung tumors in sheeps (Caporale et al. 2006); similarly, an AAV-6 (Adeno-associated virus 6) vector expressing JSRV env induced lung tumors in Rag-2 knockout mice (Wootton et al. 2006).

Molecular mechanisms of JSRV env tumorigenic activity are still unsure. Comparison of exogenous JSRV with its endogenous non-tumorigenic forms revealed differences in at least 67 amino acids, located in the membrane-spanning domain and the cytoplasmic tail (Palmarini et al. 2000). One of the most striking differences was the presence of tyrosine at position 590 (Y590) in exogenous JSRV env. Mutations of Y590 abolished both transformation of rodent cells and OPA induction in mice, (Palmarini et al. 2001; Cousens et al. 2007). Since Y590 is part of the YxxM motif which, in a phosphorylated state, represents a putative binding motif for the SH2 domain of P3Ik regulatory subunit p85, it was suggested that P3Ik/Akt signaling pathway is involved in JSRV-driven tumorigenesis (Palmarini et al. 2001). However, although Akt is active (albeit inconsistently) in tumor cells, no phosphorylation of Y590 or binding to p85 was observed (Liu, Lerman, and Miller 2003). Moreover, JSRV is able to transform p85 knockout mice-derived cells (Zavala et al. 2003). Involvement of signaling through MAPK pathways was also considered as a high activity of Ras, MEK, and MAPK was observed in transformed cells and OPA (Naoyoshi Maeda et al. 2005), and inhibitors of Ras and MEK1/2 abolished transformation of rodent cell lines. Some studies also draw attention to the possible importance of Src kinase, Hsp90, and Rac1 in JSRV driven transformation (Hull and Fan 2006; Varela et al. 2008; Naoyoshi Maeda and Fan 2008). None of the above observations, however, points to direct interconnection with env protein.

A more promising hypothesis came with the discovery of Hyal-2 (hyaluronidase-2) being the cellular receptor recognized by the JSRV env. (Rai et al. 2001). Hyal-2 functions as a tumor suppressor that binds RON/STK receptor tyrosine-protein kinase and interferes with its signaling (Ronsin et al. 1993); its deletion is often observed in human lung carcinomas. Thus, a role of JSRV env-Hyal-2 interaction in tumorigenesis was proposed (Rai, DeMartini, and Miller 2000) and the following scheme was suggested: virus-driven down-regulation of Hyal-2 results in stronger signaling from RON, which in turn activates both PI3k/Akt and Ras/Raf/MEK/MAPK signaling pathways (Danilkovitch-Miagkova et al. 2003). However, this scheme is obviously not universal since JRSV transforms mouse cells despite the fact that murine Hyal-2 does not bind JSRV env (Liu et al. 2003). Furthermore, it was shown RON can bind not just Hyal-2, but also JSRV and MuLV envs, making the whole story even more complicated (Varela et al. 2006).

Interestingly, a short splicing variant of env, called Rej, was discovered recently and shown to be indispensable for JSRV gag synthesis in vitro essays The final functional product of Rej is its signal peptide released by a signal peptidase during Rej translocation into the endoplasmic reticulum. At least a proportion of it escapes from ERAD (Endoplasmic Reticulum-Associated Degradation) and is transported to the nucleus. However, since the same signal peptide is also released from the major full-length form of env, the Rej variant maybe just inconsequential curiosity that does not significantly increase the amount of active product. In any case, the signal peptide is required for JSRV replication but does not seem to be involved in oncogenesis (Hofacre, Nitta, and Fan 2009).

A similar way of tumorigenesis was observed with ENTVs (Enzootic nasal tumor viruses, a close relative of JSRV). ENTVs induce ENA (enzootic nasal adenocarcinoma) in the sheep (ENTV-1) and goats (ENTV-2) (De las Heras et al. 2003). Similar to JSRV env, ENTV-1 and

ENTV-2 envs transform rodent cells and activate MAPK and PI3k/Akt signaling pathways (Palmarini et al. 2000; Alberti et al. 2002; Liu and Miller 2005; Naoyoshi Maeda and Fan 2008; Naoyoshi Maeda et al. 2020). Though there are significant differences between cytoplasmic tails of JSRV and ENTV env, Y590 mentioned above is also present in ENTV, and mutation of this amino acid also reduces the transformation ability of the env. Interacting partners and molecular mechanisms underlying tumorigenesis were not established.

The ability of another betaretrovirus, MMTV (mouse mammary tumor virus) to induce mammary adenocarcinomas via insertional mutagenesis of cellular proto-oncogenes has been known for a long time (Bittner 1936; Nusse and Varmus 1982). However, a later demonstration that MMTV env alone can transform murine and human cells (Katz et al. 2005) called this simple notion into question. MMTV env SU contains a putative ITAM (immunoreceptor tyrosine-based activation motif), $Yxx(L/I)x_{6-8}Yxx(L/I)$ that could potentially play a role in the transformation (Ross et al. 2006). Mutations of either tyrosine of this motif reduced transformation abilities both in vitro and in vivo,

MMTV env was also reported to bind mouse TLR4 (toll-like receptor 4), a sensor of pathogen-associated molecular patterns (Rassa et al. 2002). Later on, however, it became clear that the interaction of MMTV with TLR4 is mediated by the bacterial LPS (lipopolysaccharides) bound to the LPS receptors that have been incorporated into the MMTV envelope (Wilks et al. 2015). In any case, TLR4 stimulation by MMTV leads to the production of immunosuppressive cytokines and blockage of the antiviral response; no role in the MMTV-driven carcinogenesis is presumed.

Similarly to JSRV, a short splicing variant of env, in the case of MMTV called Rem, was found. In every way, Rem seems to be an equivalent of Rej in JSRV, including doubts about the importance of this splicing variant (Buyn 2010 - PNAS).

All the viruses discussed above have the ability to induce tumorigenesis via a fully functional envelope glycoprotein. Highly oncogenic F-SFFV (Friend spleen focus-forming virus), on the other hand, codes for a recombinant, shortened, and defective env. The env contains a 195 amino acid deletion in the middle part that connects the amino terminus of SU with the carboxy terminus of TM; the deletion eliminates SU/TM cleavage site. The TM part, moreover, lacks the whole cytoplasmic tail due to a single base insertion and premature termination. The SU part best aligns to dual tropic MCF (mink cell focus-forming virus) which itself is a recombinant of exogenous ecotropic MuLV with endogenous dual tropic MuLV, while the TM part is derived from ecotropic F-MuLV (Friend murine leukemia virus) (Amanuma et al. 1983; Wolff, Scolnick, and Ruscetti 1983; Clark and Mak 1983).

F-SFFV together with its anticipated parents were components of the Friend virus complex isolated in 1957 from a mouse injected with Ehrlich mouse carcinoma cells (Friend 1957). The complex, as well as the mixture of F-MuLV (as a helper) with F-SFFV or with a retroviral vector expressing only SFFV env, induced rapid-onset erythroleukemia in mice (Wolff and Ruscetti 1988). Development of erythroleukemia in transgenic mice expressing only SFFV env definitely proved that the env is sufficient for leukemogenesis and does not require other parts of SFFV nor the helper virus (Aizawa et al. 1990). A comparison of many deletions and recombination variants formed during SFFV passaging showed that the TM part, not the SU part, of SFFV env, is crucial for leukemogenesis (Gomez-Lucia et al. 1998).

The search for SFFV env interaction partners revealed that it binds directly to the EpoR (erythropoietin receptor). This interaction results in activation of signaling molecules typical for a response to erythropoietin: JNK (c-Jun N-terminal Kinase), Stat5 (Signal Transducer

and Activator of Transcription 5), PKC (Protein Kinase C), Ras/Raf/MEK/MAPK pathway, and PI3k/Akt pathway. This demonstrates that SFFV env mimics the action of erythropoietin which results in the proliferation of erythrocyte precursors (Ohashi, Masuda, and Ruscetti 1996, 1995; Muszynski et al. 1998). Only a small fraction of SFFV env (less than 5%) is successfully processed and is displayed on the cell surface, and none is packaged into virions. Though ER-retained majority of SFFV env makes on-site complexes with EpoR, only interaction on the cell surface activates appropriate signaling cascades. Ternary complexes SFFV env-EpoR-Epo form on the surface of erythroid cells when both Epo and SFFV are present, suggesting that different epitopes on EpoR are occupied by the two ligands (Ferro et al. 1993).

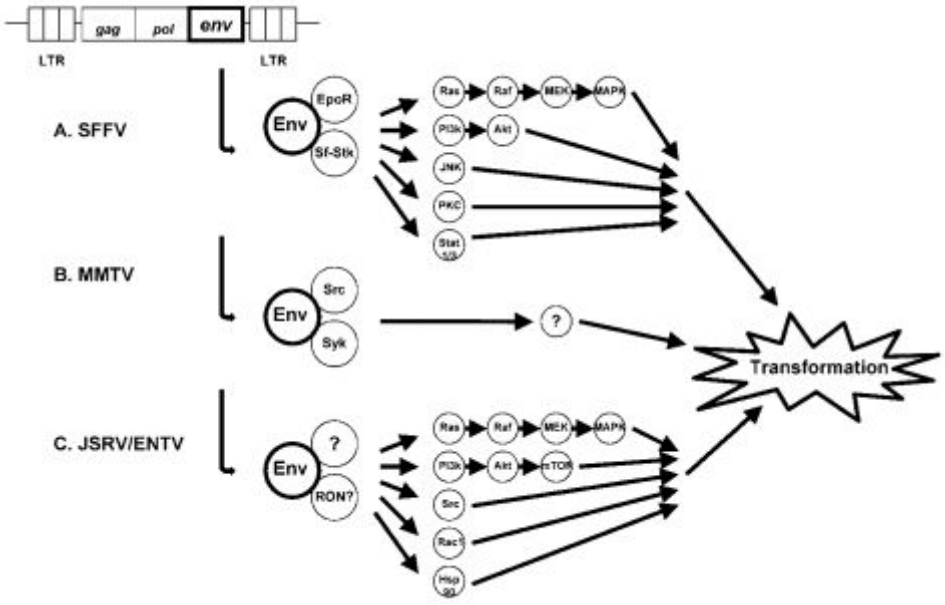


Figure 4.: Envelope glycoproteins involved in oncogenesis
Brief scheme of envelope glycoproteins of SFFV (A), MMTV (B), JSRV, and ENTV (C) and their possible interaction partners participating in env-driven oncogenesis (adapted from Naoyoshi Maeda, Fan, and Yoshikai 2008).

Envelope glycoprotein with unknown yet essential role in tumorigenesis

Among ALVs, two strains carry env with potent oncogenic activity: AHV (Avian hemangioma virus) and MAV (Myeloblastosis-associated virus). Though chicken retroviruses contributed enormously to the whole field of oncogenesis (discovery of the first cellular protooncogene - src - and discovery of the phenomenon of oncogenesis by insertional mutagenesis – myc), today's researches became somewhat uninterested in chicken models and the two ALV strains were little studied.

AHV generates vascular tumors in about 30 % of infected birds 3-10 months after infection and induces anchorage-independent growth of monkey and murine cells (Burstein et al. 1984; Alian et al. 2000). Its env retains oncogenic ability when expressed alone in a retroviral vector; even the SU alone is transforming (though on a reduced level). AHV virus as well as purified AHV SU are also able to induce a significant CPE (cytopathic effect) in chicken cells (Resnick-Roguel et al. 1989; Sela-Donenfeld et al. 1996). There is no knowledge of underlying mechanisms.

The other virus, MAV-2, was originally isolated by endpoint dilution from the complex viral stock AMV BAI A (Smith, R. E., and C. Moscovici. 1969) where it served as a helper of defective c-myb-transducing acutely oncogenic virus AMV (Avian Myeloblastosis Virus). MAV-2 proved to be a non-defective B-subgroup virus with a strong tendency to induce both nephroblastomas and osteopetrosis (a disease characterized by hyperproliferation of osteoblasts (Souza et al. 1980; R. E. Smith, Davids, and Neiman 1975). MAV variants with different potential to promote osteopetrosis, from low (less than 10%) to very high (100 %), have been isolated (Karafiát, unpublished data).

Experiments aimed to identify osteopetrosis-inducing determinants via the construction of chimeric viruses led to the conclusion that both env and LTR were involved in the osteopetrosis induction (Joliot et al. 1993). The importance of the TM region rather than the SU conforms with the observation that osteopetrosis potential is not limited to B-subgroup, as documented, e.g., by MAV-1-like virus responsible for osteopetrosis outbreak in Costa Rica in 1986 (Barbosa et al. 2010). As in the case of AHV, mechanisms underlying this disease are not known, except that very high levels of unintegrated MAV proviruses in osteoblast are a fundamental prerequisite for osteopetrosis development (Foster et al. 1994).

Aims

The envelope glycoproteins play a crucial role in the life cycle of enveloped viruses including retroviruses. The first steps of virus entry start with receptor recognition, assisted by interactions with other adhesive molecules on the cell surface, followed by the fusion of viral and cell membranes. The envelope glycoprotein is also an important template of retrovirus evolution because its endogenous expression prevents subsequent entry of superinfection virions of the same genus. Thus any changes in receptor-binding regions constitute an advantage for the new generation of viruses. Although it is widely acknowledged that retroviruses evolve through cross-species transmission and host range extension, the exact mechanism of receptor shift is still unclear.

Recently, a new group of avian leukosis virus emerged, denoted ALV-K solely on the sequence divergence to other subgroups. To support these findings, host range examination, interference analysis, and possibly receptor identification remain to be done. Therefore, we decided to check if JS11C1 represents a new subgroup of avian leukosis viruses and explore this recent example of env-receptor co-evolution.

Although such analysis of the newly emerging subgroup provides a great chance to the understanding of virus evolution, the information about shifting the receptor specificity might be missing due to the fully adapted envelope. Therefore, we resorted to the traditional model in our laboratory, a hamster cell line derived from Rous sarcoma virus-induced tumors. Mammalian cells do not support RSV replication and lack any molecule which might serve as a receptor for alpharetroviruses. Therefore, we investigated RSV provirus in the H20 cell line from the point of view of env-receptor co-evolution.

The envelope glycoprotein was also reported to be crucial for virus pathogenesis in the case of beta- and gammaretroviruses. Similarly, chicken osteopetrosis is promoted upon infection of certain ALV strains, e.g., MAV-2.O, and involvement of the envelope glycoprotein has been suggested. Having available the standard myeloblastic strain of MAV and its osteopetric derivate, we set to compare them and analyze the env evolution toward a newly emerging pathogenesis.

Materials and methods

Preparation of embryo fibroblasts and cell culture

Embryo fibroblasts of domestic chicken, ducks, and wild galliform species were prepared as described earlier (M. J. Federspiel and Hughes 1997), from embryos in the middle of incubation, i.e., 11 or 12 days postfertilization. Embryonated eggs were obtained as follows: northern bobwhite (*Colinus virginianus*) and red jungle fowl (*G. gallus* subsp. *murghi*) from South Bohemia Zoological Gardens (Hluboká nad Vltavou, Czech Republic), California quail (*Callipepla californica*), Gambel's quail (*Callipepla gambelii*), silver pheasant (*Lophura nycthemera*), white-crested kalij pheasant (*Lophura leucomelanos* subsp. *hamiltoni*), and gray jungle fowl (*Gallus sonneratii*) from the AVES Farm (Košice, Czech Republic), guinea fowl (*Numida meleagris*), turkey (*Meleagris gallopavo*), and chukar (*Alectoris chukar*) from the University of Veterinary and Pharmaceutical Science (Brno, Czech Republic), gray partridge (*Perdix perdix*), common ringed-neck pheasant (*Phasianus colchicus*), and Reeve's pheasant (*Syrmaticus reevesii*) from the Židlochovice Forest Enterprise (Židlochovice, Czech Republic), and Mrs. Hume's pheasant (*Syrmaticus humiae*) from Fasanerie Christian Möller (Erfurt, Germany). Chicken embryo fibroblasts were prepared from inbred lines CB, WA, L15, H6, and M and duck embryo fibroblasts from Khaki Campbell, which were maintained at the Institute of Molecular Genetics (Prague, Czech Republic). All embryo fibroblasts, as well as the permanent chicken cell line DF-1 (Himly et al. 1998) and the Japanese quail (*Coturnix japonica*) tumor cell line QT6 (Moscovici et al. 1977), were grown in a mixture of 2 parts Dulbecco's modified Eagle's medium and 1 part F-12 medium supplemented with 8% fetal calf serum, 2% chicken serum, and 1× antibiotic-antimycotic solution (Sigma), in a 5% CO2 humidified atmosphere at 37°C.

Construction of RCAS vector with JS11C1 *env* gene

We prepared the K subgroup RCAS vector by replacing the *env* gene in the RCASBP(D)GFP subgroup D retrovirus vector (M. J. Federspiel and Hughes 1997; Hughes 2004) with the complete *env* gene of JS11C1 virus. RCASBP(D)GFP transducing the GFP reporter gene was digested with KpnI and StuI (both from New England BioLabs), and the 1,853-bp fragment containing the 3′ end of *pol* and the entire *env* gene was discarded. The JS11C1 *env* gene (GenBank accession no. KF746200.1) was synthesized (Integrated DNA Technologies) together with adjacent RCAS sequences, including the KpnI and BsaBI sites at the 5′ and 3′ ends. Due to an additional BsaBI site present in the *env* coding sequence, a partial KpnI-BsaBI (New England BioLabs) digestion was performed to obtain the 2,120-bp fragment, which was then inserted into the RCAS backbone; the resulting vector, RCASBP(JS11C1)GFP, was used for virus propagation and infection experiments.

Construction of RCAS vectors with dsRed fluorescent marker

The RCASBP(A)dsRed and RCASBP(JS11C1)dsRed vectors used in the superinfection experiments were prepared by replacing the *gfp* gene of RCASBP(A)GFP and

RCASBP(JS11C1)GFP with *dsRed-Monomer*. The coding sequence of *dsRed-Monomer*, encoding a monomeric mutant of the fluorescent protein dsRed, was amplified with forward (5′-accactgtggcatcgatGGTCGCCACCATGGACAACAC-3′) and reverse (5′-ccgtacatcgcatcgatCTCTACTGGFGCCGGAGTG-3′) primers from the pDsRed-Monomer-N1 vector (Clontech) and was cloned into ClaI-linearized RCASBP(A)GFP and RCASBP(JS11C1)GFP vectors using the In-Fusion HD cloning kit (Clontech). The 17-bp overlapping sequences of the forward and reverse primers are shown as lowercase letters.

Virus propagation and FACS assay of virus infection

All viruses used in this study were propagated by transfection of their respective vector plasmid DNA into DF-1 cells using the XtremeGENE transfection reagent (Roche). Virus stocks were harvested on day 9 or 10 posttransfection and were cleared of debris by centrifugation at 2,000 × *g* for 10 min at 10°C. Aliquoted viral stocks were stored at −80°C. Virus titers were determined by terminal dilution, subsequent infection of DF-1 cells, and detection of GFP positivity. All RCAS-based retrovirus vectors used in this study reached titers of 10^6 IU per ml. The *in vitro* infection of GFP- or dsRed-transducing RCASBP vectors was assayed in cultured avian embryo fibroblasts, QT6 cells, or NIL-2 cells. The cells were seeded at a density of 5×10^4 cells per well in a 24-well plate; 24 h after seeding, 5×10^5 IU of the virus was added to 0.25 ml medium for 1 h. Two days postinfection, the cells were washed in phosphate-buffered saline, trypsinized, pelleted by centrifugation in the cultivation medium, and resuspended in Hoechst solution (Sigma). The percentages of GFP-positive cells, dsRed-positive cells, and GFP/dsRed-double-positive cells were quantitated by FACS analysis using an LSRII analyzer (Becton, Dickinson).

Ectopic expression of Tva in Syrian hamster NIL-2 cells

Induction of susceptibility to RCASBP(A)GFP and RCASBP(JS11C1)GFP by ectopic Tva expression was shown using Syrian hamster (*Mesocricetus auratus*) NIL-2 cells stably transfected with the Tva expression vector (Elleder et al. 2005). The NIL-2 cell line was transfected, using Lipofectamine 2000, with 2 μg of plasmid pTva together with 0.2 μg of pMC1neo poly(A) (Stratagene, La Jolla, CA), which contains the neomycin resistance gene. The transfected cells were grown for 10 days with G418 (500 μg/ml) to select for neomycin resistance. NIL-Tva was isolated as a cell clone from soft agar, expanded, and used for infection experiments.

Preparation of *tva* and *tvb* knockout DF-1 clone

We employed the DF-1-*tva*$^{-/-}$ cell clone described previously (Koslová et al. 2018) and the newly constructed DF-1ΔTvb. Briefly, we used CRISPR/Cas9 genome-editing tools and cloned the guide RNA sequences 5′-CCGACTGCTACCCGCTGGAG-3′ and 5′-GCATCCCCACCAGGAACACG-3′, matching the second exon of the chicken *tva* gene and fourth exon of chicken *tvb* gene, respectively, into the single guide RNA cloning backbone of the PX458 vector (AddGene vector pSpCas9BB-2A-GFP plasmid 48138). The

resulting construct was transfected into DF-1 cells using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's protocol. CRISPR/Cas9 indel activity was assayed by T7EI heteroduplex cleavage, and single-cell clones were derived using the single-cell sort mode of the Influx cell sorter (Becton, Dickinson). The expanded clones were then tested for resistance to RCASBP(A)GFP and frame-shifting deletions in the *tva* sequence and for resistance to RCASBP(B)GFP and frame-shifting deletions in the *tvb* sequence, respectively.

Soluble Tva immunoadhesin production and inhibition of virus infection

As a source of the soluble form of Tva receptor, we used the quail Tvas receptor fused to the constant region of a mouse IgG heavy chain, also known as immunoadhesin sTva-mIgG. The sTva immunoadhesin was expressed from the RCASBP(B)stva-mIgG vector, containing the *stva-mIgG* gene cassette in the ClaI cloning site (Sheri L. Holmen et al. 1999). The RCASBP(B)stva-mIgG vector was a kind gift from M. J. Federspiel (Mayo Clinic, Rochester, MN). Stocks of sTva-mIgG immunoadhesin were generated by transfection of RCASBP(B)stva-mIgG plasmid DNA into DF-1 cells using the XtremeGENE transfection reagent (Roche) and harvesting of the supernatant after 1-week culture of infected cells. The supernatant was cleared by centrifugation at 2,000 × *g* for 10 min at 4°C and stored at −80°C. The RCASBP(A)GFP and RCASBP(JS11C1)GFP virus aliquots were mixed with sTva-mIgG supernatant at 4°C and used for DF-1 cell infection after 30 min of preincubation.

XC and H20 cell line origin

XC and H20 are rodent tumor cell lines transformed with the PR-RSV-C strain. The rat XC cell line harbors several provirus copies per genome (Mitsialis et al. 1983), whereas the hamster H20 cell line possesses only a single provirus (Svoboda et al. 1983). Both cell lines produce either no or negligible infectious virus (Geryk et al. 1984; Svoboda et al. 1963). The XC cell line is derived from a tumor that was induced by inoculation of PR-RSV-C–infected chicken tissues into newly born rats (Svoboda 1960). The H20 cell line was prepared in a similar way with the difference that chicken sarcoma tissue induced by XC-RSV, which was rescued by transfection from XC cells, was inoculated into newly born Syrian hamsters (Geryk et al. 1984).

TM oligomerization assay

The TM oligomerization assay was performed similarly as previously described (Walther Mothes et al. 2000; J. G. Smith et al. 2004; Melder et al. 2009). The temperature threshold that triggers TM oligomerization was determined by incubating the purified virions in HN buffer [10 mM Hepes, 130 mM NaCl (pH 8.0)] at the indicated temperature for 20 min before lysis with Laemmli loading buffer (1% SDS, 10% glycerol, 0.1 M Tris-Cl, pH 6.8, 0.1% bromophenol blue, 5% β-mercaptoethanol). The pH of HN buffer with virions was adjusted with predetermined volumes of 100 mM Hepes (pH 3.8 or 7.4), and the samples were

incubated at room temperature for 30 min. The samples were then neutralized with 1 M Tris (pH 7.6 or 9.5), lysed with Laemmli loading buffer, and analyzed by immunoblotting after SDS/PAGE in 11% polyacrylamide gels with a rabbit antibody specific for the carboxyl terminus of the TM subunit of ASLV Env (1:1,000) (Walther Mothes et al. 2000).

Virus liposome binding assay

The virus liposome binding assay is a modification of the protocol previously described (Melikyan et al. 2004). A total of 10^5 GFP-transducing units of each virus in 100 µL of cultivation media was incubated with 250 µL of liposomes (5 mM) at 37 °C for 30 min. A total of 350 µL of ice-cold 80% sucrose-PBS was added to the preparation to bring it to 40% sucrose, and 700 µL of this solution was layered on 3.2 mL of 50% sucrose-PBS and overlaid with 1 mL of 30% sucrose-PBS and 100 µL of 5% sucrose-PBS. The samples were ultracentrifuged at 152,000 × *g* at 4 °C for 2 h. Three 400-µL aliquots followed by one 600-µL aliquot were taken from the air-liquid interphase. The top 400 µL and the last 600 µL (fraction above 50% sucrose, called bottom) of each sample were mixed with 5× Laemmli loading buffer, boiled for 10 min, and separated in 13% SDS/PAGE. The presence of virions was detected by immunoblotting with the anti-ASLV rabbit p27 Antiserum (Charles River Laboratories, 1:1,000).

Statistical Analysis

We performed Welch *t* test to determine significance of difference in virus infectivity. In graphs, significant differences are marked by asterisks (**P* = 0.05–0.01, ***P* = 0.01–0.001, ****P* < 0.001).

Cell cultures

The chicken fibroblast cell line DF-1, which is free of ASLV-related endogenous (*ev*) loci (Himly et al. 1998), was obtained from S. Hughes, National Cancer Institute, Bethesda. L15 cells are primary chicken embryo fibroblasts prepared from 10-d-old embryos of chicken inbred line L15, which harbors a mutation introducing a premature stop codon in Tvc receptor (Elleder et al. 2005). Hamster cell line NIL-2 is a spontaneously transformed cell line derived from Syrian hamster embryo (Diamond 1967). NIL-2 cells expressing Tvc receptor (NIL-Tvc) were derived from NIL-2 and their preparation was described previously (Elleder et al. 2005). HEK293 (human embryonic kidney cells) and RPE1-hTERT (hTERT-immortalized retinal pigment epithelial cells) were purchased from the American Type Culture Collection. DF-1, L15, NIL-2, and NIL-Tvc cells were grown in 1:1 DMEM:F-12 medium (Life Technologies) supplemented with l-glutamine, 5% calf serum, 1–5% FCS, 1% chicken serum, and 10% tryptose phosphate broth (Life Technologies). HEK293 and RPE1-hTERT cells were grown in DMEM (Sigma) supplemented with 10% FCS and 1× antibiotic–antimycotic solution (Sigma).

Virus rescue from H20 cells

X-irradiated H20 cells were fused with DF-1 by means of PEG as described before (5). Virus production in culture medium was 5–15 focus-forming units (FFU)/mL 4 d after fusion in contrast to untreated, treated, or self-fused H20 cells, which produced no infectious virus. The virus was then replicated in DF-1 cells to ~10^5 FFU. The viral particles were concentrated by ultracentrifugation through a 25% sucrose cushion at 32,000 rpm for 2 h in a Beckman SW38 rotor.

DNA extraction and RSV *env* gene amplification

The genomic DNA was isolated by phenol-chloroform extraction from the XC, H20, or XC-RSV–infected DF-1 cells. The RSV *env* gene was amplified using the forward primer PolEnd_fw (5′-TTTGGGTACCCTCTCGAAAAGT) and reverse primer EnvEnd_StuI_rv (5′-ACAGGCCTTTTGCATCTTCCTGTATTCAGTA). The following PCR conditions were used: 98 °C for 30 s, 36 cycles of 98 °C for 15 s, 65 °C for 30 s, and 72 °C for 60 s, and terminal extension at 72 °C for 7 min with fusion polymerase (NEB). The resulting PCR product of 1,869 bp in length was sequenced from both sides with the primers used in the PCR amplification.

Construction of virus reporter vectors and virus propagation

The original virus bearing PR-RSV-C was expressed from plasmid pPrC, which was obtained from K. Beemon, Johns Hopkins University, Baltimore (Ogert, Lee, and Beemon 1996) and amplified in DF-1 cells with a resulting titer ~10^5 FFU. The viral particles were concentrated by ultracentrifugation through 25% sucrose cushion at 32,000 rpm for 2 h in a Beckman SW38 rotor. RCAS(C)GFP and RCAS(H20)GFP were prepared by env replacement from retroviral vector RCASBP(C)GFP (M. J. Federspiel and Hughes 1997) transducing the green fluorescent protein (GFP) reporter gene. env from H20 cells and from pPrC was amplified using the forward primer PolEnd_fw and reverse primer EnvEnd_StuI_rv by fusion polymerase with the same conditions as mentioned above. PCR products were adenylated and cloned in pGEM-T Easy plasmid vector (Promega). The resulting pGEM-EnvC and pGEM-EnvH20 as well as vector RCASBP(C)GFP were digested with KpnI and StuI restriction enzymes and the 1,853-bp fragment containing the 3′ end of pol and the entire env gene from RCASBP(C)GFP was replaced with equal-length fragments from pGEM-EnvC or pGEM-EnvH20. Chimeric constructs EnvC-H20Parts1–4 were prepared by replacement of one specific part of EnvC by EnvH20. pGEM-EnvC and pGEM-EnvH20 were digested with NheI and AgeI for EnvH20Part1, AgeI and HindIII for EnvH20Part2, HindIII and Xma for EnvH20Part3, and Xma and StuI for EnvH20Part4. The cleaved fragment from pGEM-EnvC was replaced with the equal-length fragment from pGEM-EnvH20. The resulting chimeric pGEM-EnvC-H20Parts1–4 was digested with KpnI and StuI and inserted into cleaved RCASBP(C)GFP in the same way as described for RCAS(C)GFP. EnvC-L378S, EnvC-G464S, and EnvC-L503V are constructed harboring EnvC with one specific mutation and were prepared by PCR mutagenesis and In-Fusion cloning. Fifty nanograms of pGEM-EnvC were amplified with primers bearing mutation, primers

5′-CGCAAGCCTCAAGAGAAATTGAGAGAC and 5′-TTCTCTTGAGGCTTGCGCAGCT for EnvC-L378S, 5′-TAAGATCAGCGTGGACAGCGACC and 5′-GTCCACGCTGATCTTATTGACATGTTTC for EnvC-G464S, and 5′-ATTGCTAGTGGTGTGCCTGCCTT and GGCACACCACTAGCAATAAAATAACTACAAGCC for EnvC-L503V. The following PCR conditions were used: 98 °C for 30 s, 36 cycles of 98 °C for 15 s, 70 °C for 30 s, and 72 °C for 3 min, and terminal extension at 72 °C for 7 min with fusion polymerase (NEB). The resulting PCR product (4,900 bp) was cleaved with DpnI to get rid of the original pGEM-EnvC and recircularized by In-Fusion HD Cloning Kit (Clontech) according to the manufacturer's protocol. pGEM-EnvC with the particular mutation was digested with KpnI and StuI and inserted into cleaved RCASBP(C)GFP in the same way as described for RCAS(C)GFP. EnvB-H20Part4 was prepared from RCASBP(B)GFP (M. J. Federspiel and Hughes 1997). Part of the env gene encoding the surface subunit of EnvB was amplified with primers 5′-TCGATTTTTGGGTACCCTCTCGGA and 5′-CTTGTGCAGCTGCTACCC from template RCASBP(B)GFP with fusion polymerase. The resulting PCR product was inserted into pGEM-EnvC-H20Part4, which was cleaved with KpnI–XmaI using In-Fusion HD Cloning Kit. The resulting vector pGEM-EnvB-H20Part4 as well as vector RCAS(C)GFP were cleaved with KpnI and BstBI, and the 1,797-bp fragment from RCAS(C)GFP was discarded and replaced with same-length fragments from pGEM-EnvB-H20Part4. All viruses were propagated in the same way. DF-1 cells were transfected with viral constructs using Lipofectamine 3000 (Thermo Fisher Scientific) according to the producer's protocol and the viruses were amplified for 2–3 weeks. The medium was then collected, centrifuged (3,850 × g, 20 min, 4 °C), and filtered through a 0.45-μm filter. Aliquots were stored at −80 °C.

Quantification of proviral DNA in infected cells

NIL-2 and NIL-Tvc cells were seeded at a density of 5×10^3 per well in a 96-well plate. Five hours after seeding, the cells were pretreated for 30 min with 50 μL of medium with polybrene (20 μg/mL) and infected with 50 μL of medium containing virus. Before infection, all viral stocks were treated with DNase for 30 min at 37 °C to get rid of residual viral DNA. On days 1, 3, 6, and 9 after infection, the cells were washed with PBS and resuspended in 100 μL of lysis buffer (10 mM Tris·HCl pH 8.0, 1 mM EDTA, 0.2 mM CaCl2, 0.001% Triton X-100, 0.001% SDS, 1 mg/mL proteinase K). The resuspended cells were incubated at 58 °C for 1 h and then the protease was heat-inactivated at 95 °C for 10 min. Aliquots (4 μL) were analyzed by real-time quantitative PCR based on the MESA GREEN qPCR MasterMix Plus for SYBR Assay Kit (Eurogentec) and a CFX96 system for qPCR detection (Bio-Rad). Quantifications of viral transcripts were performed with primers 5′-GCCAGGGAACCTTTGGATTA and 5′-CCCTTAAAATCACCTTCGGAAA in env gene or 5′-ACGTAAACGGCCACAAGTTC and 5′- TGCAGATGAACTTCAGGGTCAG in gfp. Results were normalized to the amount of mGAPDH genomic locus, which was measured with primers 5′-AACTTTGGCATTGTGGAAGG and 5′-ATCCACAGTCTTCTGGGTGG. The volume of the reaction mixture was 20 μL with 400 nM final concentration of each primer. Cycling conditions were 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 61 °C for 20 s, and 72 °C for 20 s. Calibration curves were prepared by amplification of diluted plasmid samples ranging from 10^2 to 10^7 copies per reaction.

Determination of viral titers

PR-RSV-C and H20-RSV viruses were titrated by the infectious center assay described previously (Svoboda and Dourmashkin 1969). RCAS-EnvC-GFP, RCAS-EnvH20-GFP, and all other env-mutant viruses derived from these two vectors were titrated using flow cytometry. DF-1 and HEK293 cells were seeded at a density of 5×10^4 per well in a 24-well plate; NIL-2, NIL-Tvc, and RPE1-hTERT were seeded at a density of 2.5×10^4 per well in a 24-well plate. Five hours after seeding, the cells were pretreated for 30 min with 200 µL of medium with polybrene (20 µg/mL) and infected with 200 µL of the virus-containing medium. The next day, the medium was replaced and in the case of DF-1 cells, AZT (final concentration 5 µM) was added into the new media 24 h postinfection. The percentage of GFP+ cells was quantified by fluorescence-activated cells sorting (FACS) using an LSRII analyzer (Becton, Dickinson) 2 d (in the case of DF-1 and HEK293 cells) or 3 d (in the case of NIL-2, NIL-Tvc, and RPE1 cells) postinfection. The cells were trypsinized and washed with PBS before the analysis. The viral titer was determined from the following formula: TU/mL = (1/dilution) × (1/volume used to infect) × (cells per well at the time of infection) × [−ln (1 − positive fraction)] (Rainey et al. 2003).

Virus purification

Viruses used for the pH inactivation assay, PMB inhibition, and TM oligomerization assays were concentrated from culture supernatants by ultracentrifugation through a 25% sucrose cushion in HN buffer [10 mM Hepes, 130 mM NaCl (pH 8.0)], and then resuspended in a small volume of HN buffer overnight at 4 °C.

Temperature-sensitivity assay of virions

Virus stocks in the culture medium were diluted to a similar titer and aliquoted into five samples, which were incubated for 0, 2, 4, 6, or 8 h at 44 °C and transferred to ice. DF-1 cells and NIL-Tvc cells were infected as described above. The next day, DF-1 cells were harvested for quantification of newly made viral DNA. NIL-Tvc cells were analyzed for GFP expression by flow cytometry 3 d after infection.

pH inactivation assay

Virus inactivation by low pH was performed as described previously (Mitsialis et al. 1983). To modify the pH, virions in the HN buffer were diluted 100-fold in a medium that was kept at pH 7.4 or adjusted to pH 5.0 with HCl. The samples were then incubated either on ice or at 37 °C for 30 min before neutralization with an equal volume of medium buffered with 25 mM Hepes (pH 7.4). The virus was then added to DF-1 cells in 24-well plates, and the next day the medium was replaced and AZT (final concentration 5 µM) was added. Two days after infection, GFP expression was analyzed by flow cytometry.

PMB inhibition

PMB inhibition of virus infection was performed as described previously (Walther Mothes et al. 2000). PMB (Thermo Fisher Scientific) was dissolved in the HN buffer and added into the virus preparation in the HN buffer to the indicated final concentrations. Samples were incubated at 37 °C for 30 min, cooled on ice, diluted in growth medium supplemented with 40 mM Hepes, and added on ice to 24-well plates of DF-1 cells that were washed with chilled PBS. Plates were centrifuged for 2.5 h at 1,520 × g, 4 °C. The medium was changed on ice and plates were then transferred to a 37 °C incubator. The next day, the medium was changed and AZT (final concentration 5 μM) was added. Two days after infection, GFP expression was analyzed by flow cytometry.

Production of liposomes

Liposomes were produced by a modification of the protocol described previously (Earp et al. 2003). Liposomes were always freshly prepared before each experiment as a 2:1 molar ratio mixture of egg phospholipids and cholesterol (Sigma) in chloroform. Hen egg phospholipids were isolated according to method 2 by Gladkowski et al. (Gładkowski et al. 2012). The concentration of phospholipids was determined by molybdenum blue reaction (Rouser, Fkeischer, and Yamamoto 1970). The lipid mixture was dried down to a thin film in a round-bottom glass tube under a constant stream of nitrogen at room temperature. After resuspension in PBS by extensive vortexing, liposomes were sonicated twice for 60 s at 43 °C in a water bath sonicator and then extruded 25 times through a 0.1-μm pore size polycarbonate membrane (Avestin) in a LiposoFast Basic apparatus (Avestin).

Product-enhanced reverse transcriptase assay

The product-enhanced reverse transcriptase (PERT) assay was performed as described previously (Fábryová et al. 2015). In each run, one positive control (ASLV of defined titer) and one negative control (culture medium of uninfected cells) were included.

Inoculation of chicken embryos

Outbred Brown Leghorn chickens were used (Plachý and Hála 1997). On day 12 of embryonal development, we have injected 0,1 ml of virus stocks into the chorioallantoic vein. The animals were sacrificed and examined when signs of suffering were spotted or when the experiment was ended. Upon sacrifice, chickens were examined for bone transformation.
For time dependent experiments, we inoculated embryos on days 10, 13, or 17 or at day 0 or 7 post-hatching. For the dose-dependent experiment, we have used only embryos at day 12 for inoculation of diluted viruses.
All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council (U.S.). Institute of Laboratory Animal Resources (U.S.). Committee on Care and Use of Laboratory Animals 1985) and approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic.

Virus sequencing and comparative analysis

Genomic DNA of osteopetrotic bones was obtained by freezing the samples in liquid nitrogen, pulverized, mixed with DNA lysis solution (1% SDS, 250 mmol/L EDTA, and 1 mg/mL proteinase K) and incubated 50°C o/n. The solution was extracted by phenol-chloroform (1:1), DNA containing phase was mixed with 96% EtOH, spun, washed by 80% EtOH and resuspended in TE buffer (10 mmol/L Tris-Cl (pH 8.3) and 1 mmol/L EDTA). Extracted DNA served as a template for PCR under following conditions: AccuTaq polymerase (Sigma) with AccuTaq buffer supplemented with 500 µmol/l of deoxynucleotide triphosphates was mixed with forward (5′-TGTAGTCTTAATCATAGGTTAACATGTA-3') and reverse primer (5′-CAGAATACTCCCTCTAACCATAGCCACTGCG-3') for amplification of LTR-gag region, forward (5′-ATCCATGGGATAGGAGGGGGAATT-3') and reverse primer (5′-TGCTGGTCTGGGGACGAGGTTATGC-3') for amplification of pol region and forward (5′-CTATTAGCCAAGGCAGTGTATGCCCTTAAT-3') and reverse (5′-CAAATAAGGGAATCGCCTGATGCACC) primers for amplification of env-LTR region. Fragments were purified by EtOH standard protocol. Samples were sequenced using Sanger sequencing protocol by Seqme with following primers: (5′-TGTAGTCTTAATCATAGGTTAACATGTA-3'), (5′-ACTCCCCGGGGTCCTGGGATC-3') and (5′-TAACTTGGATCGCTTAAAGGGTCTAG-3') for sequencing of LTR-gag fragment, (5′-ATCCATGGGATAGGAGGGGGAATT), (5′-GTACGTATGTAGCACCCGTAGG-3') and (5′-TGCTGTGGCCGACAACGCCC-3') for sequencing pol region and (5′-CTATTAGCCAAGGCAGTGTATGCCCTTAAT-3'), (5′-ACTCACCGGTTAGTCTCGAGAGGT-3') and 5′-(AGCTTACCATGTTAGCACCCAACC-3') for sequencing of env-LTR region.
Obtained sequences were compared to MQNCSU (GenBank accession no. DQ365814.1), LR-9 (GenBank accession no. AY350569.1), Pr-C (GenBank accession no. V01197.1), RSA (GenBank accession no. M37980.1), MAV-2 (GenBank accession no. L10924.1), and MAV-2.O and MAV-1/2.O as presented by (Joliot et al. 1993).

MAV-2.O substitutions-derived vectors

Selected substitutions were introduced into MCAS(B) GFP vector employing the Phusion PCR two-step protocol (NEB) with the following PCR conditions for all reaction: 98 °C for 30 s, 35 cycles of 98 °C for 15 s, 60 °C for 30 s, and 65 °C for 2 min, and terminal extension at 72 °C for 5 min. For LTR-derived 160, 194, and 372, we have used forward (5′-TTTATTACCAAGCGAAGCGCCATTC-3′) and reverse primers (5′-CCCAGGACCCCGGGGAGTATAAG-3′) to flank the LTR target area for PCR-driven mutagenesis and forward (5′-CCTTCCTCATCAGATCATGTACGCGGCAGAG-3′) and reverse (5′-CTCTGCCGCGTACATGATCTGATGAGGAAGG-3′) primers for introducing 160 substitution, forward (5′-ATGATTGGATAACTGGATGGCACCATTCAT-3′) and reverse (5′-ATGAATGGTGCCATCCAGTTATCCAATCAT-3′) primers for introducing 194 substitution and forward (5′-ACGACTACGAGCACCTGCATGAAGCA-3′) and reverse (5′-TGCTTCATGCAGGTGCTCGTAGTCGT-3′) primers for introducing 372 substitution. We have used pMAV-left plasmid as a template for this reaction.

For pol-derived A683V and M765V substitutions, we have used forward (5′-GCACCACTGCTGCCTTGGAGCG-3′) and reverse (5′-TGTTGTGTGGAATTGTGAGCGGA-3′) primers to flank the pol target area for PCR-driven mutagenesis and forward (5′-CTACGGCTATCGCCGTTTTGGGAAGACC-3′) and reverse (5′-GGTCTTCCCAAAACGGCGATAGCCGTAG-3′) primers for introducing A683V substitution and forward (5′-CTATTAGCCAAGGCAGTGTATGCCCTCAAT-3′) and reverse (5′-ATTGAGGGCATACACTGCCTTGGCTAATAG-3′) primers for introducing M765V substitution. We have used pMAV-left plasmid as a template for this reaction.
For env-derived A28T, (also pol-derived S880N) L387S and A398T substitutions, we have used forward (5′-TGGCAGTAATTTGGGTAATGTC-3′) and reverse (5′-TGTCTTTCCTGCCTTGCTCGATGG-3′) primers to flank the TM target area and forward (5′-TTTATTACCAAGCGAAGCGCCATTC-3′) and reverse (5′-CCCTGGGTCGGTCGAAAGGATGTG-3′) primers to flank the signal peptide target area and forward (5′-GCTGCGCAAGCCTCAAGAGAAATCGAG-3′) and reverse (5′-CTCGATTTCTCTTGAGGCTTGCGCAGC-3′) primers for introducing L387S substitution, forward (5′-GGTCCGTTAAACAGACTAATTTGACAACATC-3′) and reverse (5′-GATGTTGTCAAATTAGTCTGTTTAACGGACC-3′) primers for introducing A398T substitution and forward (5′-GAAGAAACCGCCAGCAACAAGCAAGAAAGA-3′) and reverse (5′-TCTTTCTTGCTTGTTGCTGGCGGTTTCTTC-3′) primers for introducing A28T substitution. We have used pMCAS-right GFP plasmid as a template for this reaction.
All amplified sequences were purified by LMP agarose gel, excised, and isolated by the standard phenol extraction protocol (“Current Protocols in Molecular Biology” 1993) and mixed for the next step of PCR amplification according to Phusion PCR two-step protocol (NEB) with following PCR conditions for all reaction: 98 °C for 30 s, 15 cycles of 98 °C for 15 s, 60 °C for 30 s, and 65 °C for 3 min, and terminal extension at 72 °C for 5 min. All samples were purified by standard ethanol precipitation protocol (“Current Protocols in Molecular Biology” 1993) and digested as follows: LTR-derived substitutions fragments, together with pMAV-left plasmid were digested by PvuII-SalI, pol-derived substitutions fragments together with pMAV-left plasmid were digested by PmlI-KpnI, TM-derived substitutions fragments together with pMCAS-right GFP plasmid were digested by SmaI-SacI and leader peptide-derived substitutions fragments together with pMCAS-right GFP plasmid were digested by PvuII-BamHI and purified by LMP agarose gel as described above. Fragments were ligated with respective digested plasmids following T4 ligase protocol (NEB). For the generation of vectors with multiple MAV-2.O-derived genes, multiple steps of Phusion PCR or digestion, as described above, were taken. To produce the final vector for virus production, pMAV-left and pMCAS-right GFP were digested by KpnI-BsiWI, purified by LMP agarose gel, and env-LTR fragment of pMCAS-right GFP was ligated into pMAV-left backbone.

Introduction of MAV-2.O env-derived substitutions into A, C and K subgroups

For the production of A subgroup-derived vector, we have digested plasmids pAT-MAV-1 (plasmid with pAT153 backbone containing MAV-1 (GenBank accession no. L10922.1) and pMAV-left by KpnI-SacI, purified by LMP agarose gel protocol described above and ligated by T4 ligase. For the production of C and K subgroups-derived vectors, we have employed

Phusion PCR protocol using forward (5′-AGTCAGCCACCTCCCCTTTT-3′) and reverse (5′-TGCCTGAGCTCTAGACTGCTCCGCCCTGCAT-3′) primers for subgroup C (template pPrC) and forward (5′-AGTCAGCCACCTCCCCTTTT-3′) and reverse (5′-TGCCTGAGCTCTAGACCGCCCCATTTTCAGGCTG-3′) primers for subgroup K (template pRCAS(JS11C1)GFP to introduce SacI restriction site (site underlined) at the end of env gene under following PCR conditions: 98 °C for 30 s, 35 cycles of 98 °C for 15 s, 60 °C for 30 s, and 65 °C for 2 min, and terminal extension at 72 °C for 5 min. Amplified sequences were purified by EtOH standard protocol and digested by SmaI-SacI. Next, we have digested pMCAS-B-right GFP plasmid by KpnI-SacI and pPrC and RCAS(JS11C1)GFP by KpnI-SmaI and purified them by LMP agarose gel. We have mixed digested fragments obtained from Phusion PCR reaction with digested pMCAS(B)-right-GFP and pPrC for production of pMCAS(C)GFP-right or RCAS(JS11C1)GFP for production of pMCAS(K)-right GFP together with T4 ligase. To introduce MAV-2.O env-derived substitutions, we have employed the Phusion PCR two-step protocol (NEB) with the following PCR conditions for all reaction: 98 °C for 30 s, 35 cycles of 98 °C for 15 s, 60 °C for 30 s, and 65 °C for 2 min, and terminal extension at 72 °C for 5 min with forward (5′-AGTCAGCCACCTCCCCTTTT-3′) and reverse (5′-TGTCTTTCCTGCCTTGCTCGATGG-3′) primers to flank the TM target area and forward (5′-GCTGCGCAAGCCTCAAGAGAAATTGAG-3′) and reverse (5′-CTCAATTTCTCTTGAGGCTTGCGCAGC-3′) primers to introduce L387S substitution into A and C-derived vectors (as template served MCAS(A)-right GFP and pMCAS(C)-right GFP, respectively) and forward (5′-GCTGCGCAAGCCTCAAGAGAAATCGAG-3′) and reverse (5′-CTCGATTTCTCTTGAGGCTTGCGCAGC-3′) primers for introducing L387S substitution into K-derived vector (as template served pMCAS(K)-right GFP). To introduce A398T substitution, we have used forward (5′-GGTCCGTTAAACAGACTAACTTGACAACATC-3′) and reverse (5′-GATGTTGTCAAGTTAGTCTGTTTAACGGACC-3′) primers for A, C, and K-derived vectors, with respective templates. All respective fragments were mixed and underwent the second step of two-step Phison PCR reaction under the following conditions: 98 °C for 30 s, 15 cycles of 98 °C for 15 s, 60 °C for 30 s, and 65 °C for 3 min, and terminal extension at 72 °C for 5 min. All obtained fragments were purified by standard EtOH protocol, digested by SmaI-SacI together with respective vectors, purified by LMP agarose gel, and ligated by T4 ligase. To introduce multiple substitutions, multiple steps were repeated with different primers.

Construction of pMCAS vector with multiple cloning site and P2A self-cleaving peptide

To construct MCAS based vector with neomycin resistance gene, we have introduced multiple cloning site (containing ClaI, MreI, NotI, AsiSI, SpeI, AscI, PmeI and SgrI unique restriction sites) using forward (5'-ATCGATCGCCGGCGGCCGCGATCGCACTAGTGGCGCGCCGTTTAAACGTCGACGAT-3') and reverse (5'-TAGCAGCTGCAAATTTGCCGCGCGGTGATCACGCTAGCGCCGGCGGCCGCTAGCTA-3') oligomers, which were hybridised digested by ClaI together with pMCAS(B)-right GFP and ligated by T4 ligase, creating pMCASCS(B)-right GFP To add P2A self-cleaving peptide, we have employed Phusion PCR protocol using pMCASCS(B)-right GFP as template and

forward (5′-TCTGTGTTCTGCAGGGCAGCATGGTGAGCAAGGGCGA-3') and reverse (5′-GGCCGCCGGCGATCGATGGGTCCCGGATTCTCTTCCACATCTCCAGCCTGCTTGAG CAATGAAAAATTGGTTGCTCCTGACCCCTTGTACAGCTCGTCCATGCCGA-3') primers under following conditions: 98 °C for 30 s, 3 cycles of 98 °C for 15 s, 65 °C for 30 s, and 68 °C for 4 min, 25 cycles of 98 °C for 15 s, 65 °C for 30 s, and 72 °C for 4 min, and terminal extension at 72 °C for 10 min. The amplified fragment was introduced into pMCASCS(B)-right GFP digested by BamHI using In-Fusion HD cloning kit (Clontech), producing pMCASII(B)-right GFP. For introducing neo gene, we have employed Phusion PCR protocol using forward (5′-CCCATCGATCAAGATGGATTGCACGCAGGTTCT-3') and reverse (5′-AGCGGCCGCTCAGAAGAACTCGTCAAGAAGGCG-3') primers, introducing ClaI and NotI restriction sites (underlined) into the amplified fragment. We used pcDNA3 plasmid (Invitrogen) as a template and PCR was performed under the following conditions: 98 °C for 30 s, 35 cycles of 98 °C for 15 s, 60 °C for 30 s, and 65 °C for 2 min, and terminal extension at 72 °C for 5 min. We have purified amplified fragments by standard EtOH protocol and digested them together with pMCAS(B)II-right-GFP vector using ClaI-NotI. Both were purified by LMP agarose gel procedure and ligated by T4 ligase. Vector then served as a template for site-directed mutagenesis as described above.

Comparative analysis of MAV-2.O and selected ALV strains

Genomic DNA of osteopetrotic bones was obtained by freezing the samples in liquid nitrogen, dusting, mixed with DNA lysis solution (1% SDS, 250 mmol/L EDTA, and 1 mg/mL proteinase K) and incubated 50°C o/n. Solution was extracted by phenol-chloroform (1:1), DNA containing phase was mixed with 96% EtOH, spun, washed by 80% EtOH, and resuspended in TE buffer (10 mmol/L Tris-Cl (pH 8.3) and 1 mmol/L EDTA). Extracted DNA served as a template for PCR under following conditions: AccuTaq polymerase (Sigma) with AccuTaq buffer supplemented with 500 µmol/l of deoxynucleotide triphosphates was mixed with forward (5′-TGTAGTCTTAATCATAGGTTAACATGTA-3') and reverse primer (5′-CAGAATACTCCCTCTAACCATAGCCACTGCG-3') for amplification of LTR-gag region, forward (5′-ATCCATGGGATAGGAGGGGGAATT-3') and reverse primer (5′-TGCTGGTCTGGGGACGAGGTTATGC-3') for amplification of pol region and forward (5′-CTATTAGCCAAGGCAGTGTATGCCCTTAAT-3') and reverse (5′-CAAATAAGGGAATCGCCTGATGCACC) primers for amplification of env-LTR region. Fragments were purified by EtOH standard protocol. Samples were sequenced using Sanger sequencing protocol by Seqme with following primers: (5′-TGTAGTCTTAATCATAGGTTAACATGTA-3'), (5′-ACTCCCCGGGGTCCTGGGATC-3') and (5′-TAACTTGGATCGCTTAAAGGGTCTAG-3') for sequencing of LTR-gag fragment, (5′-ATCCATGGGATAGGAGGGGGAATT), (5′-GTACGTATGTAGCACCCGTAGG-3') and (5′-TGCTGTGGCCGACAACGCCC-3') for sequencing pol region and (5′-CTATTAGCCAAGGCAGTGTATGCCCTTAAT-3'), (5′-ACTCACCGGTTAGTCTCGAGAGGT-3') and 5′-(AGCTTACCATGTTAGCACCCAACC-3') for sequencing of env-LTR region. Obtained sequences were compared to MQNCSU (GenBank accession no. DQ365814.1), LR-9 (GenBank accession no. AY350569.1), Pr-C (GenBank accession no. V01197.1), RSA (GenBank accession no. M37980.1), MAV-2 (GenBank accession no. L10924.1), and MAV-2.O and MAV-1/2.O as presented by (Joliot et al. 1993).

Infection of cells with MAV-2.O-derived substitutions vectors

Cells were analysed using flow cytometry. Cells were seeded at a density of 5×10^4 per well in a 24-well plate. Eight hours after seeding, the cells were infected with 100 µl (original protocol) or with 1 ml (adjusted protocol) of the virus-containing medium. Cells were harvested by PBS wash and trypsinization two days later The percentage of GFP+ cells was quantified by fluorescence-activated cells sorting (FACS) using an LSRII analyzer (Becton, Dickinson).

Temperature-Sensitivity Assay of vectors

Virus stocks in the culture medium were aliquoted into three samples, which were incubated for 0, 12, or 24 hours at 44 °C and transferred to ice. DF-1 cells were infected as described above. three days later, DF-1 cells were harvested and analyzed for GFP expression by flow cytometry. Same setup was used in the case of incubation of virus stocks at 33°C, 37°C or 40°C.

Results

ALV-K subgroup definition and Tvk identification

RCAS(JS11C1)GFP host range

To investigate the host range of postulated subgroup K (Wang X., Zhao, and Cui 2012), we cloned JS11C1 ALV strain env into an ALV-based RCAS vector encoding green fluorescent protein (GFP). Env was synthesized according to the published sequence (GenBank accession no. KF746200) and replaced the env gene of RCAS(D)GFP, resulting in RCAS(JS11C1)GFP. Virus titer of $10^{\wedge 6}$ infectious units (UI) per ml was produced by transfection of DF-1 cells. Replication competence of the resulting virus was confirmed by the virus spread in DF-1 cells and increased percentage of GFP-positive cells.

In order to delineate the host range of JS11C1, we cultured and infected embryo fibroblasts of multiple galliform birds to test them for susceptibility or resistance to the JS11C1. Our panel contained species susceptible or resistant to subgroups A-E and J which enables comparison of the host ranges. We infected cells with RCAS(A)GFP in parallel as a control. As expected, cells were infected with different efficiency by both viruses, with highest rates in the case of QT6 cells derived from Japanese quail, then gradually lowering for both viruses. Three species of New World quails, ring-neck pheasant and gray partridge, displayed the lowest positivity for virus infection in the case of RCAS(JS11C1)GFP and lowest to none positivity in the case of RCAS(A)GFP. As previously described, New World quails bear a W48S substitution in the Tva receptor, probably disrupting the subgroup A virus binding site, thus rendering quails resistant. A similar effect was observed in the case of red jungle fowl, as cells were susceptible to RCAS(JS11C1)GFP infection while nearly resistant to RCAS(A)GFP. The resistance of red jungle fowl was explained previously by intronic deletion of the branch point signal in the Tva receptor lowering the expression of the protein (Reinišová et al. 2012). Guinea fowl exhibited a reverse pattern, as derived cells were highly positive for infection of RCAS(A)GFP, but completely resistant to RCAS(JS11C1)GFP. Embryo fibroblasts derived from the domestic duck were resistant to both viruses.

We also investigated embryo fibroblasts derived from inbred lines, as their receptors for ALV viruses had been already characterized. We observed infection in lines WA, L15 and H6, though RCAS(JS11C1)GFP infected 2-10× more cells than RCAS(A)GFP. In case of M and CB lines, we observed low to none infection by RCAS(JS11C1)GFP and nearly complete resistance to infection by RCAS(A)GFP. These findings were in accordance with the intronic deletion found in red jungle fowl and C40W substitution, as described previously.

In summary, the host range of RCAS(JS11C1)GFP differs from that of other subgroups, as compared to RCAS(A)GFP in our experiment or to other subgroups from previous studies. At the same time, we observed similarities in the host range of the viruses analyzed in our study and similar reaction to the Tva depletion or structure changes, suggesting that RCAS(J11C1)GFP may utilize the same receptor as the subgroup A viruses.

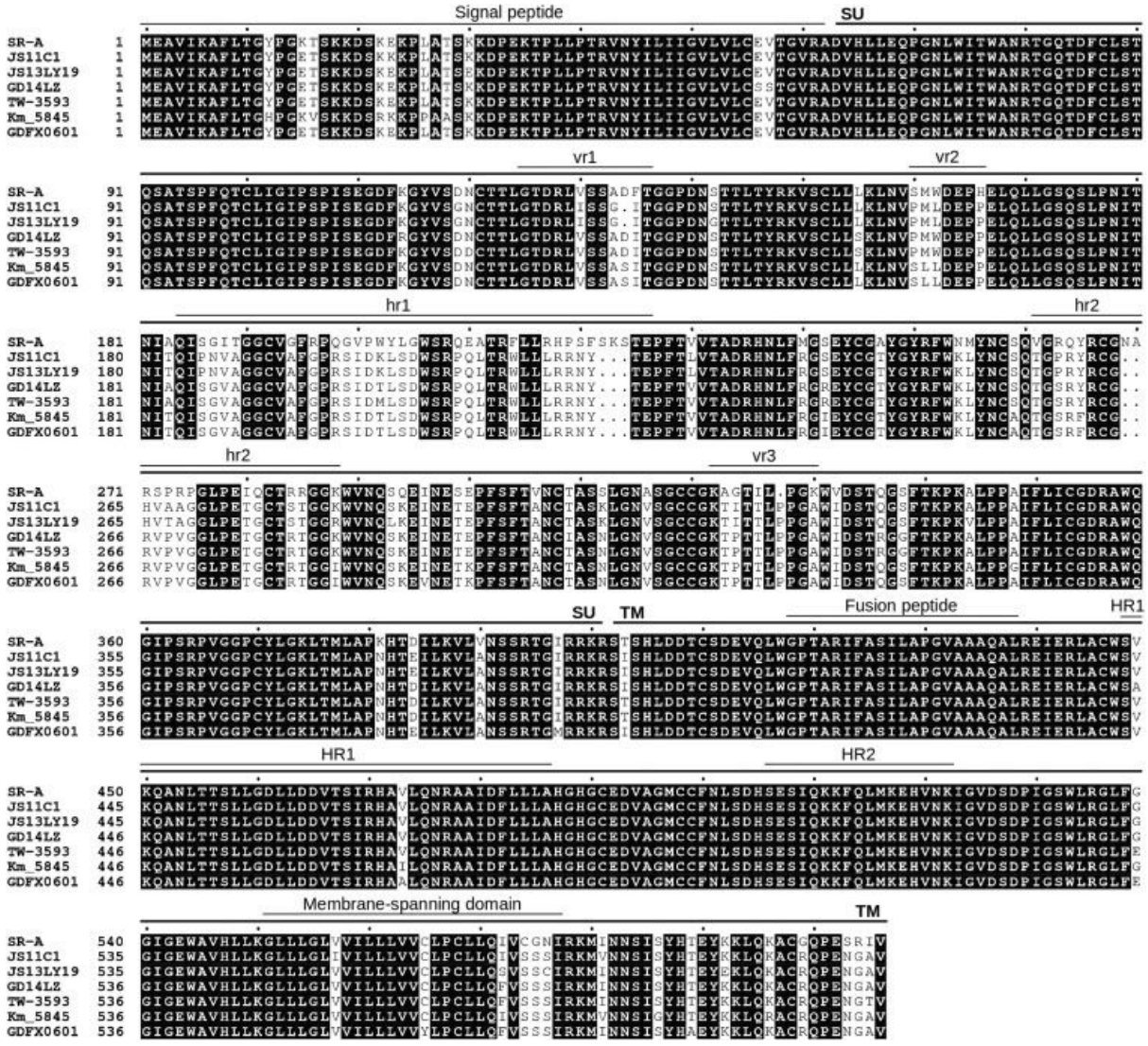


Figure A1.: Alignment of the amino acid sequences of ALV subgroup A and subgroup K envelope glycoproteins

Amino acid sequences deduced from the available GenBank data are shown. Subgroup A is represented by the Schmidt-Ruppin A (SR-A) strain (GenBank accession no. NP040548), and subgroup K is represented by the Chinese and Taiwanese nonrecombinant strains JS11C1 (GenBank accession no. KF746200), JS13LY19 (GenBank accession no. AWO14321), GD14LZ (GenBank accession no. ANW72067), TW-3593 (GenBank accession no. ADP21276), Km_5845 (GenBank accession no. BAL70358), and GDFX0601 (GenBank accession no. AKP18446). Identical amino acids are highlighted in black. Gaps are indicated by dots. The main functional domains of envelope glycoproteins are delineated above the sequence. SU, surface subunit; TM, transmembrane subunit; vr1, vr2, and vr3, variable regions 1 to 3; hr1 and hr2, hypervariable regions 1 and 2; HR1 and HR2, heptad repeats 1 and 2. (Přikryl et al. 2019)

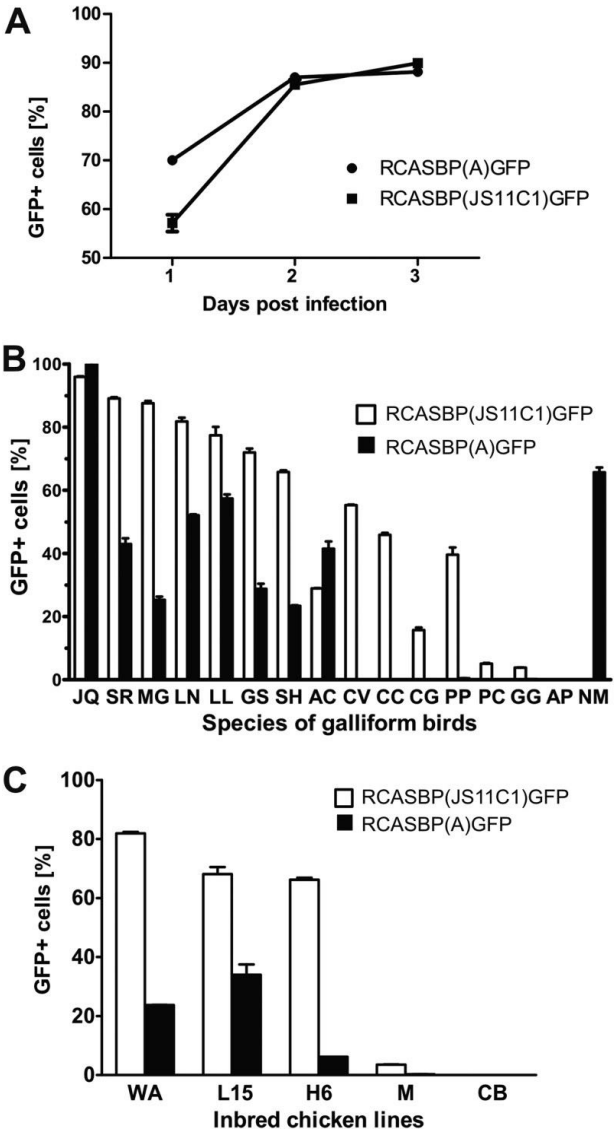


Figure A2.: Host range of RCASBP(JS11C1)GFP in galliform species and inbred lines of domestic chickens

(A) Time courses of RCASBP(JS11C1)GFP and RCASBP(A)GFP infection in DF-1 cells. The percentages of GFP-positive cells in the three consecutive days after infection are shown as means and standard deviations of triplicate assays. (B) Efficiency of RCASBP(JS11C1)GFP and RCASBP(A)GFP infection, measured as the percentages of GFP-positive cells three days after infection for cultured embryo fibroblasts of 14 galliform species (Reeve's pheasant [SR], turkey [MG], silver pheasant [LN], white-crested kalij pheasant [LL], gray jungle fowl [GS], Mrs. Hume's pheasant [SH], chukar [AC], northern bobwhite [CV], California quail [CC], Gambel's quail [CG], gray partridge [PP], ringed-neck pheasant [PC], red jungle fowl [GG], and guinea fowl [NM]), QT6 cells representing the Japanese quail (JQ), and embryo fibroblasts of the domestic duck (AP). Results are shown as means ± standard deviations of duplicate assays. (C) Efficiency of RCASBP(JS11C1)GFP and RCASBP(A)GFP infection in cultured embryo fibroblasts of five inbred chicken lines. Results are shown as means ± standard deviations of duplicate assays. (Přikryl et al. 2019)

RCAS(JS11C1)GFP interferes with the A subgroup

We substituted GFP for red fluorescent marker dsRed in our RCAS vectors creating RCAS(A)dsRed and RCAS(JS11C1)dsRed. Vectors encoding different fluorescent dyes enable examination of infection of cells pre-infected with different viruses using FACS to analyze GFP-positive, dsRed-positive, and GFP/dsRed-double-positive cells.

We pre-infected DF-1 cells with RCAS(A)GFP, RCAS(B)GFP, and RCAS(J)GFP in parallel and superinfected the cells with RCAS(A)dsRed or RCAS(JS11C1)dsRed after one week. Preinfection of the cells resulted in about 85% GFP-positive cells for subgroup A, about 100% for subgroup B and about 50% for subgroup J. The inability of RCAS(A)GFP and RCAS(J)GFP to infect all cells may be due to the fact that GFP-defective virus emerged during the infection using low multiplicity of infection (MOI). Superinfection by subgroups A and K resulted in the absence of dsRed- or double-positive cells pre-infected by subgroup A, roughly 100% of double-positive cells pre-infected by subgroup B and 50% of dsRed-positive and 50% of double-positive cells pre-infected by subgroup J.

As superinfection of subgroups A and K gave the same result, strong interference with subgroup A preinfection, we suggest that they belong to the same interference group, sharing the same receptor.

Tva is sufficient for RCAS(JS11C1)GFP infection

As we observed a similar host range and interference of RCAS(A)GFP and RCAS(JS11C1)GFP, we tested whether expression of ectopic Tva in cells of resistant species is sufficient for RCAS(JS11C1)GFP infection.

We stably transfected NIL-2 cells of Syrian hamster (species resistant to all ALV subgroups) with chicken Tva or Tvc in parallel as a control. NIL-Tva and NIL-Tvc clones, together with the control chicken DF-1 cell line, were infected with RCAS(A)GFP, RCAS(JS11C1)GFP, RCAS(B)GFP and RCAS(C)GFP. DF-1 cells were infected with similar efficiency by all viruses (77-95% of GFP-positive cells). NIL-Tva cells were susceptible to RCAS(A)GFP and RCAS(JS11C1)GFP but resistant to RCAS(B)GFP and RCAS(C)GFP. Finally, NIL-Tvc cells were susceptible only to RCAS(C)GFP and resistance to the rest of the viruses.

As an expression of Tva rendered resistant NIL-2 cells susceptible, we conclude that Tva is sufficient for the RCAS(JS11C1)GFP infection.

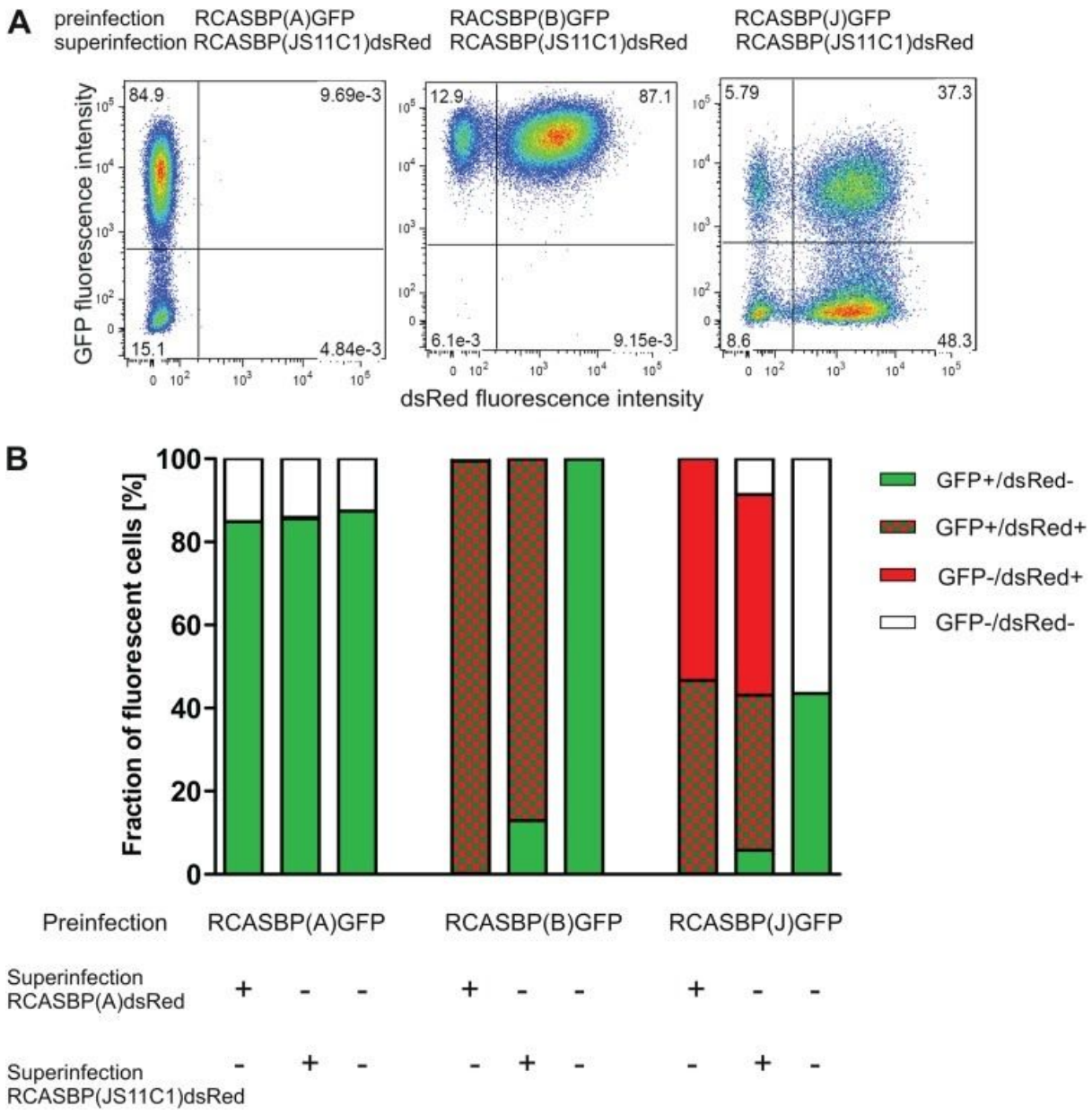


Figure A3.: Superinfection interference between ALV-A and ALV-K subgroups
(A) Results of interference experiments shown as FACS dot plots. Preinfection of DF-1 cells with RCASBP(A)GFP blocks superinfection with RCASBP(JS11C1)dsRed (left), whereas preinfection with RCASBP(B)GFP (middle) or RCASBP(J)GFP (right) permits subsequent superinfection with RCASBP(JS11C1)dsRed. Horizontal axes, dsRed positivity; vertical axes, GFP positivity. (B) The efficiency of superinfection interference between RCASBP(A)GFP, RCASBP(B)GFP, and RCASBP(J)GFP as preinfection viruses and RCASBP(A)dsRed and RCASBP(JS11C1)dsRed as superinfection viruses. The results are shown as mean percentages of GFP-negative/dsRed-negative, GFP-positive/dsRed-negative, GFP-negative/dsRed-positive, and GFP-positive/dsRed-positive cells, measured in triplicate. (Přikryl et al. 2019)

Tva is required for RCAS(JS11C1)GFP infection

Having demonstrated that the ALV-A receptor, Tva, is sufficient for RCAS(JS11C1)GFP infection, we tested the effect of its depletion in chicken cells.

We employed the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system to prepare chicken DF-1 cells bearing homozygous frameshift deletion within the *tva* gene, resulting in the DF-1-tva-/- cell clone. We infected this clone with RCAS(A)GFP, RCAS(JS11C1)GFP, RCAS(B)GFP and RCAS(C)GFP. While we observed a high level of GFP-positive cells of the DF-1-tva-/- cell clone infected by RCAS(B)GFP and RCAS(C)GFP, we observed none infected by both RCAS(A)GFP and RCAS(JS11C1)GFP.

Taken together with previous findings of the similar host range, interference, sufficiency of Tva, and now necessity of Tva for RCAS(A)GFP and RCAS(JS11C1)GFP entry, we conclude that they share the same receptor while being separate subgroups, supporting the establishment of novel subgroup K represented by the JS11C1 isolate.

Soluble form of tva receptor interferes with RCAS(JS11C1)GFP infection

In order to investigate the direct interaction of the Tva receptor with the JS11C1 envelope, we used its soluble form (sTva) fused with a mouse immunoglobulin heavy chain (mIgG), termed sTva-mIgG, as described previously.

Both RCAS(JS11C1)GFP and RCAS(A)GFP viruses were incubated with sTva-mIgG or mock control and then used for infection of naive DF-1 cells. Incubation of sTva-mIgG with RCAS(A)GFP resulted in total abrogation of infection, while incubation with RCAS(JS11C1)GFP resulted only in 77% inhibition of infection. To rule out the possible nonspecific interaction with mIgG, we repeated the experiment employing the soluble form of ALV-C subgroup receptor Tvc (sTvc) fused with mIgG (sTvc-mIgG).

Incubation of sTvc-mIgG with RCAS(A)GFP or RCAS(JS11C1)GFP resulted in no inhibition of infection of naive DF-1 cells, showing that both RCAS(A)GFP and RCAS(JS11C1)GFP interact only with the ALV-A receptor, Tva, bringing additional evidence to sharing the same receptor.

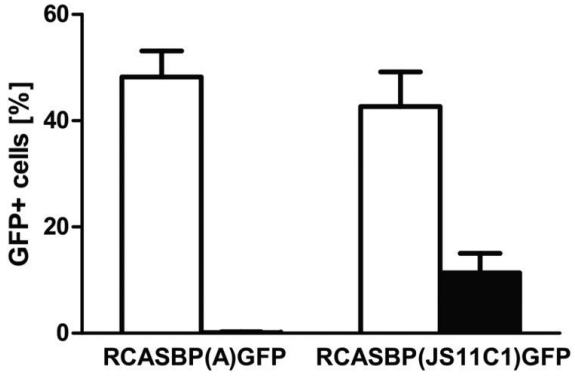


Figure A4.: Soluble Tva interference with ALV-A and ALV-K infection in susceptible cells

RCASBP(A)GFP and RCASBP(JS11C1)GFP viruses were preincubated (filled columns) or mock-incubated (empty columns) with sTva-mIgG immunoadhesin and used for infection of DF-1 cells. Infection efficiencies are shown as means ± standard deviations of percentages of GFP-positive cells; the experiment was performed in triplicate. (Přikryl et al. 2019)

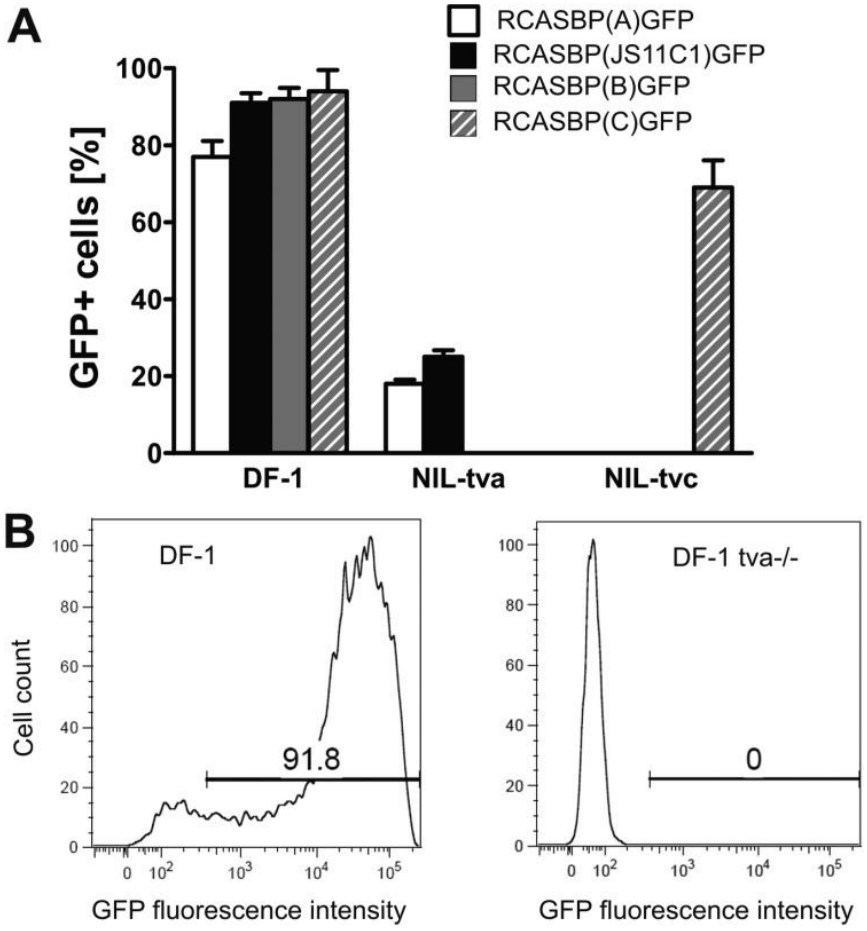


Figure A5.: Expression of the *tva* gene is sufficient and necessary for RCASBP(JS11C1)GFP infection

(A) Efficiency of RCASBP(JS11C1)GFP and RCASBP(A)GFP infection in the Syrian hamster cell line NIL-2 ectopically expressing the *tva* gene (NIL-Tva). DF-1 cells were used as a susceptible control. Viruses with B and C specificity, i.e., RCASBP(B)GFP and RCASBP(C)GFP, respectively, and NIL-2 cells ectopically expressing the *tvc* gene were used as independent controls. Infection efficiencies are shown as percentages of GFP-positive cells and are presented as means ± standard deviations of triplicate assays. (B) The efficiency of RCASBP(JS11C1)GFP infection in intact DF-1 cells (left) and DF-1 cells with a frameshift mutation in the *tva* gene (right), presented as FACS histograms of GFP-negative and GFP-positive cells. The relative GFP fluorescence is plotted against the cell count, and the percentage of GFP-positive cells is indicated. Typical results from triplicate assays are shown. (Přikryl et al. 2019)

RCAS(JS11C1)GFP induced pathology and lethality

To investigate the pathology of the newly emerging subgroup, we inoculated 17 and 16 brown leghorn embryos 12 days post fertilization with either RCAS(A)GFP or RCAS(JS11C1)GFP, respectively. Hatched chickens were maintained till sudden death, in case of severe pathology or termination of experiment, they were euthanized. Corpses were dissected with any abnormalities in morphology or neoplasia recorded.

We observed rapid mortality of chickens infected with RCAS(JS11C1)GFP, culminating between days 10 and 23, with a total of 10 dead chickens coupled with the stunning disease of the remaining chickens. Dissected animals displayed hemorrhage in the liver and muscles, hypertrophied heart and disruption of bone marrow integrity. The rest of the surviving chickens (7) were euthanized. Mortality induced by RCAS(A)GFP culminated between days 19 and 46, with a total of four dead chickens. One of the dissected animals displayed disruption of the integrity of bone marrow and some suffered from ascites in the abdomen (data not shown).

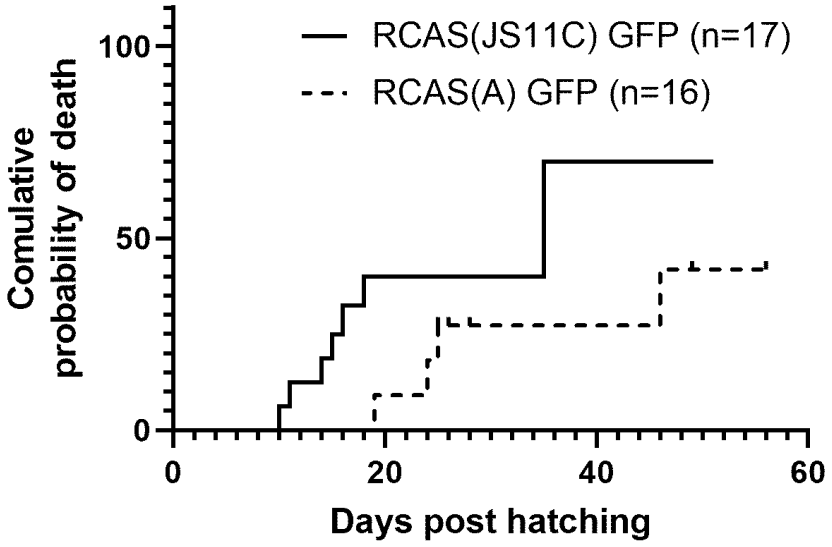


Figure A6.: Subgroup K induces rapid death in infected animals

We inoculated 12-day-old embryos with RCAS(JS11C1) GFP or RCAS(A) GFP virus stocks and observed differing lethality. While more than half of RCAS(JS11C1) GFP-infected chickens rapidly died, RCAS(A) GFP-infected chickens showed lethality of about 40% and the first death was observed on day 19 (compared to day 10 of RCAS(JS11C1) GFP-infected animals).

Mechanism of the host range extension

RSV passage through mammalian cells induces env adaptation and host range extension

To tackle the question of env-based host range extension, we analyzed the env sequence of PR-RSV-C passaged twice through mammals and compared it to the wt strain. The first passage was done by injection of tumor (generated by injection of PR-RSV-C into a chicken embryo) mince into rats, resulting in growth of a single tumor, labeled XC tumor and containing the XC-RSV strain. The second passage was done by injection of tumor (generated by injection of XC-RSV into a chicken embryo) mince into a Syrian hamster, resulting in growth of a single tumor, denoted H-20, containing the H20-RSV provirus.

We identified eight amino acid substitutions in H20-RSV env sequence compared to wt PR-RSV-C, member of subgroup C. We also analyzed the XC-RSV env sequence and again observed all eight substitutions in all acquired env sequences in concert with H20-RSV, although the XC tumor contained multiple proviruses, in contrast to the H-20 tumor containing a single provirus. All found substitutions are dispersed over the whole env gene, some are located at the fusion peptide and heptad repeat regions, regions responsible for fusion of virus and host membrane with previously identified substitutions affecting the fusion machinery (see figure C1).

As mammalian cells are normally nonpermissive for ALV because they lack specific receptors used by ALV subgroups, we infected Syrian hamster cell line NIL-2 and its derived cell line NIL-Tvc expressing the C subgroup-specific receptor, Tvc, by wt PR-RSV-C and H20-RSV and measured the amount of proviral DNA at different times. We observed similar amounts of proviral DNA in NIL-Tvc infected by both viruses. However, we observed nearly a two-log decrease of signal in NIL-2 cells infected by H20-RSV and more than a three-log decrease of signal in NIL-2 cells infected by PR-RSV-C. In all cases, we observed a decrease of signal over time in the infected cells.

Since we detected a higher amount of H20-RSV DNA compared to PR-RSV-C DNA in the NIL-2 cell line, while the amounts of both viruses in the cells expressing the receptor is similar, we conclude that the H20-RSV strain exhibits an extended host range while retaining its original affinity to the Tvc receptor.

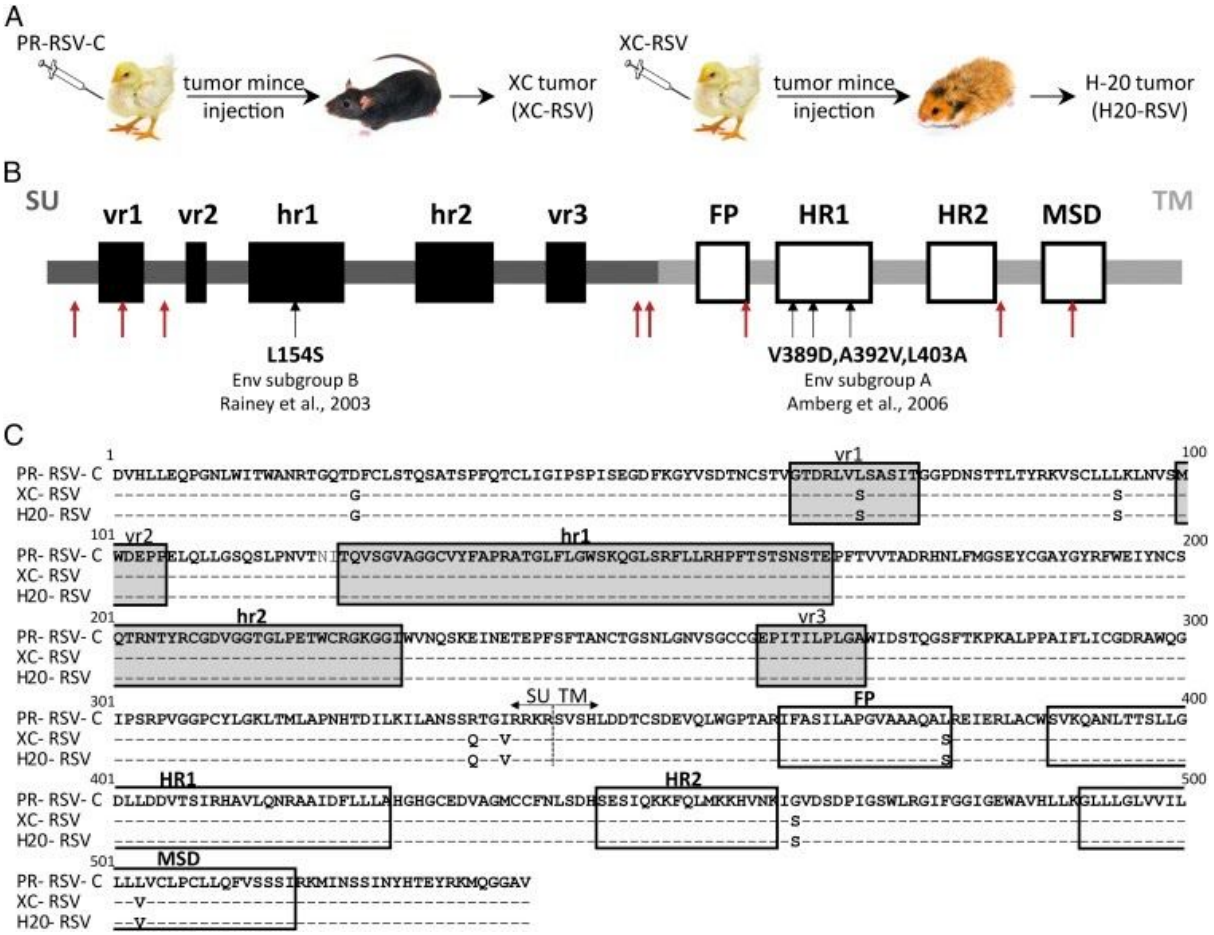


Figure C1.: Virus transmission to mammalian cells and envelope glycoprotein alteration

(A) Scheme of rat and hamster infection with original PR-RSV-C virus. The PR-RSV-C virus was injected into a chicken, the resulting tumor was minced and injected into a rat, which developed a tumor caused by XC-RSV. The virus rescued from XC cells was again multiplied in a chicken and injected into a Syrian hamster, where the H20 tumor harboring H20-RSV was obtained (for details see Materials and Methods). (B) Diagram of the envelope glycoprotein domain structure with positions of described mutations responsible for mammalian tropism (black arrows) (Rainey et al. 2003; Amberg et al. 2006) and found amino acid mutations (red arrows). Host-range regions (hr1-2) and variable regions (vr1-3) in the surface subunit (SU) are depicted in gray boxes. The fusion peptide (FP), heptad repeats (HR1-2), and membrane-spanning domain (MSD) in the transmembrane subunit (TM) are shown in white boxes. (C) Comparison of gp85 amino acid sequences from original parental virus PR-RSV-C (sequence from GenBank V01197.1) (Schwartz, Tizard, and Gilbert 1983) and viruses rescued from RSV-transformed rat (XC) or hamster (H20) cells. h and vr regions in SU are depicted in gray boxes. FP, HR1, HR2, and MSD in TM are shown in white boxes. Their position is depicted according to refs. (Aydin, Smrke, and Lee 2013; Bova, Olsen, and Swanstrom 1988). (Lounková et al. 2017)

Analysis of amino acid substitutions in H20 env

For further analysis of H20-RSV env substitutions, we substituted RCAS(C)GFP env for H20-RSV env, creating RCAS(H20)GFP. To confirm the ability of H20-RSV env to enter mammalian cell lines is untouched in the new vector, we repeated the experiment described above using the new vector. While we observed a high amount of GFP-positive NIL-Tvc cells infected by both viruses, we detected GFP-positive NIL-2 cells infected only by RCAS(H20)GFP, though the signal was decreased by two logs, as observed in the previous experiment (figure C2).

To investigate the role of individual substitutions, we divided the H20-RSV env into four regions using unique restriction sites and replaced the corresponding regions of RCAS(C)GFP. The following infection showed that only vectors bearing region 1 (containing D23G substitution) or 4 (containing L378S, G464S and L503V substitutions) can infect the NIL-2 cell line, as we observed GFP signal above the limit of detection.

As region 4 contains three mutations, we used site-directed mutagenesis to introduce these mutations into RCAS(C)GFP. The following infection showed that only L378S substitution, located at the end of the fusion peptide, was sufficient to promote entry into Syrian hamster cells.

To further investigate the effect of D23G and L378S substitutions on mammalian tropism, we infected human embryonic kidney cells (HEK293) and human telomerase reverse transcriptase (hTERT)-immortalized retinal pigment epithelial cells (RPE1-hTERT). Despite the fact that HEK293 cells were significantly more sensitive to infection with RCAS(C)GFP compared to NIL-2 and RPE1-hTERT, RCAS vectors containing the whole H20-RSV env, region 1, region 4, or L378S substitution showed the highest infection efficiency (figure C3).

Furthermore, we confirmed the same results using a chicken cell line lacking Tvc receptor L15, with the highest infection efficiency mediated by the whole H20-RSV env, region 4, L378S, or region 1, respectively.

In summary, both D23G and L378S enable virus entry into mammalian cells, both in concert or separately.

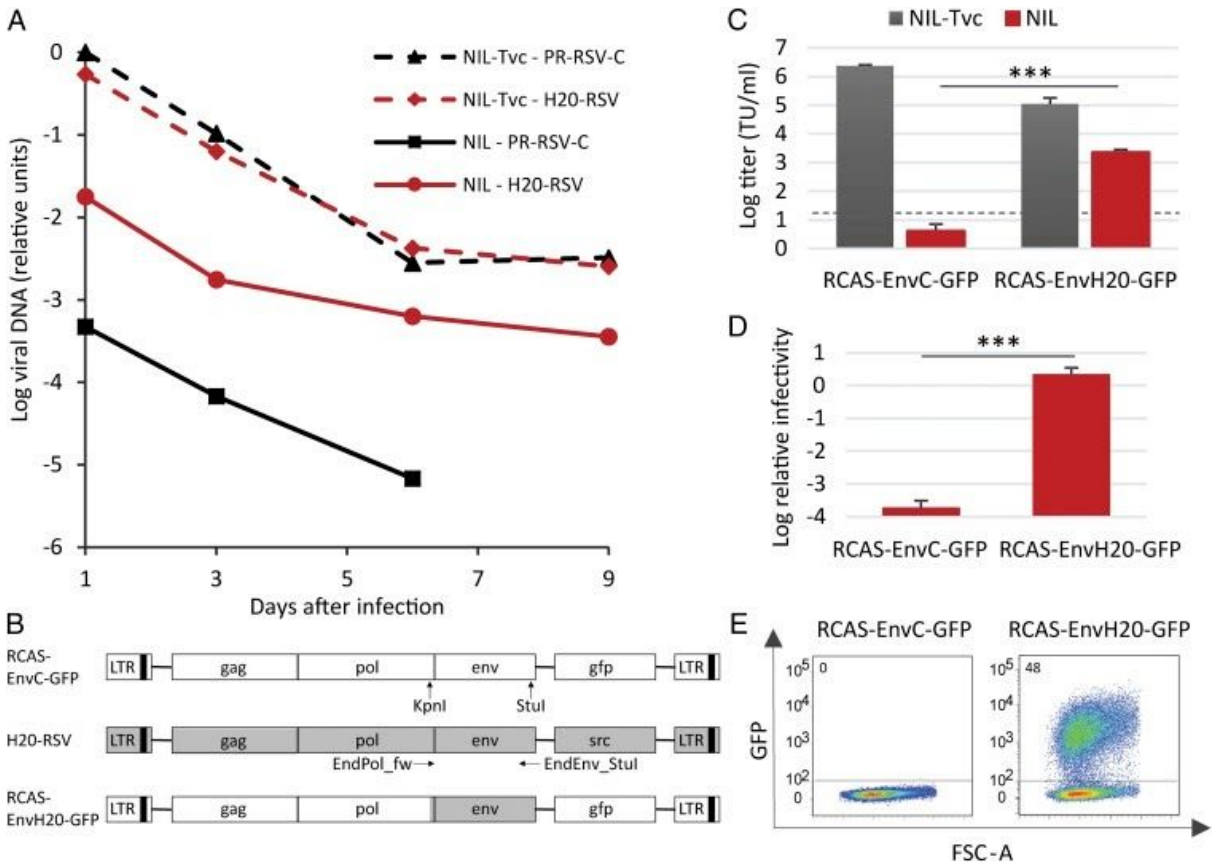


Figure C2.: Viruses equipped with EnvH20 are able to infect hamster cells

(A) The amount of newly produced *env* viral DNA in infected cells (NIL or NIL-Tvc) was quantified at different time points post-infection. The results were normalized to chGAPDH expression and are presented relative to the sample of NIL-Tvc-infected PR-RSV-C on day 1. (B) Schematic diagram of chimeric reporter vector RCAS-EnvH20-GFP prepared from RCAS-EnvC-GFP and H20-RSV virus. Restriction sites and the position of primers used for the *env* gene substitution are depicted. (C) Infection of NIL and NIL-Tvc cells with RCAS-GFP viruses containing EnvC or EnvH20 was scored by flow cytometry 3 d later. Titers in GFP-transducing units were determined as described in Materials and Methods. Error bars show the SD of two independent experiments in parallel. The limit of detection (signal from uninfected NIL cells) is marked with a dashed line. Significant differences are marked by asterisks (***P < 0.001). (D) Relative infectivity of NIL cells infected with RCAS-EnvC-GFP and RCAS-EnvH20-GFP. Infectivity is expressed as a percentage of the viral titer in NIL-Tvc cells. (E) Examples of FACS diagrams showing the percentages of GFP+ cells after infection of NIL cells with RCAS-EnvC-GFP and RCAS-EnvH20-GFP viruses. (Lounková et al. 2017)

Premature activation of H20 env

We observed significant loss of infectivity of RCAS(H20)GFP in our experiments with cells expressing the Tvc receptor compared to RCAS(C)GFP. This phenomenon can be explained either by lower production of virions or by decreased infectivity of the produced virions. To challenge this question, we measured reverse transcriptase activity and virus-like particle formation and found very small differences when comparing the viruses; therefore, we excluded the possibility of altered virion production and concentrated solely on the possibility of decreased virus infectivity (figure C4).

Differences in infectivity in our RCAS-based system can be explained only by different stability or conformation of the respective env constructs. We tested the stability of RCAS(C)GFP and RCAS(H20)GFP envs by incubating viruses at 44°C at different times. Analysis of infected cells by both FACS and qPCR revealed a decreasing signal in cells infected by RCAS(H20)GFP. As env is relatively stable to heat and pH until it is primed by a receptor, we also tested its sensitivity to low pH. While RCAS(C)GFP was nearly completely resistant to pH 5, RCAS(H20)GFP was inhibited more than four-log.

As our observation suggested that RCAS(H20)GFP env is in its receptor-primed state regardless of the receptor, we exploited biochemical assays to test the conformations of the TM subunit of envs. First, we analyzed them by SDS/PAGE at both neutral and low pH. We detected the signal for the non-primed state (approximately 30 kDa) in all samples, but the signal for the primed state (70-150 kDa) was observed only in samples containing TM subunits isolated from RCAS(H20)GFP, both at neutral or low pH. In our next experiment, we showed that RCAS(H20)GFP is able to bind liposomes regardless of the presence of a receptor, unlike RCAS(C)GFP. After mixing virions and liposomes and using density gradient centrifugation, we detected signals of virions at the bottom, while the signal of RCAS(H20)GFP was also detected at the top of the gradient since the liposomes could not be spun down and remained at top of the gradient. Virion-liposome complexes could be explained by exposure of the fusion peptide of the env TM subunit. To confirm that, we incubated virions with thiol-specific alkylating reagent PEG-maleimide-biotin (PMB), as the maleimide group reacts specifically with reduced thiols to form stable thioether bonds. It was shown that PMB selectively inhibits virions in a receptor-primed state. While RCAS(C)GFP was inhibited only by a high dosage (10mM) of PMB, RCAS(H20)GFP was fairly inhibited by a lower dosage (1mM) and nearly completely by a high dosage (10mM) of PMB.

Taken together, our results suggest that env of H20-RSV has similar features to receptor-primed env.

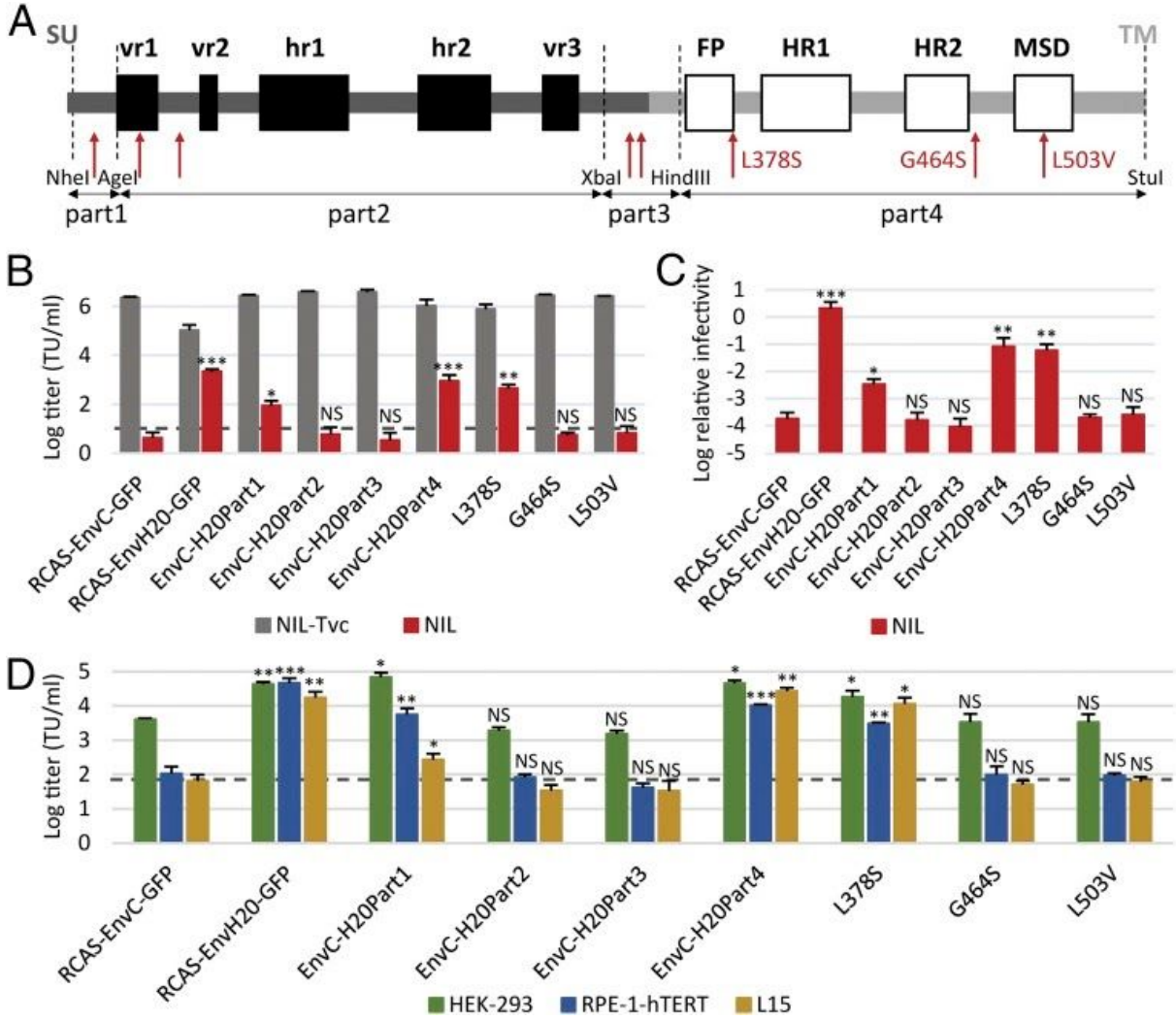


Figure C3.: Mutations in the first part of SU (D23G) and in the fusion peptide (L378S) are responsible for the virus-extended host range

(A) Schematic diagram showing restriction sites (dashed lines) that were used to divide EnvH20 into four parts, which were tested separately as chimeras with EnvC. Positions of the found amino acid mutations are shown with red arrows. (B and D) Infection of hamster cell lines NIL and NIL-Tvc (*B*), human cell lines HEK293, RPE1-hTERT, and chicken L15 cell line (Tvc−) (D) with RCAS-GFP viruses harboring EnvC-H20 chimeras and RCAS-EnvC-GFP single mutants L378S, G464S, and G464S was scored by flow cytometry two (HEK293) or three (NIL, RPE1-hTERT, L15, NIL-Tvc) days later. Titers were determined as described in Materials and Methods. The limit of detection (signal from uninfected cells) is marked with a dashed line. (C) Relative infectivity of hamster NIL cells with different RCAS-GFP viruses is expressed as the percentage of the viral titer in NIL-Tvc cells. Error bars show the SD of two independent experiments in parallel. Significant differences are marked by asterisks (*P = 0.05–0.01, **P = 0.01–0.001, ***P < 0.001). NS, not significant. (Lounková et al. 2017)

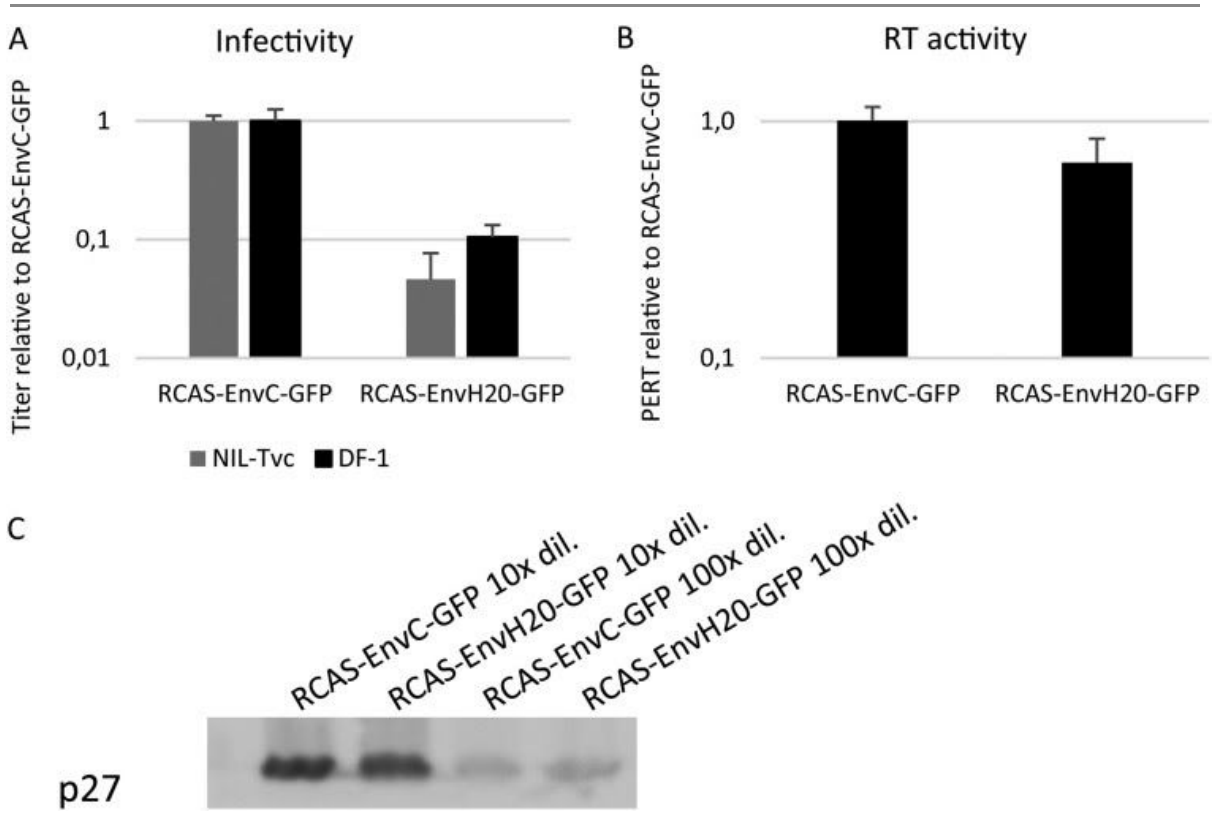


Figure C4.: Virus harboring EnvH20 has a lower titer than the virus with original EnvC despite the same RT activity and VLP formation

(A) Infection of NIL-Tvc and DF-1 cells with RCAS-EnvC-GFP and RCAS-EnvH20-GFP viruses was scored by flow cytometry two (DF-1 cell) or three (NIL-Tvc) days later. Titers were determined as described in Materials and Methods. The results are presented as titers relative to RCAS-EnvC-GFP. (B) RT activity of RCAS-EnvC and RCAS-EnvH20-GFP was measured using the PERT assay described in Materials and Methods. The results of the PERT assay are presented relative to RCAS-EnvC-GFP. (C) VLP production of DF-1 cells infected with RCAS-EnvC-GFP or RCAS-EnvH20-GFP was estimated by Western blot analysis. Expression of Gag product p27 in the medium was determined with anti-p27 antibody. (Lounková et al. 2017)

Receptor-independent entry correlates with instability of env

To compare the ability to enter mammalian cells and sensitivity to low pH and PMB inhibitor, we expanded our analysis to RCAS(C)GFP bearing previously described regions of H20-RSV env. We observed that the virus bearing D23G substitution of region 1, coupled with mild ability to enter mammalian cells, was inhibited by low pH or PMB treatment only partially. The virus bearing L378S, G464S and L503V substitutions of region 4, coupled with high ability to enter mammalian cells, was highly inhibited by low pH or PMB treatment. Regions 2 and 3 showed inhibition similar to the wt PR-RSV-C env (figure C6).
Taken together, we conclude that the level of inhibition correlates with the ability to enter Tvc null cells.

Mutations of TM activate env regardless of the subgroup

Since the most potent substitution for receptor-independent entry L378S was localized at the end of the fusion peptide of the TM subunit, we wondered whether the same substitution could promote receptor-independent entry of other subgroups. By cloning region 4 of H20-RSV env into the env of B subgroup, we created RCAS(B-H20part4)GFP and together with RCAS(B)GFP virus of subgroup B, we analyzed its ability to enter RPE1-hTERT cells and its inhibition by PMB. RCAS(B-H20part4)GFP showed similar levels of entry into mammalian cells and inhibition by PMB, which were not observed in RCAS(B)GFP (figure C7).
These results suggest that the L378S substitution of TM can mediate premature activation of env regardless of the subgroup and support our hypothesis that viruses in our study can enter the cells in a receptor-independent way instead of binding to a non-specific ubiquitous receptor present on surfaces of avian and mammalian cells.

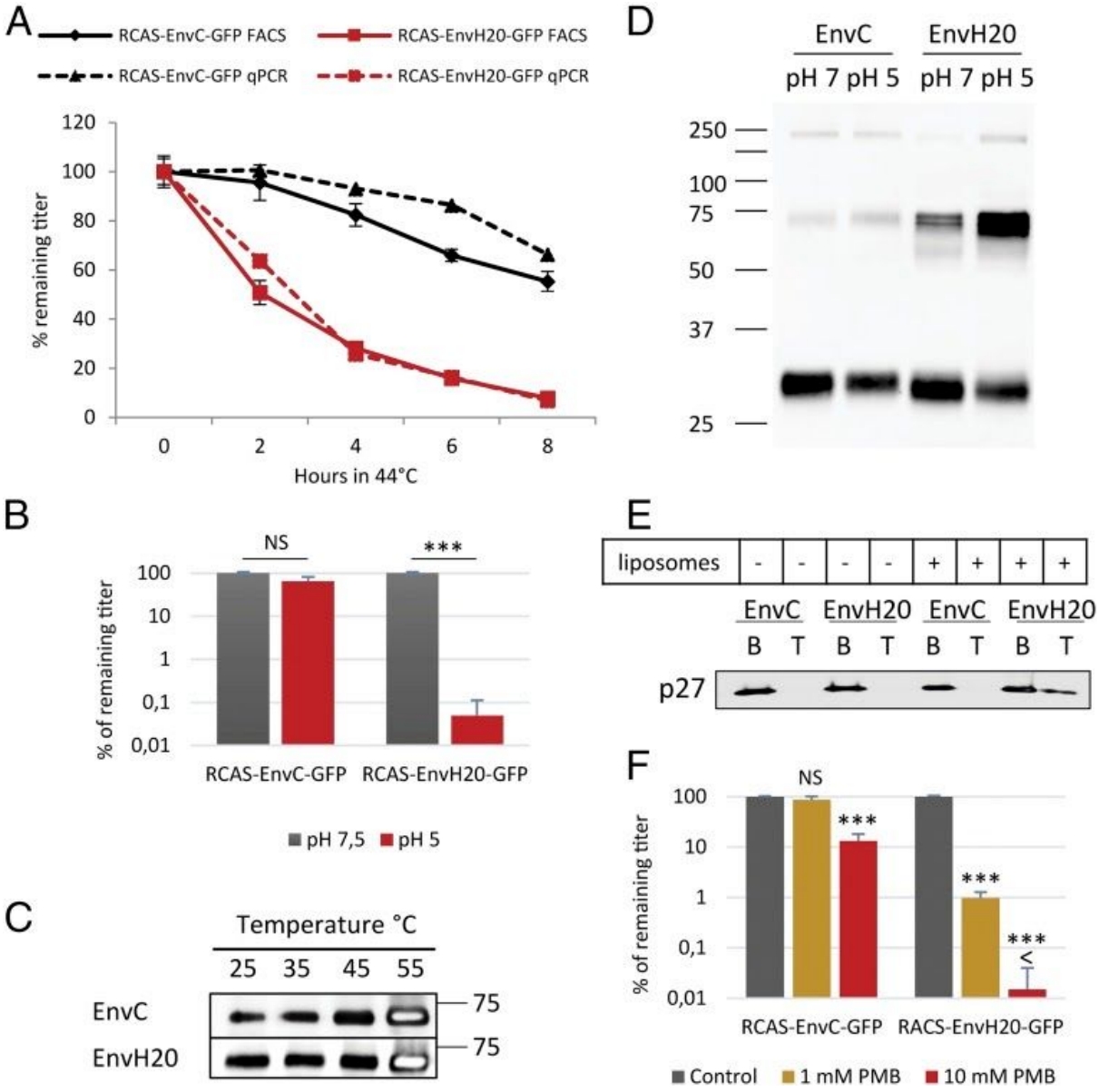


Figure C5.: EnvH20 has similar features as a receptor-primed Env

(A) The virus with EnvH20 is thermosensitive. Virus stocks of similar titers were incubated at 44 °C for the indicated time. The titers were determined on NIL-Tvc 3 d after infection using flow cytometry. The amount of proviral DNA in infected DF-1 cells was measured 1 d after infection using RT-PCR. The results are presented as the percentage of the original titer (full lines) or the amount of DNA (dashed lines) remaining after heating. (B) The virus with EnvH20 is inactivated by low pH. Purified viruses of similar titers were incubated with low (pH 5) or neutral (pH 7.5) treatment at 37 °C for 30 min before infection of DF-1 cells. The titers were determined by flow cytometry 2 d later. (C) Formation of TM oligomers triggered by increasing temperature. The virus was incubated for 20 min at the indicated temperature. Samples were lysed in the Laemmli loading buffer and analyzed by SDS/PAGE without boiling. TM was detected by immunoblotting with an antibody against its C-terminal part. Only the 70-kDa isoform of TM is shown. (D) Formation of TM oligomers at low pH. The virus was incubated for 30 min at the indicated pH at room temperature. Samples were

neutralized, lysed in Laemmli loading buffer, and analyzed by SDS/PAGE without boiling. TM was detected by immunoblotting with an antibody against its C-terminal part. (E) The virus with EnvH20 binds liposomes. Viruses of similar titers were incubated with or without liposomes at 37 °C and then the mixture was separated by sucrose gradient centrifugation. Sample fractions were collected and analyzed by Western immunoblotting using anti-p27 antibody. T, top; B, bottom. (F) The virus containing EnvH20 is inhibited with PMB. Purified viruses of similar titers were incubated at 37 °C for 30 min with increasing concentrations of PMB. Viruses were diluted in medium and spinoculated on DF-1 cells. The percentage of GFP+ cells was measured by flow cytometry 2 d after infection. Titers below the limit of detection are marked with "<". Error bars show the SD of two independent experiments in parallel. Significant differences are marked by asterisks (*P = 0.05–0.01, **P = 0.01–0.001, ***P < 0.001). NS, not significant. (Lounková et al. 2017)

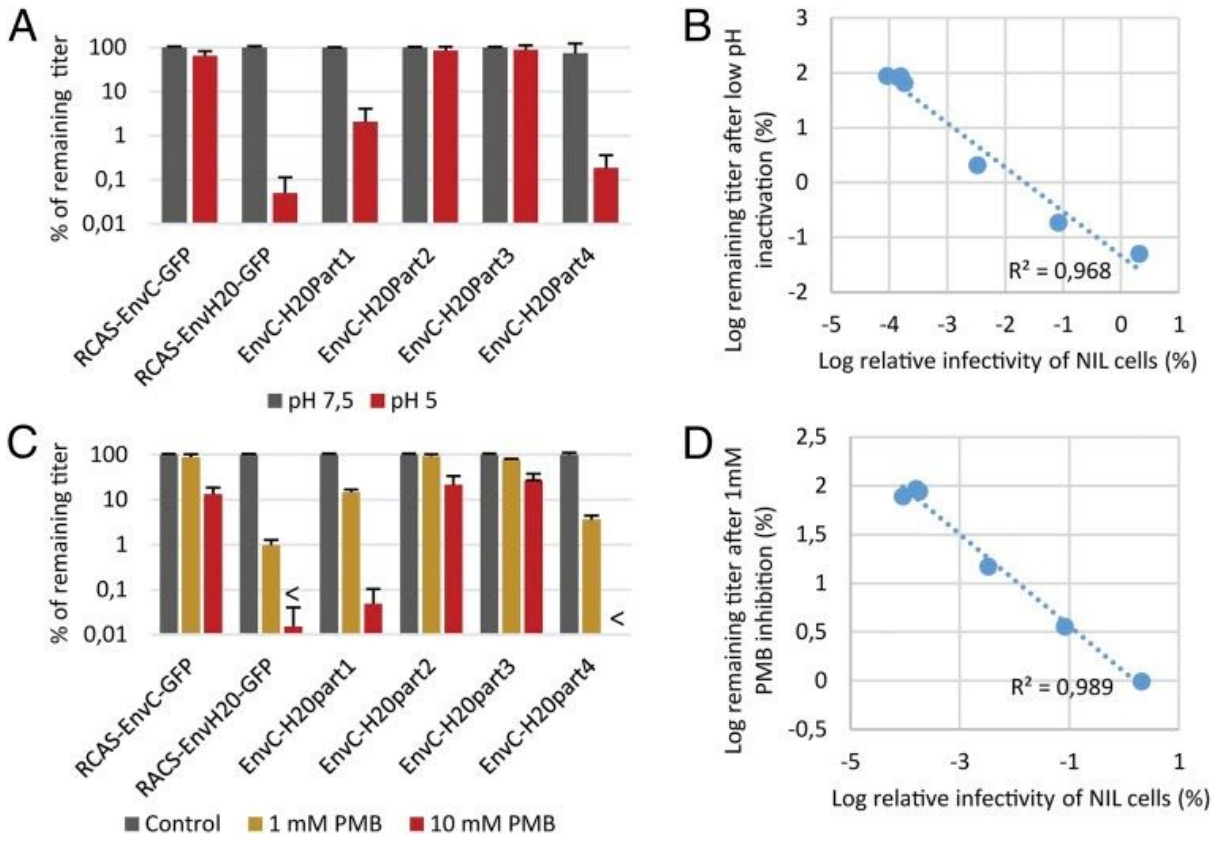


Figure C6.: The level of Env activation correlates with the efficiency of receptor-independent entry

(A) Virus inactivation by low pH. Purified viruses harboring EnvC-H20 chimeras were incubated with low (pH 5) or neutral (pH 7.5) treatment at 37 °C for 30 min before infection of DF-1 cells. The titers were determined by flow cytometry 2 d later. (C) PMB inhibition of virus infection. Purified viruses harboring EnvC-H20 chimeras were incubated at 37 °C 30 min with increasing concentrations of PMB. The titers were determined on DF-1 cells by flow cytometry 2 d after infection. Titers below the limit of detection are marked with "<". Error bars show the SD of two independent experiments in parallel. (B and D) Correlation between relative NIL infectivity and virus inactivation at low pH (*B*) or PMB inhibition of virus infection (expressed as residual infectivity after 1 mM PMB) (*D*). (Lounková et al. 2017)

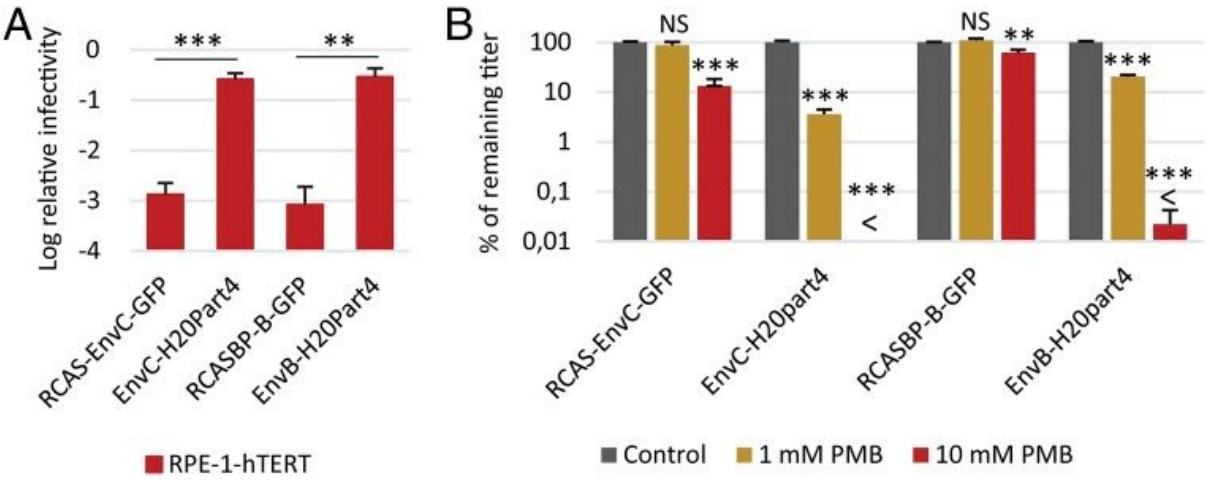


Figure C7.: Mutation in TM facilitates entry into mammalian cells independently of SU
(A) Relative infectivity of human RPE1-hTERT cells with RCAS-EnvC-GFP, RCASBP-B-GFP, and RCAS-GFP viruses bearing chimeric Envs (SU-C or SU-B with TM from EnvH20) is expressed as the percentage of the viral titer on DF-1 cells. The percentage of GFP+ cells was scored by flow cytometry two (DF-1) or three (RPE1-hTERT) days after infection. (B) PMB inhibition of virus infection. Purified viruses were incubated at 37 °C for 30 min with increasing concentration of PMB. The titers were determined on DF-1 cells by flow cytometry 2 d after infection. Titers below the limit of detection are marked with "<". Error bars show the SD of two independent experiments in parallel. Significant differences are marked by asterisks (**P = 0.01–0.001, ***P < 0.001). NS, not significant. (Lounková et al. 2017)

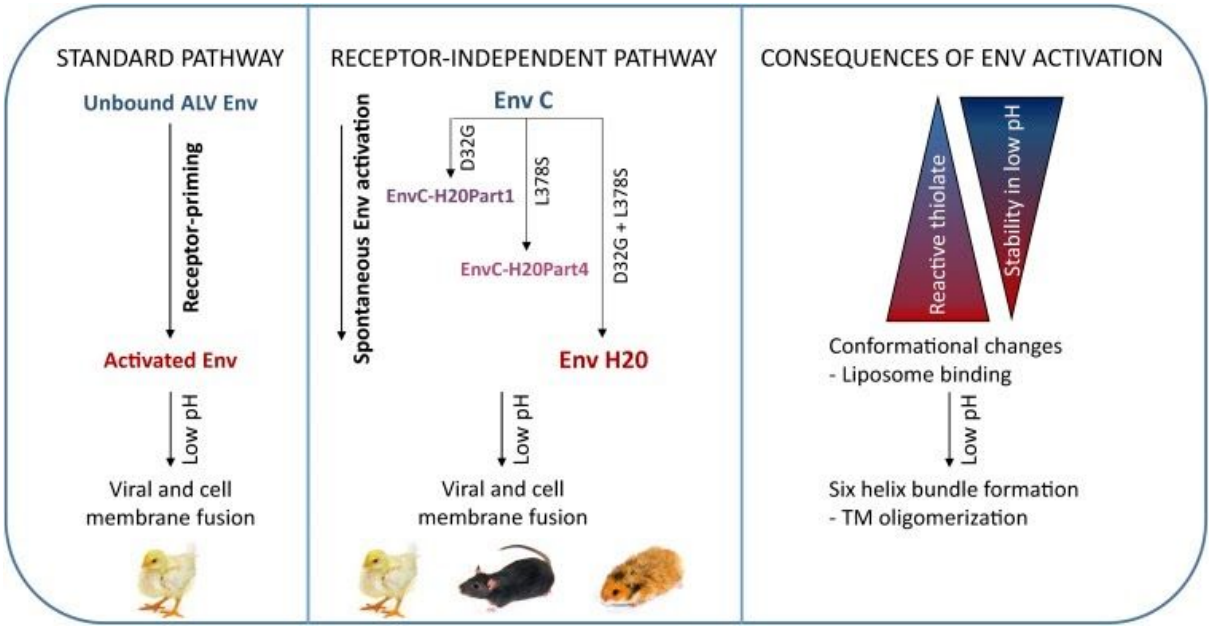


Figure C8.: Proposed model of receptor-independent entry

(Left) Viral Env is normally activated by binding with the receptor. Activated Env takes a conformation enabling further changes that occur at low pH in endosomes and lead to viral and cell membrane fusion. (Middle) Mutations D32G and L378S change the EnvC conformation and shift Env close to the active state, which normally follows receptor priming. These viruses are therefore able to be spontaneously activated with efficiency, depending on the mutation type. After low pH exposure, activated Env facilitates viral and cell membrane fusion. (Right) The consequences of Env activation can be observed in different steps. The level of Env activation positively correlates with increased formation of reactive thiolate and decreased stability at low pH. After activation, the conformation is changed: heptad repeats are exposed and the fusion peptide is inserted into the liposome membrane. After low pH treatment, the formation of the six-helix bundle can be determined by TM oligomerization assay. (Lounková et al. 2017)

Envelope glycoprotein-driven osteopetrosis

Two strains of MAV-2.O induce severe osteopetrosis, in contrast to its derivate, MAV-B

Our studies took advantage of possession of three different stocks of MAV-2 viruses that differed extremely in their osteopetrotic potential. Two stocks (marked MAV-2.O and MAV-2.P) were derived from the original osteopetrotic MAV-2 stock and were (after a few passages in cell cultures and chickens) kept frozen for over 40 years. The third one (marked MAV-2.N) has originated from the same stock of MAV-2 but has undergone many passages in chickens when the virus obtained from nephroblastomas or adjacent cysts in one chicken generation was used to infect the next chicken generation; the virus has lost most of its osteopetrotic potential during the passages. MAV-2.N was molecularly cloned (the cloned virus was named MAV-B) and then engineered to carry fluorescent marker GFP (the product was named MCAS(B) - Přikryl 2015). Virus stocks were prepared by transfection DF-1 cells with relevant molecular clones and virus-containing medium was collected two weeks later.

To compare quantitatively the oncogenic potential of our MAV strains, we have inoculated 12-day chicken embryos with MAV-2.O, MAV-2.P, and MCAS(B) viruses. We observed only a low incidence of very mild osteopetrosis in chickens infected with MCAS(B) GFP even after more than two months, while both MAV-2.O and MAV-2.P induced rapid osteopetrosis in infected chickens starting 13 days after hatching and culminating 18 and 19 days after hatching, respectively. MAV-2.O, compared to MAV-2.P, induced remarkably more severe osteopetrosis and acted with higher penetrance (61% versus 42% of chickens, figure B1). Therefore, we have decided to continue our experiments only with two strains with the most divergent pathogenicity, MAV-2.O, and MCAS(B) GFP.

We also tested the transforming ability of viruses in vitro by infecting CEF (chicken embryo fibroblasts) and overlaying them with agar. We observed no focus-forming colonies during more than three weeks, indicating neither MAV-2.O nor MCAS(B) GFP has the ability to transform chicken fibroblasts in vitro.

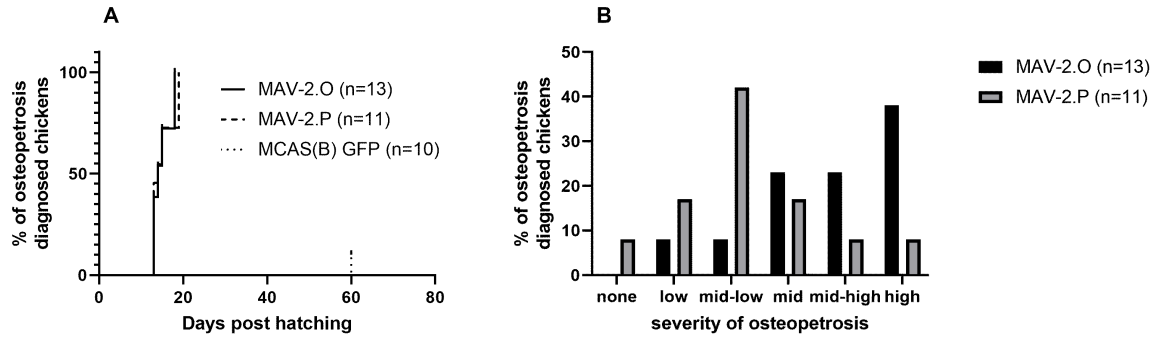


Figure B1.: Osteopetrosis-inducing strains of MAV-2
(A) Relative number of brown leghorn chickens diagnosed with osteopetrosis upon inoculation at day 12 of embryonic development with MAV-2.O, MAV-2.P and MCAS(B) GFP. (B) The severity of osteopetrosis induced by MAV-2.O or MAV-2.P viruses. Severity is expressed in multiple categories based on the number of bones affected and the stage of bone transformation (enlargement).

Osteopetrosis induced by MAV-2.O is not time- but dose-dependent

Next we examined at which stage of chicken development the target cells of oncogenesis (presumably osteoblasts or their precursors) are available. We inoculated three groups of embryos at the different embryonic developmental stage: on day 10 (E10), 13 (E13), and 17 (E17) and two groups of hatched chickens on the day of the hatch (K0) and 7 days later (K7) with MAV-2.O and analyzed the incidence and severity of osteopetrosis.
Group K7 developed no osteopetrosis but suffered from severe anemia, probably as a result of a fully functional immune system reaction to the virus. Other groups established osteopetrosis at a similar rate, with the first manifestation in group E10 (15 days post-inoculation), followed by K0 (18 dpi), E13 (20 dpi), and E17 (22 dpi). We also compared the severity of osteopetrosis of groups E17 and K0, to find out that the K0 group developed mild-to-intermediate osteopetrosis while the E17 group developed severe osteopetrosis (figure B2).
Furthermore, we inoculated chicken embryos on day 12 with MAV-2.O of different virus doses ($10^{\wedge 7}$, $10^{\wedge 5}$, $10^{\wedge 4}$, $10^{\wedge 3}$, $10^{\wedge 2}$, $10^{\wedge 1,}$ and with the virus-free medium) and analyzed the hatched chickens. Although we detected osteopetrosis in all chickens infected with dose $10^{\wedge 3}$ and higher, a manifestation of osteopetrosis was delayed in chickens infected by dose $10^{\wedge 5}$ (day 37), $10^{\wedge 4}$ (day 56), and $10^{\wedge 3}$ (day 71), while only 75% of chickens inoculated by virus dose $10^{\wedge 2}$ and 20% of chickens inoculated by virus dose $10^{\wedge 1}$ developed osteopetrosis by the end of the experiment (day 71). Also, the severity of the disease went down along with the dosage of virus used for infection (figure B2).
Taken together, our data suggest that there is no specific stage of embryonic development though infection must be carried out shortly after hatching at the latest to avoid the animal's immune reaction. As expected, the efficacy weakens with lowering the dosage of the virus.

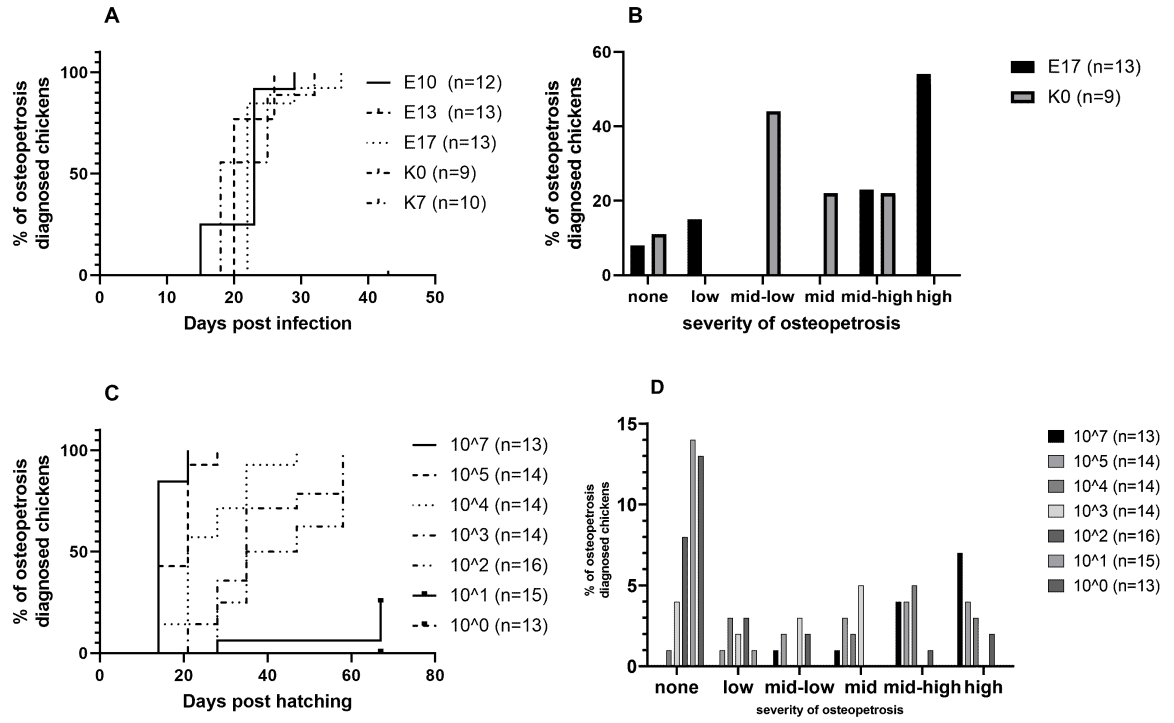


Figure B2.: Osteopetrosis induced in a time- and dose-dependent way

(A) Relative number of chickens diagnosed with osteopetrosis regardless of the severity induced by MAV-2.O inoculation at different stages of embryonic development, starting at day 10 (E10), followed by days 13 and 17 (E13, E17), the day of hatch (K0) and even days post-hatching (K7). (B) Chickens infected before hatching displayed a higher number of affected bones and the affection was greater. (C) Relative number of chickens diagnosed with osteopetrosis regardless of the severity induced by inoculation of MAV-2.O of different titers ($10^{\wedge 7}$ and $10^{\wedge 5}$ to $10^{\wedge 0}$) with a low incidence of disease by titer $10^{\wedge 2}$ and lower. (D) The severity of osteopetrosis is dose-dependent, as chickens with non-diluted virus struggled with a high amount of transformed bones, while viruses of titer $10^{\wedge 2}$ and below were able to transform only a small number of bones.

Comparative analysis of MAV-2.O and MAV-B

To look for genetic variations responsible for the strong osteopetrosis potential of some MAV strains, we sequenced proviruses in DNA from osteopetrotic tissue and normal liver from several chickens infected with MAV-2.O and compared them with MAV-B (molecularly cloned MAV-2.N). We identified a number of nucleotide substitutions in the whole genome of MAV-2.O. To select substitutions crucial for osteopetrosis induction, we applied the following criteria: 1) the substitution generates missense mutation and/or is inside known regulation regions, i.e., LTR NRS (a negative regulator of splicing), gag enhancer, env SA (env splicing enhancer), 2) the substitution is present in the majority of MAV-2.O samples (as with all retroviruses, uncloned MAV-2 is composed of many quasispecies), 3) (where applicable) the substitution is present in the majority of other sequenced ALV strains with high osteopetrotic potential while it is not present in the majority of ALV strains with low osteopetrotic potential. Selected substitutions are presented in Table B1. We found multiple substitutions within U3 (nucleotides 160 and 194), U5 (nucleotide 372), integrase (A683V and M765V - numbers according to the whole pol protein), TM (L387S and A398T - numbers according to processed env protein), and one substitution affecting both integrase and signal sequence of env (S880N and A28T, respectively). For more informations, see table B1.

MAV-2.O analysis					osteopetrotic virus			nonosteopetrotic virus					
position	locus	AA change (ori --> O)	correlation	MAV-2.O	MAV-2(O)	MAV-1/2(O)	MAV-B	MAV-2(N)	MAV-1(N)	SR-A	Pr-C	MQ-NCSU	LR-9
94	U3	-	-	A	A	A	G	A	A	-	-	-	-
160	U3	-	+	R (G>A)	A	A	G	G	G	-	-	-	-
194	U3	-	+	W (T~A)	T	T	A	A	A	-	-	-	-
291	U3	-	++	T	T	T	G	T	T	-	-	-	-
372	U5	-	++	C	C	C	A	G	A	C	A	T	C
566	UT5	-	++	G	G	G	A	G	G	A	-	-	-
2983	POL	-	+	G	G	G	A	G	G	G	G	G	G
3796	POL	-	+	C	C	C	T	C	C	C	T	C	C
3967	POL	-	+	G	A	A	A	A	A	A	A	A	A
4842	POL	ala --> val	++	T	T	T	C	T	T	T	T	T	T
5087	IN	met --> val	+++	G	G	G	A	A	A	A	A	A	A
5098	IN	-	+++	T	T	T	C	C	C	C	C	C	C
5146	IN	-	+++	T	T	T	C	C	C	C	C	C	C
5353	IN	-	-	G	A	A	G	G	G	G	G	G	G
5355	IN	ser --> asn	-	G	G	G	G	G	G	G	G	G	G
5399	IN + SP	-	-	G	G	G	G	G	G	G	G	A	G
5433	IN + SP	ser --> asn, ala --> thr	++	A	A	A	G	A	A	A	A	A	A
5530	SU	val --> ala	-	T	C	T	T	C	G	T	T	T	-
6089	SU	-	-	C	C	T	T	C	G	T	T	T	C
6467	SU	-	++	T	T	T	C	T	T	T	T	T	T
6530	SU	ile --> met	++	K (G~T)	T	T	T	T	T	T	T	T	T
6688	TM	leu --> ser	+++	C	T	T	T	T	T	T	T	T	T
6729	TM	ala --> thr	+++	R (A>G)	G	G	G	G	G	G	G	G	G
6983	TM	-	++	G	G	G	A	G	G	A	G	G	A

Table B1.: Comparative analysis of multiple ALV strains and selection of osteopetrotic potential of MAV-2.O-derived substitutions

We sequenced the genome of MAV-2.O and compared it to osteopetrotic MAV-2(O) and recombinant MAV1/2(O) strains (Joliot et al. 1993) and nonsteopetrotic strains MAV-2(N) and MAV-1(N) (Watts and Smith 1980) and other ALV strains (see Materials and Methods). The level of the potential association of substitutions with osteopetrosis is expressed from "-" (no correlation) to "+++" (strong correlation). Gray highlighted substitutions appear to have no effect on osteopetrosis induction or do not alter the amino acid sequence, while substitutions highlighted with green and red have a possible effect on the disease induction and progression. For our initial experiments, we introduced substitutions highlighted with green color into our vectors.

Osteopetrosis induced by vectors bearing MAV-2.O-derived substitutions

To test the significance of the selected substitutions, we have introduced them, one by one as well as in combinations, into the MCAS(B) clone using fusion PCR mutagenesis; for the list and specification, see figure B3. We started the evaluation with constructs containing all substitution (LPE), all substitutions in LTR+pol (LP), in pol+env (PE), and in LTR+env excluding the one in int/env signal peptide overlap (LE) or including it (LE+). We inoculated 12-day chicken embryos with mutagenized viruses (collected from transfected DF-1 cells) together with MAV-2.O (positive control) and observed osteopetrosis development for 49 days after which the chickens were euthanized. As we have seen before, MAV-2.O induced rapid onset of severe osteopetrosis. LP virus was able to induce only mild osteopetrosis starting to appear in 50 % of chicken between days 35 and 42. All constructs bearing env substitutions induced rapid and severe osteopetrosis in all animals starting with LPE (presumably between day 7 and day 14), followed by LE+ (day 14), PE and LE (both between days 14 and 21), indicating a principal role of the env gene in osteopetrosis induction (figure B3). Comparison of these numbers also demonstrates that substitutions in LTR and pol, though they have only a moderate effect, both presumably synergize with env substitutions.

As most of the animals infected with mutagenized MCAB(B) died or were euthanized within two months after hatching, we observed no other tumors than osteopetrosis with the exception of chickens infected by MCAS(B)-LP GFP developing a similar panel of tumors as wt MCAS(B) GFP (data not shown).

A

locus	LTR			pol		pol/env	env	
substitution	160	194	372	A683V	M765V	S880N/A28T	L387S	A398T
LP	x	x	x	x	x	x	-	-
LE	x	x	x	-	-	-	x	x
LE+	x	x	x	-	-	x	x	x
PE	-	-	-	x	x	x	x	x
LPE	x	x	x	x	x	x	x	x

B

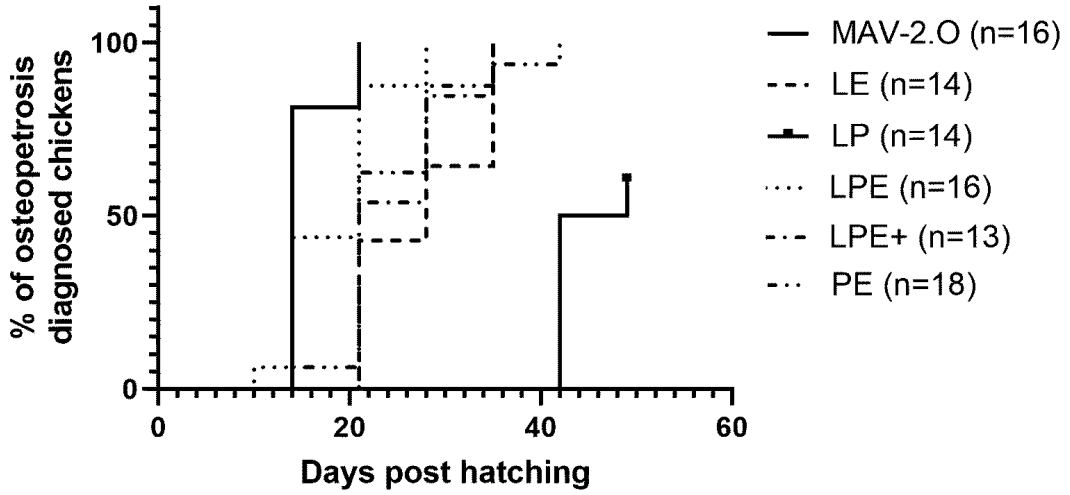
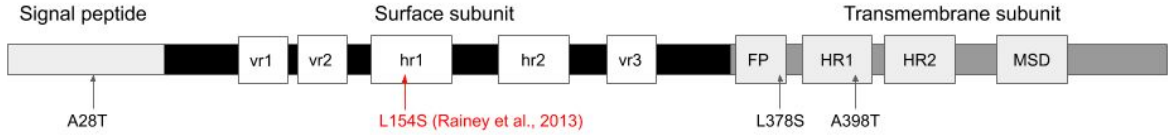


Figure B3.: Osteopetrosis induced by MAV-2.O-derived genes
(A) Table of all selected substitutions and acronyms used for vectors. (B) All vectors bearing MAV-2.O-derived envelope glycoprotein proved their ability to affect the infected chickens in a similar fashion as the MAV-2.O virus regardless of additive regions of MAV-2.O. The vector bearing MAV-2.O-derived LTR and pol (LP) was able to transform only about half of the chickens with a mild outcome.

MAV-2.O env-derived substitutions render the virus unstable, more sensitive to temperature, less infectious and capable of infecting receptor-less cells

Further experiments were done using cell cultures instead of animals and were aimed to elucidate how the substitutions changed the basic properties of the virus, like the stability and efficiency of infection and propagation. Constructs carrying individual substitutions in env and all their possible combinations were included in the experiment so that we were able to assess the importance of individual substitutions. Vectors containing A28T substitution are coded P3 (for pol/env shared locus), L378S containing vectors are coded T1 (for TM locus) and vectors containing A398T are coded T2. For more info, see figure B4.

A



B

	P3	T1	T2	P3T1	P3T2	T12	P3T12	WT
A28T	x	-	-	x	x	-	x	-
L378S	-	x	-	x	-	x	x	-
A398T	-	-	x	-	x	x	x	-

Figure B4. Scheme of substitutions and produced vectors.
(A) Schematic representation of envelope glycoprotein. Variable regions (vr1-3, hr1-2) within the surface subunit are depicted in white boxes, the fusion peptide (FP), heptad repeats (HR1-2), and membrane-spanning domain (MSD) of transmembrane subunit are depicted in grey boxes. Positions of selected MAV-2.O env-derived substitutions are marked with black arrows. Substitution responsible for mammalian tropism is marked with a red arrow.
(B) Table of MAV-2.O env-derived substitutions and names of constructed vectors.

Only a low proportion of DF-1 cell become GFP-positive after infection by T1 (16%), P3T1 (25%), and P3T12 (6%) viruses compared to other viruses (average 72%), suggesting corruption of envelope glycoprotein containing L387S substitution alone or in combinations with others (Figure B4).
Env destabilization may be a manifestation of e.g. decreased activation energy for env priming, which would lead to spontaneous self-priming and, in addition to decreasing env longevity, it would make the virus able to enter cells lacking the respective receptor. To attest this possibility we generated, using CRISPR/Cas9 system, DF-1 knockout cell line without functional Tvb gene and attempted to infect this cell line, denoted DF-1ΔTvb, with our mutagenized viruses. In the first experiment we observed no infection, exactly as with wt MCAS(B). Since we realized that some of our viruses poorly infected even cells with the functional receptor, we adjusted our infection protocol by increasing both the amount of the virus preparations and the duration of the treatment. Using this protocol (from now on called

adjusted infectivity test), we could detect a low GFP signal in DF-1ΔTvb cultures infected with any of our viruses (0,48% of cells in culture on average, figure B4) with exception of wt MCAS(B) GFP which failed to infect DF-1ΔTvb cells even under adjusted infectivity conditions, while the virus was able to infect DF-1 cells (for more details, see figure B5).
Since the data supported our conjecture, we tested the constructs for another symptom of facilitated priming: increased temperature sensitivity. We exposed the viruses to 44°C for 12 or 24 hours prior to infection of DF-1 cells and compared remaining virus infectivity with untreated viruses (figure B6). MCAS(B) GFP sustained the highest infectivity (50% after 24 hours of incubation at 44°C), while most vectors bearing substitutions sustained only partial infectivity, starting with the most stable T12 (34%), followed by P3 (17,5%), P3T12 (14%), T1 and T2 (both 10%), P3T1 (9%) and ending with the least stable P3T2 (6,5%).
Taken together, we have shown that changed properties of the viruses with substitutions in env (decreased stability and infectivity as well as acquired ability to infect receptor-less cells) all comply with the concept of facilitated priming.

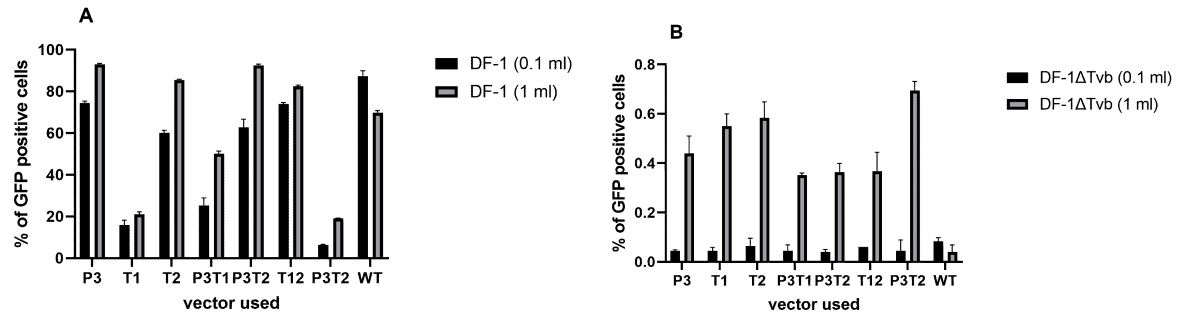


Figure B5.: Infectivity of DF1 and DF-1ΔTvb by different vectors

We have cloned all selected substitutions into the MCAS(B) GFP vector, alone or in combination, and used them for infection of cells expressing or lacking the Tvb receptor. (A) Vectors infected DF-1 cells with differing efficiency. We observed the lowest GFP-positive cells infected by the vector with all substitutions or only T1 substitution. We repeated infection using 10-times more virus to observe an enhancement of infection in most cases, while the vector with T1 substitution displayed only minor enhancement. (B) We infected DF-1ΔTvb in the same fashion and observed infection only by vector bearing MAV-2.O-derived substitutions and only with high amounts of the virus. All vectors proved significant differences ($P < 0.05$).

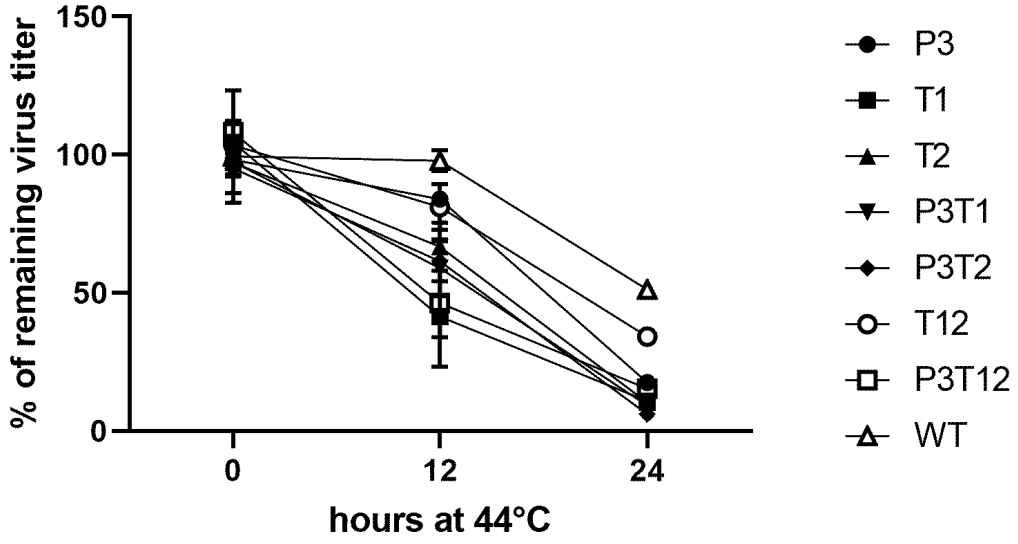


Figure B6.: MAV-2.O-derived substitutions provide raised thermosensitivity

We incubated vectors at 44°C for 0, 12 or 24 hours and used them for infection of DF-1 cells. The numbers of GFP-positive cells infected by vectors incubated for 12 or 24 hours were normalized to non-treated vectors.

MAV-2.O env-derived substitutions affect A and C subgroups envelope glycoproteins similarly to B subgroup env

To further scrutinize MAV-2.O-specific env substitutions and to see whether their effect is restricted to subgroup B, we introduced the substitutions into a new context of subgroup A, C and K envelope glycoproteins using MCAS(A), MCAS(C) and MCAS(K) vectors with structure analogical to that of MCAS(B). Subgroup specificity resides in variable parts of SU subunit while TM subunits are highly homologous, thus the TM-localized L387S and A398T amino acid residues (the sites of T1 and T2 substitutions) together with their vicinity are conserved in A, C, and K subgroups. The A28 residue in the env signal peptide (the site of P3 substitution) was not examined since A28T is already present in subgroups A, C and K.

First we tested infectivity on DF-1 cells. According to the numbers of GFP-positive cells, A subgroup virus with T1 (A-T1) and C subgroup virus with T1 and T2 (C-T1, C-T2) substitutions showed approximately 10-times lower infectivity compared to the respective wt virus. As for the double substitution in env C, infectivity was reduced 100-times. Neither T2 (A-T2) substitution in A subgroup nor any substitution in K subgroup affected the infectivity of the virus (figure B7).

Next, we performed the same test using receptor-less DF-1 cells, using DF-1ΔTva and DF-1ΔTvc cells, cell lines with an off-frame deletion in Tva or Tvc genes induced by CRISPR/Cas9, respectively, by subgroups A and K viruses (DF-1ΔTva) and by subgroup C viruses (DF-1ΔTvc). C subgroup T1 virus was able to enter about 1% of receptor-less cells, while neither C subgroup T2 (C-T2) or T12 (C-T12) were able to infect these. None of the substitutions provided subgroup A and K viruses with the ability to infect receptor-less cells.

To test thermal stability, mutagenized A, C and K subgroup viruses were incubated at 44°C for 12 and 24 hours prior to DF-1 cell infection. Significant decrease of infectivity was observed in all cases. The A subgroup viruses retained only ca 75% after 12 hours of incubation and less than 25% after 24 hours, for the K subgroup the numbers were 25%/~0% and for the C subgroup 10% /~0%.

Temperature 44°C in our experimental setup was chosen based on the literature and our previous experiments. For very unstable viruses (like our mutagenized subgroups K and C), however, it might be too high. To better compare the stability of the respective viruses, we soften the test conditions for C and K subgroup viruses to 24 hour incubation at 33°C, 37°C, or 40°C (figure B8). At these temperatures we could observe differences between individual substitutions. For the C subgroup, we found the greatest decrease of infectivity with T12 virus (retention of 50%, 15%, and 5% after incubation at 33°C, 37°C, and 40°C, respectively), while single substitutions separately showed infectivity reduction 50%, 40%, and 10%, respectively. Surprisingly, we observed no infectivity reduction with any of our K subgroup viruses under the above conditions which contrasted sharply with our results when using incubation temperature 44°C (see above).

Taken together, our data suggest that MAV-2.O env-derived substitutions, when introduced into subgroup A and C envelope glycoproteins, have some effects similar to those we described for subgroup B: they make the protein less stable which reduces virus infectivity. The third symptom, ability to infect receptor-less cells, was observed only with mutagenized constructs of the C subgroup. Subgroup K demonstrated none of the above-mentioned effects regardless of the substitution we introduced.

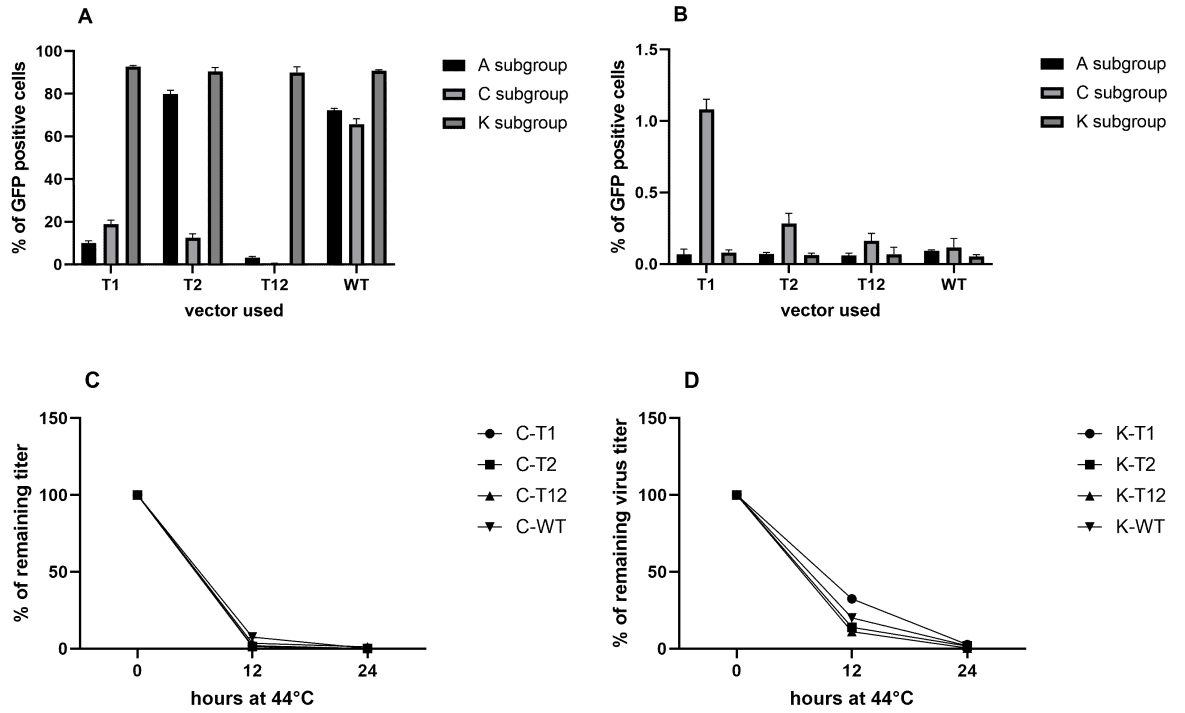


Figure B7.: Infectivity of different subgroup based vectors

We introduced MAV-2.O-derived substitutions into A, C and K subgroup envelope glycoproteins and used them for infection of DF-1 cells (A) and cells lacking subgroup-specific receptors (B). Only the C subgroup T1 substitution proved a significant difference to the wt C subgroup ($P < 0.01$). Next, we incubated C subgroup (C) or K subgroup (D) vectors for 0, 12, and 24 hours at 44°C and used them for infection of DF-1 cells, numbers of GFP-positive cells are expressed in comparison to infection of untreated respective viruses.

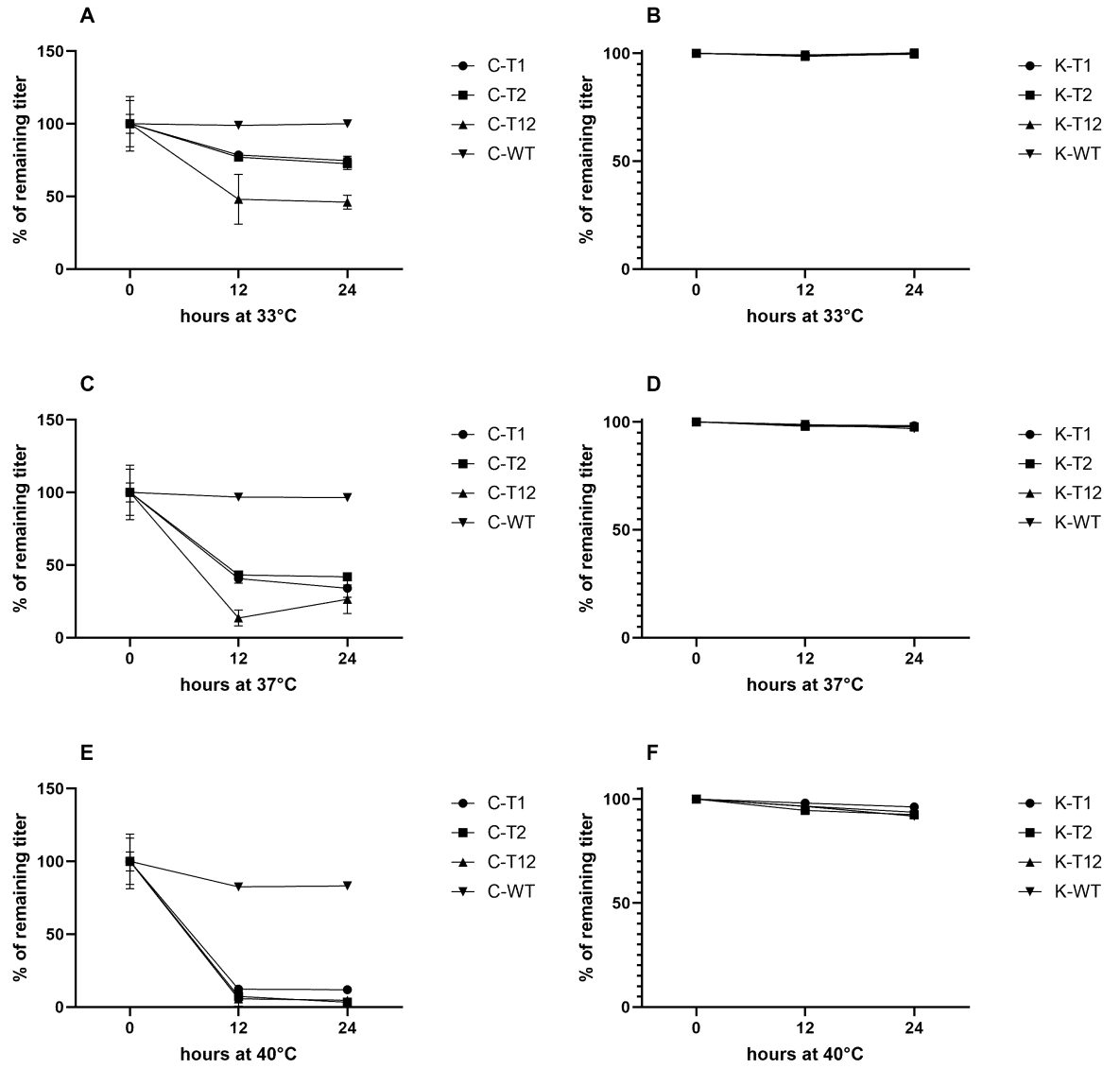


Figure B8.: C and K subgroup vectors show different thermosensitivity
We incubated C subgroup-derived vectors at 33°C (A), 37°C ©, or 40°C (E) for 12 or 24 hours and used them for infection of DF-1 cells. The same scheme was used for K subgroup-derived vectors (B, D, E).

Additional substitution L154S in envelope glycoprotein enhances infection of receptor-less cells

We can presume that the osteopetrosis connected env destabilizing substitutions scrutinized above are not the only ones that can incite the phenomena. In our previous work (Přikryl 2015) we have analyzed the L154S substitution in B subgroup envelope glycoprotein, selected on the basis of its ability to mediate infection of receptor-less cells ((Rainey et al. 2003)). We have shown that this substitution also incites env destabilization and promotes (though very mild) osteopetrosis. To expand our results and compare effects of L154S substitution with those described above, we introduced it into our MCAS(B)-based constructs denoted "E", for "extended host range".

Test of infectivity of these new constructs on DF-1 cells showed a high number of GFP-positive cells after infection with E (60%) or P3E (87%) vectors. Other viruses (ET1, ET2,...) managed to infect DF-1 cells only with an average of 9% GFP-positive cells. Vector ET12 was not used for infection as we failed to propagate the virus in tissue culture. Since the failure might be caused by excessive damage to envelope glycoprotein due to the introduced substitutions, we constructed the ET12neo vector with the neomycin resistance gene connected to the GFP gene via P2A self-cleaving peptide. After three-week-long selection by G418, we harvested the virus and used it together with other vectors, using our adjusted infecting protocol to infect DF-1 cells. We observed a high amount of GFP-positive cells infected by E (94%) and P3E (67%) constructs, mediate amount of GFP-positive cells infected by ET1 (42%) or P3ET1 (32%) and low amount of GFP-positive cells by P3ET2 (13%), ET2 (10%), ET12 (10%) or P3ET12 (4%) constructs (figure B9).

Next we examined virus infectivity on DF-1ΔTvb cells using both standard and adjusted infectivity tests. We observed the most efficient infection with E (54%), followed by ET1 (42%) and P3ET1 (39%) when infected by our adjusted protocol. Other vectors infected the DF-1ΔTvb cells in 10% of GFP-positive cells on average (figure B9).

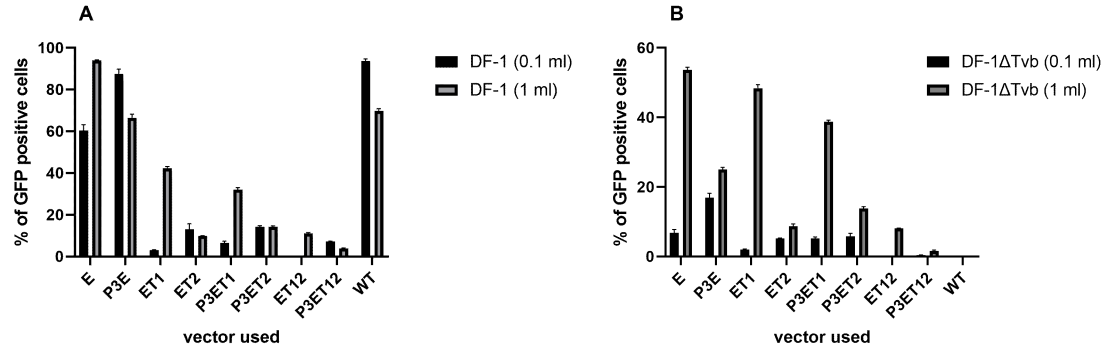


Figure B9.: Infectivity of MAV-2.O-derived substitutions coupled with L154S substitution-bearing vectors

We introduced the L154S substitution into our vectors and used them for infection of DF-1 cells (A) or DF-1ΔTvb cells (B). We used both a low (0.1 ml) and high (1 ml) amount of virus to counter the low efficiency of some viruses.

Conclusions

The aim of this thesis was to analyze envelope glycoproteins with possible receptor recognition shifts. We have tested JS11C1, the prototype isolate of the postulated new K subgroup, RSV-H20, provirus successfully integrated into a mammalian cell line, and MAV-2.O, highly osteopetrotic strain. All investigated viruses present unique insights into the virus-host evolution arm race, as they managed to overcome the obstacle of occupied receptors or absent specific receptor in non-permissive species.

JS11C1 switched receptor specificity due to extensive changes in domains responsible for receptor recognition. We have synthesized its envelope glycoprotein, cloned it into a RCAS vector, creating RCAS(JS11C1) GFP and:

- found unique host range, differing from other ALV subgroups
- observed interference between RCAS(JS11C1)GFP and RCAS(A)GFP
- identified a sufficient and necessary receptor used by JS11C1, Tva, shared with subgroup A viruses
- observed differing inhibition efficiency between JS11C1 and RCAS(A)GFP by soluble Tva receptor
- observed rapid mortality in chickens infected by RCAS(JS11C1)GFP

Our observations verify the existence of new ALV subgroup K and its importance for further research of avian retroviruses evolution and pathogenicity.

RSV-H20 invaded mammalian cells through either interaction with the unknown cell receptor or env destabilization. To distinguish between these two possibilities we have:

- sequenced and cloned env of RSV-H20 into a RCAS vector, resulting in the RCAS(H20)GFP vector and identified amino acid residues coupled with the cross-species transmission
- verified the capacity of selected substitutions to activate the envelope glycoprotein independently on the receptor and thus providing association with artificially made liposomes
- discovered RCAS(H20)GFP high temperature and low pH sensitivity, along with the sensitivity to a selective inhibitor typical for activated prefusion state env

Taken together, these findings support the assumption of receptor-independent cell entry of RSV H20.

MAV-2.O induces chicken osteopetrosis by an unknown mechanism involving the envelope glycoprotein and LTR. We studied the characteristics of osteopetric variants of env and conclude that:

- identified substitutions of amino acid residues within env are associated with MAV-2.O induced chicken osteopetrosis
- mutations in nucleotide sequence of LTR and substitutions of amino acid residues within pol are not directly responsible for the disease induction but potentiate the disease progress when coupled with MAV-2.O env-derived substitutions

- env-derived substitutions destabilize the envelope glycoprotein and predispose the virus to infect receptor-less cells
- MAV-2.O env-derived substitutions are not subgroup-specific, providing similar capacity to A and C subgroups
- envelope glycoprotein-provided receptor-less entry and observed destabilization is further enhanced by SU-derived L154S substitution

We suggest that the envelope gene destabilization is involved in osteopetrosis induction. However, further experiments are required to fully understand the nature of the MAV-2O pathogenesis.

Discussion

In our studies, we focused on retroviral variants and their changed interaction with permissive and nonpermissive cells. Three unrelated chicken viruses were investigated: JS11C1 virus postulated to be the prototype virus of the newly emerged ALV-K subgroup, RSV-H20, a variant of RSV-C, C-subgroup virus found integrated into hamster-derived cell line and MAV-2.O, a B-subgroup virus capable of inducing osteopetrosis. In the case of JS11C1, we sought to identify the receptor for subgroup K viruses. Regarding RSV-H20 we questioned the possibility of the virus infection via a novel molecule present both at avian and mammal hosts as RSV-C enters the avian cells using the Tvc receptor. Concerning MAV-2.O, we looked for virus genetic determinants for osteopetrosis induction, which, incidentally, converged to the same issue: the possibility of involvement of its envelope glycoprotein in tumorigenesis via an unknown mechanism. We employed classical virological methods such as superinfection interference assay and infectivity assay using different cell lines and primary cell cultures together with biochemical methods such as lipid-binding assay and pH based neutralization assay.

The K-subgroup virus study

In the first study, we aimed to verify that the viruses assigned to the K subgroup really represent a new subgroup since this definition was based solely on *env* gene sequences. The first step was to verify if these viruses actually do not fit into some of the older subgroups. For this reason, we infected a panel of cell lines and primary cultures derived from a set of outbred or inbred chicken strains as well as multiple non-chicken Galliformes fibroblasts with a retroviral vector carrying JS11C1 env, with the intent to compare the sensitivity of these cells to subgroup K versus subgroup A. The experiment showed a good (but not absolute) correlation of host range between A and K subgroups, suggesting both subgroups use the same receptor. The evidence was supported by parallel experiments with Tva-null chicken cells and mammalian cells expressing Tva (Tva is ALV-A specific receptor) showed that JS11C1 is able to enter cells via this molecule. Either RCAS(A) or MCAS(A) GFP vectors were used as ALV-A viruses; both gave the same results.

To further confirm that A and K subgroups share the same receptor, we carried out a superinfection interference assay. The principle of the assay consists in the ability of env glycoprotein, produced by infected cells, to occupy the receptor and/or to block its env-binding site so that no further virus of the same subgroup can enter the cell. We found that pre-infection with ALV-A or with JS11C1 viruses rendered the cells resistant to superinfection with both ALV-A and JS11C1 viruses, while susceptibility to other subgroups was maintained, in full agreement with the above-stated assumption.

To tell the whole story, we have to mention some observations that did not conform to the theory. We observed that the ratio between infection efficiency of ALV-A versus JS11C1 was different in different cells and there were even cells that were resistant to ALV-A but not to JS11C1 and vice versa. The ALV-A-resistant/JS11C1-susceptibility cells carried already described alleles with known resistance-rendering substitutions. This discrepancy can be explained in two ways. Either, A and K env glycoproteins bind different epitopes on the receptor so that the affinities to the two glycoproteins evolved independently.

Further experiments tested the infectivity of the two viruses in the presence of a selective inhibitor of ALV-A entry - soluble form of Tva (sTva). Contrary to ALV-A, infection with the JS11C1 virus was not fully inhibited by sTva. A plausible explanation of this discrepancy is that the bulky IgG domain hampers sTva interaction with env-K more than with env-A. That would imply, in turn, that the two glycoproteins bind either to different epitopes or indifferent ways. The results provide no clue as to which of the above scenarios is true.

In summary, we postulate JS11C1 and related viruses as an independent K subgroup since they display host range different from other subgroups. Although they share the receptor (Tva) with A subgroup, they recognize different epitopes or recognize the same but contact it in a different way. Using the same receptor, however, manifests itself by reciprocal interference between A and K viruses and by similar (but not identical) host range.

Finally, we investigated the pathological effects of infection with K subgroup viruses in vivo. Brown Leghorn chickens, after being inoculated with JS11C1 as embryos, exhibited significantly higher mortality compared to chickens infected with ALV-A. After dissection, we observed severe pathology in JS11C1-infected chickens, represented by muscle hemorrhages, enlarged heart, ascites in the abdomen and disrupted the integrity of bone marrow (data not shown). These symptoms were similar to the symptoms caused by the FGV (fowl glioma virus) group of viruses, so far classified to A subgroup (Tomioka et al. 2003; Nakamura et al. 2011). Surprisingly, when we compared env glycoprotein sequences from K subgroup viruses with those of many other viruses, we found members of the FGV group to be significantly closer to K than to A (data not shown), indicating that FGV viruses might belong to the K subgroup.

Furthermore, new isolates are continuously emerging (X. Zhou et al. 2019; Su, Li, Li, et al. 2018; Zhao et al. 2018b) with the potential to cause harm to the farming industry similar to subgroup J (Lv et al. 2019). The problem of receptor specificity appears to be even more complex since some strains of K subgroup exhibited unusual behavior in receptor recognition and interfering with A subgroups viruses (Mingzhang et al. 2018) and distinction interaction of some ALV-A strains with Tva were shown (Melder et al. 2015). Also, isolate GDFX0601 of K subgroup appears to have mixed receptor specificity (Jianyong, unpublished data), questioning its interaction with Tva receptor, therefore, appears crucial and priority in the future. The same analysis should be performed for FGV viruses-derived env. Since both K subgroup and FGV group viruses are strongly pathogenic, obtained information could be of great practical use, e.g. for the development of resistant chicken strains via modification of the Tva gene.

RSV-H20 virus study

In the second study, we have investigated the RSV-H20, the C subgroup RSV variant present, as a provirus, in hamster cell line transformed with Pr-RSV-C. First, we examined whether this ability is limited to hamster or, more widely, to rodent cells. We generated an RCAS vector carrying RSV-H20-derived env (RCAS(H20) GFP) and used it to infect several mammalian cell lines, including human ones. In all cases, we observed a greatly increased proportion of infected cells with RCAS-H20 compared to wt RCAS-C, even in cells like HEK293 which showed a high background of infection with wt RCAS-C. However, mammalian cells engineered to express the chicken Tvc receptor showed much higher infectivity by both constructs, the wt RCAS-C being more efficient than RCAS-H20 in this case.

Next, we looked for genetic determinants in H20 env that are responsible for its unique properties. We have sequenced the H20 env gene and identified multiple substitutions compared to wt RSV-C. With this knowledge, we divided the H20 env into four parts each carrying a proportion of the identified substitutions and by swapping them in RCAS-C we generated four chimeras each carrying only a single part of the H20 env. Then we examined the infectivity of the chimeras on mammalian cell lines. We found that the infectivity correlated with the presence of the first or fourth part while swapping the second or third part had no effect. The first part contained only one substitution, D32G. In the fourth part, there were three substitutions, L378S, G464S, and L503V. Therefore, we separated these substitutions and investigated them separately. Only L378S substitution rendered the env glycoprotein capable of infecting mammalian cells; the effect was stronger than in the case of D32G substitution.

Then we asked how the virus managed to enter mammalian cells known to lack the functional receptor for the C subgroup. We considered the possibility that substitutions in env provided it with the ability to interact with mammalian versions of chicken Tvc, or it could use quite different molecules on the mammalian cells. However, since RCAS-H20 indiscriminately infected all tested cells and infection was enhanced by mammalian cells expressing Tvc receptor, we favored the hypothesis that it enters mammalian cells via a receptor-independent pathway. Under normal circumstances, envelope glycoprotein is in a metastable state with fusion peptides hidden inside the envelope´s trimeric structure. The glycoprotein has the competence to undergo conformational rearrangements leading to the fusion peptides exposure (so-called prefusion state). This process, however, requires lowering its activation energy, rendered by an interaction with a receptor; this process is thus called receptor-mediated priming. The decrease of activation energy can also be achieved by some mutations in env, leading to its destabilization (which is meant as the propensity to undergo transitions to prefusion and then postfusion states, not propensity to denature) so that it gradually and spontaneously undergoes rearrangement; this we will call self-priming. Spontaneous rearrangements make the virus able to fuse with cells regardless of a receptor. On the other hand, it decreases env stability since prematurely activated env undergoes further spontaneous rearrangements (most probably corresponding to the transition from the prefusion state into the postfusion state) thus causing depletion of infectious particles.

To confirm our assumption we tested characteristics of env. One of them is the decreased stability at elevated temperature (44°C) or in an acidic environment (pH 5) (Bova-Hill, Olsen, and Swanstrom 1991; W. Mothes et al. 2000). Indeed, H20 env, as well as chimeric envs, shown to infect mammalian cells, manifested a faster decrease of infectivity at 44°C as well as in low pH in comparison to wt env-C. Another parameter is the ability to associate with liposomes which was probed using the so-called lipid-binding essay. The virus was incubated with liposomes and the association was monitored by ultracentrifugation in sucrose gradient where the virus-liposome complex sediments to intermediate density, separately from free liposomes and free virus. The assay showed that RCAS-H20 as well as chimeric mammalian cells-infecting viruses associated with liposomes, contrary to wt RCAS-C. Finally, we used PMB, which reacts selectively with cysteine thiol groups on fusion peptides that become accessible only after priming, thus acting as the inhibitor of env-driven membrane fusion. PBM effectively inhibited infection of mammalian cells with RCAS-H20 and derived viruses, thus proving that virus entry into mammalian cells is preceded by fusion peptide exposure.

When we compared all measured properties of our viruses we found that they perfectly correlated - the viruses ranked in the same order when we compared infectivity on mammalian cells, instability at increased temperature or low pH, and sensitivity to PBM: wt RCAS-C < RCAS-C(D32G) < RCAS-C(L378S) < RCAS-H20.
Therefore we believe that the phenomenon of avian retroviruses entry into mammalian cell lines is based on a receptor-independent process that proceeds via self-priming of variant env glycoproteins.

MAV-2.O virus study

In the last, yet unfinished study, we examined the MAV-2.O virus which is unique by its ability to induce osteopetrosis in infected chickens. Avian osteopetrosis is a disorder characterized by enlarged and thickened bones, caused by virus-induced hyperproliferation of osteoblasts. We took advantage of possessing both MAV-2.O and its direct descendant, MAV-2.N, which has lost most of the osteopetrotic potential during passaging *in vivo* and *in vitro*. MAV-2.N induced late-onset osteopetrosis with barely detectable symptoms in ca 15% of chickens compared to 100% of severe rapid-onset osteopetrosis induced in chickens by parental MAV-2.O. In the starting experiments, we also examined a close relative of MAV-2.O, named MAV-2.P, which has shown to have slightly reduced osteopetrotic potential compared to MAV-2.O. We then concentrated solely on comparing MAV-2.O versus MAV-2.N (the latter after molecular cloning, generating MAV-B virus and then after inserting GFP gene, generating MCAS(B) virus (Přikryl et al., 2015)).
After a comparative analysis of the two viruses *in vivo* and adjusting the experimental conditions for osteopetrosis induction, we turned our attention to comparative genetic analysis. The two viruses were sequenced and sequences aligned. We revealed multiple point substitutions from which we, in the first step, selected those lying in important regulatory regions (e.g. LTR) as well as missense mutations residing inside the genes (encoding integrase, signal peptide, and a transmembrane subunit of envelope glycoprotein). In the next step, we pinpointed potentially important substitutions in this collection by comparing them with corresponding sequences of other ALVs for which both the sequence and ability or inability to induce osteopetrosis was known, including several close relatives of MAV-2. We ended up with three substitutions in LTR, two substitutions in integrase, two substitutions in TM domain of env, and one substitution affecting both integrase and signal peptide of env.
Pinpointed substitutions were introduced into MCAS(B) virus, first individually (using fusion PCR mutagenesis), and then in different combinations (with the help of swapping or additive fusion PCR mutagenesis). MCAS(B) variants generated this way were then scrutinized for the ability to induce osteopetrosis in infected chickens; wt MCAS(B) and MAV-2.O served as a negative and positive control, resp. We started with variants carrying all MAV-2.O-specific substitutions in LTR, in integrase, or in env and continued with variants carrying all possible combinations of these three groups of substitutions. Substitutions in env turned up to be by far the most effective since the results of infection with MCAS(B) variants carrying a combination of substitutions in TM with substitutions in integrase or in LTR were quite consistent: adding more substitutions always increased the osteopetrotic potential though the auxiliary effect of substitutions in LTR and/or in integrase was only mild. However, though MCAS(B) variant carrying all selected substitutions proved to be very potent, it did not fully reproduce the osteopetrotic potential of MAV-2.O, indicating that further mutations in MAV-2.O, which did not get through our selection process, might have a supportive effect.

The missed mutations might also be present only in a subpopulation of viruses in MAV-2.O stock, which was not produced from the molecular clone and thus is a mixture of quasispecies. Such mutation would not be recorded by Sanger sequencing.

In our previous work (Přikryl et al., 2015) we examined the MAV-2-derived virus-carrying L154S mutation in the SU domain of env. The mutation was originally identified by Rainey and his colleagues (Rainey et al. 2003; Rainey and Coffin 2006) because of its ability to enter mammalian cells; the phenomenon was named "extended host range". We found that this virus also promotes osteopetrosis (with high penetrance but very mild symptoms) and carries destabilized env glycoprotein. We realized that all these properties might be connected (and might ensue from the phenomenon of self-priming). Therefore, we switched from experiments on living animals to in vitro essays and explored the possible effect of MAV-2.O-specific substitutions on env stability and virus potential to infect receptor-less cells. For this purpose, we constructed a chicken cell line (DF-1ΔTvb) lacking Tvb, a subgroup B-specific receptor. Then we generated MCAS(B) variants carrying individual MAV-2.O TM-specific substitutions, individually and in combinations, and tested their effect on virus entry into DF-1 versus DF-1ΔTvb cells. Compared to what we have seen previously for L154S substitution, all MAV-2.O-specific substitutions showed decreased infectivity on DF-1 cells and only very low infectivity on DF-1ΔTvb cells. To see it at all we had to use an adjusted infection protocol with increased both virus dose and treatment period; yet, it was still meaningful since wt MCAS(B) had nearly zero infectivity on DF-1ΔTvb cells under the same conditions.

We assume that the low infectivity of our MCAS(B) variants results from decreased stability of the respective env glycoproteins. To verify it, we performed a temperature stability assay (incubation of the viruses at 44°C for 12 or 24 hours) and looked for a decrease in infectivity. As expected, all substitution made the env more temperature-sensitive. This result supports our assumption that the ability to induce osteopetrosis correlates with the destabilization of the env glycoprotein as well as with the ability to infect receptor-less cells and that all these properties are just signs of the same phenomenon - env self-priming.

To examine whether L154S substitution synergizes with the substitutions found in MAV-2.O TM, we constructed viruses carrying individual MAV-2.O-specific substitutions together with L154S substitution and tested their infectivity on wt and receptor-less cells. The L154S substitution alone provided strong receptor-independent infectivity compared to wt env while preserving infectivity on DF-1 cells, which suggests that the mutated env remained relatively stable. When added to other substitutions it also strongly enhanced receptor-independent infection; however, some of the triple or quadruple substitution variants displayed low infectivity on both types of cells alike the relevant substitution alone, meaning that the env-destabilizing effect of relevant substitutions was retained in the presence of L154S substitution. Infectivity of one triple substitution variant was so attenuated that we were forced to produce the virus with the help of a selection marker to be able to propagate and harvest the virus, yet we could clearly detect its infection of the receptor-less cells. These results show, again, that all three observed phenomena (extended host range or the ability to infect receptor-less cells, env destabilization, and ability to induce osteopetrosis) are connected and may have a common ground - self-priming.

Destabilization of A, C, and K subgroups

Many previous works, including our own, showed that the receptor-less entry is not limited to a specific subgroup. Therefore, we used receptor-less cells DF-1ΔTva and DF-1ΔTvc and

constructed A, C, and K subgroup viruses bearing MAV-2.O TM-specific substitutions L378S and A398T (we could not make A28T substitution since, in A, C and K subgroups, T is already present at the position 28). Then we tested the infectivity of these viruses on wt versus receptor-less cells (see figure B7).

The strongest effect was observed in the A and C subgroup vectors with L378S substitution present in the envelope glycoprotein. This substitution disrupted the viruses ability to efficiently infect DF-1 cells while providing successful infection of Tvc-null cells by the C subgroup virus. Though this finding was in conjunction with our previous project since L378S substitution is present in the H20-derived provirus, we observed no impact on the K subgroup viruses. Through incubation of our vectors in different temperatures, we also confirmed the destabilization of our C subgroup vectors. The fact we failed to detect any anomalies in the K subgroup substituted vectors implies this subgroup is special in terms of both stability and SU-TM cross-talk, offering a unique opportunity to further understanding of envelope glycoprotein mechanisms.

Possible role of self-priming in coevolution of env and receptor

Co-evolution of ALVs and their receptors still remains to be better documented. Nowadays envelope glycoproteins and glycoproteins of endogenous proviruses, together with their corresponding receptors, are the only remaining traces of the past evolution. A group of closely related glycoproteins that evolved from a common precursor can be most informative. By comparing such a set of glycoproteins with a corresponding set of receptors and by delineating their productive interactions, and by taking into account the presence, in relevant hosts, of endogenous proviruses carrying related envs, we can track down how arms race between the virus and its host proceeded.

Our results provided useful material for the study of env-receptor interactions. An example is our finding that the newly emerging subgroup K utilizes the same receptor as A subgroup but A and K env glycoproteins interact with Tva in a different way. A similar situation is represented by subgroups B, D, and E that recognize the same receptor, Tvb (Heather B. Adkins, Brojatsch, and Young 2000). In all these cases, a plausible scenario of how subgroups recognizing the same receptor originated is the following: to prevent interaction with the specific virus subgroup, the env-binding epitope on the host´s receptor has changed. The virus reacted by adapting its receptor-binding domain so that the interaction with the receptor was restored, binding the different epitope. This might also result in changes in host range, as different alleles are found across species, and thus fulfilling the criteria for establishing a new subgroup by an adapted virus. Such a scenario might apply not only on the changes in recognition of different epitopes of the same receptor, but also on the changes in different receptors recognition, as was simulated by Mark Federspiel (Mark J. Federspiel 2019)

Similarly, the phenomena of self-priming or extended host range, which we studied using RSV-H20 and MAV-2.O viruses, could play an essential role in the virus-host arms race. Uncoupling virus entry from receptor recognition would facilitate virus evolution as it provides the chance for the virus to adapt to changes in cells that attempt to prevent virus entry. The uncoupling, however, has a negative impact on virus viability as it is connected with env destabilization. Therefore, as soon as the virus is sufficiently adapted, natural selection favors more stable variants that, concurrently, lose the ability to enter the cells lacking a functional receptor.

In our experiments, we have found substitutions with different effects on envelope glycoprotein stability as well as on virus infectivity on receptor-less cells. The following table compares viruses carrying these substitutions with regard to the infectivity on DF-1 and DF-1ΔTvb cells as well as titer remaining after incubation at 44°C for 12 hours. By normalizing the infectivity on DF-1ΔTvb cells to infectivity on wt DF-1 cells we can set the effect of self-priming apart from the decrease of stability; the number obtained tells us what infectivity on DF-1ΔTvb cells we would see if the virus remained stable. The results clearly show the following: First, the reduced infectivity on DF-1 cells (row 1) correlates well with decreased stability as measured at 44°C (row 4). Second, the ability of self-priming (row 3) correlates well with env destabilization (row 4).

vector	P3	T1	T2	P3T1	P3T2	T12	P3T12	L154S	WT
DF-1	93%	21%	85%	50%	92%	82%	19%	94%	70%
DF-1 Δ Tvb	0.44%	0.55%	0.58%	0.35%	0.36%	0.37%	0.69%	54%	0.04%
DF-1 Δ Tvb normalized	0.47%	2.62%	0.68%	0.70%	0.39%	0.45%	3.63%	57%	0.06%
12h at 44°C	85%	37%	71%	60%	64%	84%	33%	n.d.	100%

We presume that there exists a perfect grade of env glycoprotein stability (in chemical terms, the optimal level of activation energy for priming). There are two reasons for this supposition. First, the trigger for receptor priming must be sensitive enough so that env, after interaction with the receptor, undergoes proper rearrangement with sufficient speed and efficiency. Second, there is a clear evolutionary advantage in having env glycoprotein just on the verge of instability; then, when functional receptors become unavailable, a single or a few substitutions can generate env capable of receptor-independent entry into the cells.

In this context, we have to take into account yet another factor. Retroviruses have a high rate of mutation and recombination, therefore every retroviral population is a mixture of many pseudo species. Under conditions of a high multiplicity of infection (a condition which is fulfilled in the infected chickens and especially in osteopetrotic bone) two pseudo species, one carrying a destabilizing mutation in env can meet the same cell. Such cells then produce env trimers which contain both stable and destabilized env. It is plausible to assume that the destabilizing effect is attenuated in heterotrimer which enables the destabilized env to escape selection pressure and maintain its presence (at a certain level) in the virus population. Moreover, the high recombination rate of retroviruses leads to a perpetual exchange of individual mutations between viruses; if destabilization requires more than one mutation, a pool of such mutations may remain in the virus population.

Connection between osteopetrosis and env destabilization

To explain how self-priming relates to osteopetrosis induction we have to combine two facts. Firstly, the virus capable of entering receptor-less cells is naturally also able to enter the cells that already have been infected with the same virus, in other words, to escape superinfection resistance. As a result, an unlimited number of viral particles can enter each cell. Secondly, hyperproliferative osteoblasts from osteopetrotic tissue are characterized by an extremely high load of unintegrated viral DNA (up to hundreds of copies). Of course, there are more questions than answers but no question remains a complete mystery. To

some questions we can bring forward plausible explanations, about some we can at least speculate and all of them can be solved experimentally.

We failed to conclude whether the excessive accumulation of viral DNA in osteopetrosis is a cause or consequence. We also failed to analyze the nature of LTR- and pol-derived substitutions on viral expression and integration. Furthermore, since the extended host range provided by SU- and TM-derived substitutions could be explained by env destabilization, we are failing to understand the nature of signal peptide-derived substitutions effects. The mutated env-B-Tvb interaction might also provide some information, as the Tvb receptor is reported to be a functional death receptor antagonized by the NF-κB pathway (Jürgen Brojatsch et al. 2000; Chi et al. 2002). Finally, it is also to be determined why viruses selected for the ability to enter receptor-less cells have relatively stable env (e.g. the env with L154S substitution), while viruses selected for their osteopetrotic potential mostly possess quite unstable env (e.g. the MAV-2.O env). The answer may lie in different conditions under which the two viruses replicate. Virus released by cultured cells diffuses into the media, spends most of its life far away from any cell, and needs plenty of time to succeed in contacting another cell; the long lifespan of env glycoprotein thus confers a great selective advantage. In contrast, the virus released by osteoblast is imprisoned in a small lacuna around the osteoblast and comes repeatedly into contact with the same cell from which it was released; thus, even short-lived env can fulfill its task. Indeed, electron microscopy pictures of osteopetrotic bone show lacunas densely packed with virions (Foster et al. 1994). Furthermore, MAV-2.O-derived may prove to be more fusogenic compared to L154S. Hyperfusogenocity was proved to be the cause of some measles-related fatal syndromes in humans (Jurgens et al. 2015). Since other substitutions in the TM subunit coupled with the extended host range (Amberg et al. 2006) and osteopetrosis (Barbosa et al. 2010) were reported, we expect to encounter even more promising substitutions worth analysis in the future.

Involvement of the student in publications involved in his Ph.D. thesis

Publication 1: Přikryl et al.:The Novel Avian Leukosis Virus Subgroup K Shares Its Cellular Receptor with Subgroup A. J. Virol. 93(17):e00580-19, 2019

First author (shared)

Analysis of infection of DF-1, DF-1-tva-/- and NIL-Tva cells, analysis of soluble Tva inhibition, manuscript preparation (materials and methods, results).
Infection of avian fibroblasts panel and interference assay were performed by Jiří Plachý, experiments *in vivo* were performed by Vít Karafiát.

Publication 2: Lounková et al.: Retroviral host range extension is coupled with Env-activating mutations resulting in receptor-independent entry. Proc. Natl. Acad. Sci. USA. 114(26):5148-5157, 2017

Collaborating author (third)

qPCR analysis, FACS analysis, thermo sensitivity assay, PERT assay.
Sequencing, cloning, infections, and acidic pH and PMB inhibition assay were performed by Anna Lounková, liposome binding assay was performed by Jan Kosla.

Data prepared for publication: MAV-2.O envelope-driven osteopetrosis

First author

PCR amplification of samples, virological assays, participation in DNA sequence analysis, DNA cloning, vector production, site-directed mutagenesis, thermo sensitivity assays, PERT assay.
Samples sequencing and comparative analysis was performed by Vladimír Pečenka, experiments *in vivo* were performed by Vít Karafiát.

I hereby confirm that the author of the thesis, David Přikryl, has substantially and inventively contributed to the publications listed above. In the case of his first-author publication, his role was seminal, he performed the major part of experimental work and contributed to the manuscript preparation.

RNDr. Jiří Hejnar, CSc.

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