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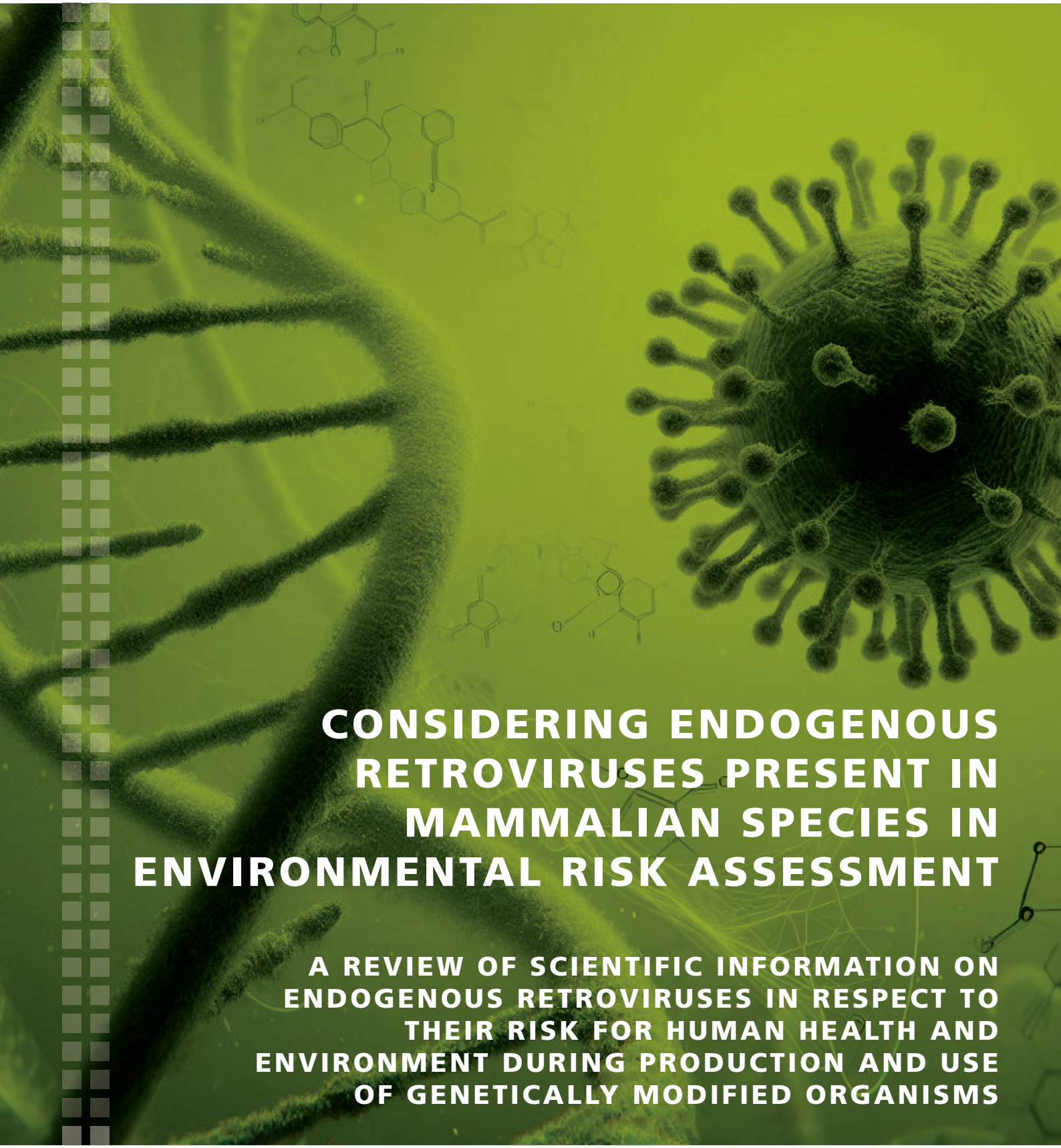

CONSIDERING ENDOGENOUS RETROVIRUSES PRESENT IN MAMMALIAN SPECIES IN ENVIRONMENTAL RISK ASSESSMENT

A REVIEW OF SCIENTIFIC INFORMATION ON ENDOGENOUS RETROVIRUSES IN RESPECT TO THEIR RISK FOR HUMAN HEALTH AND ENVIRONMENT DURING PRODUCTION AND USE OF GENETICALLY MODIFIED ORGANISMS

ONAFHANKELIJKE WETENSCHAPPELIJKE ADVISERING EN SIGNALERING VAN DE RIJKSOVERHEID

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Considering endogenous retroviruses present in mammalian species in environmental risk assessment

A review of scientific information on endogenous retroviruses in respect to their risk for human health and environment during production and use of genetically modified organisms

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Foreword

The recent COVID-19 pandemic and bird flu outbreaks have ensured that almost everyone knows how efficiently some viruses can spread. For most viruses with an RNA genome, like the COVID-19-causing coronavirus and the bird flu-causing influenza A virus, recovery is normally associated with clearance of the virus by the immune system of the infected host. For retroviruses, which also have an RNA genome, such a complete cure is however never reached. These viruses have the unique capacity to produce a DNA copy of their RNA genome and to integrate this DNA copy into the chromosomes of the host cells. As long as these cells survive or multiply, the integrated viral DNA persists and can be used as template for the production of new virus particles. A well-known example of such a retrovirus is the human immunodeficiency virus (HIV) that is responsible for the AIDS pandemic. While current antiretroviral drugs can effectively inhibit HIV replication and prevent progression to AIDS, the integrated virus is never completely cleared. As a consequence, people with HIV require life-long antiretroviral therapy to prevent viral rebound.

A special situation occurs when a retrovirus integrates in cells that are part of the germline, as the virus DNA will be inherited to the offspring of the host. Such germline integration events are likely to happen at a very low frequency and have thus far not been described for HIV in humans. However, it is generally accepted that such integrations happened during evolution for more ancient retroviruses in most - if not all - vertebrate species, including humans and other mammals. As a result, the genomes of these species have accumulated DNA fragments corresponding to the infecting retroviruses. We generally refer to these inheritable DNA fragments as endogenous retroviruses (ERVs) or endogenous retroviral elements, whereas retroviruses that are transmitted by production of virus particles in one host and subsequent infection of other hosts are referred to as exogenous retroviruses.

The presence of (nearly) complete ERVs and endogenous retroviral elements may affect the environmental risks associated with activities involving genetically modified organisms (GMOs) in a laboratory setting, or with the application of GMO medicinal products in a clinical setting. Therefore, COGEM commissioned a literature review that inventorizes scientific knowledge about ERVs and endogenous retroviral elements in human and animal cells, which can be used for environmental risk assessments and for the advisory reports that COGEM produces.

The literature review is presented in this extensive report. The advisory committee greatly appreciated the pleasant and constructive interaction with the authors. Recognizing the vast amount of predominantly descriptive information about endogenous retroviruses that can be found in literature, the committee is pleased that the authors succeeded in generating an overview of relevant information, which will be valuable for COGEM and others interested in these intriguing DNA sequences present in our and other vertebrate genomes.

Atze T. Das, chair of the advisory committee

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Summary

The presence of endogenous retroviruses (ERVs) is potentially a complicating factor in environmental risk assessments and may impact the risk classification of certain laboratory work. In order to facilitate future risk assessments on (materials containing) ERVs, **this study aimed at providing an overview of the scientific knowledge on ERVs, with particular focus on how they may become activated, what ERV products are typically formed upon activation and, subsequently, what the potential impact of this could be in view of biosafety**.

ERVs are present in the genome of vertebrates as assumed remnants of an infection with exogenous retroviruses that took place during evolution and for which integration has occurred in the genome of a germ cell. Depending on their time of integration and the mutations that occurred, different forms of ERVs have been identified. These range from solitary long-terminal repeats (solo LTRs) to complete proviral genomes in which most or even all retroviral elements are present. The majority of ERVs no longer code for functional proteins and are unable to form replication-competent virus. On the other hand, also ERVs exist with a role in mammalian physiology and pathology by affecting gene regulation and expression.

Taking the abundant presence of ERVs in vertebrates into account, it is highly likely that much of the biological material routinely used in biotechnology, including material used for genetic modification, carries ERVs. Depending on the intrinsic characteristics of the vertebrate host (cell), the type of ERV present and the experimental conditions, the ERV sequence may become activated. Activation could ultimately result in the production of infectious virus-like particles or replication-competent viruses. If such would occur, a subsequent risk may be the release of the particles and potential exposure of people or the environment.

A literature review was undertaken according to the typical consecutive steps of a systematic review. Based on the selected scientific publications, an extensive secondary search was performed to retrieve additional relevant publications. Most publications presented information on the role of specific ERVs in health and disease or on the evolution of ERVs. While some studies described features that may be relevant in view of risk assessment (e.g., formation of replication-competent virus (RCV), change in host tropism), none had the intention to study biosafety as such. Apart from the extensive literature review, additional risk considerations were identified in different advices and recommendations previously issued by COGEM, as well as on the websites of (inter)national organizations related to (bio)safety.

It was confirmed that ERVs are present in different forms in biological material routinely used in biotechnology. In brief, ERVs were described in nearly 100 types of cells, cell lines and tissues originating from different vertebrates including chickens, mice, hamsters, cats, pigs, ruminants, koalas, non-human primates, and humans. Activation of ERVs has been described to occur spontaneously or triggered by an external factor such as a chemical or physical agent, a non-retroviral infection or long-term passaging of host cells.

Most studies did not analyse ERVs beyond early stages of the retroviral life cycle as indicators for activation. Only in 30 of the described cases, particles were identified. In 10 out of 30 cases, infectious virus-like particles, i.e. particles capable of infecting cells, were identified. In 7 of these cases, the particles were also replication-competent, i.e. capable of producing additional virus particles / viral progeny. Activation of incomplete proviral sequences rarely led to the production of infectious virus-like particles or RCVs, because this would require complementation (protein level) and/or recombination (genetic sequences) with other ERVs or exogenous retroviruses.

Inversely, ERVs can complement or recombine with wildtype retroviruses which in turn may affect the characteristics of the wildtype retrovirus, such as its tropism. Finally,

complementation or recombination of ERV elements with replication-incompetent retroviral vectors may result in regaining replication competence.

Taking into account that most ERVs are not known to be pathogenic to their hosts and, under natural conditions, cannot form exogenous viruses or infect other hosts, a pathogenicity class 1 may be considered. However, in practice risk assessors typically should consider additional factors for a risk assessment, such as:

- Replication competence
- Full length versus partial presence
- Gain-of-function mutations
- Tropism
- Impact on (cellular) physiology of the host
- Insertional mutagenesis
- Immunosuppressive effect linked to a high viral load

Regulatory frameworks require considering the kind of ERVs being present in the source material. Different guidelines stipulate a cautionary, fact-based testing approach for the assessment and clearance of ERV particles in biological materials used in medicinal formulations. Steps have been taken both in research and development as well as in medical applications, to limit the expression and the presence of -relatively complete- ERVs as much as possible. These efforts are focused on specific ERV in well-defined activities e.g. in vaccine production, acknowledging that for a major part of ERVs, no function and/or negative effect has been identified so far.

Taking all of the above into account, following conclusions are made:

- Endogenous retroviruses (ERVs) are omnipresent in the animal kingdom and are therefore likely present in all sources of material (e.g., tissues, primary and established cell lines);
- Whether incomplete or intact, ERVs contained within an organism are unlikely to cause harm to the host and environment by themselves;
- Through manipulations (e.g., culturing, xenotransplantation) ERV sequences may become activated which could ultimately result in the production of infectious virus-like particles and replication-competent viruses, though the potential environmental impact remains unclear
- A thorough risk assessment (provided in this report) can aid in assigning the appropriate pathogenicity group to the ERV(s) and its originating source material.

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Samenvatting

De aanwezigheid van endogene retrovirussen (ERV's) is een potentieel complicerende factor bij de milieurisicobeoordeling en kan gevolgen hebben voor de risico-inschaling van bepaalde laboratoriumwerkzaamheden. Om toekomstige risicobeoordelingen van (materialen die) ERV's bevatten te vergemakkelijken, heeft **deze studie als doel een overzicht te geven van de wetenschappelijke kennis omtrent ERV's, met in het bijzonder aandacht voor de manier waarop ze geactiveerd kunnen worden, welke ERV-producten typisch gevormd worden bij activering en wat hiervan de potentiële impact zou kunnen zijn vanuit bioveiligheidsoogpunt.**

In het genoom van gewervelde dieren komen ERV's voor als vermoedelijke restanten van infecties met exogene retrovirussen die in het verleden hebben plaatsgevonden en waarvan de integratie in het genoom van een kiemcel heeft plaatsgevonden. Afhankelijk van het tijdstip van integratie en de mutaties die in de loop van de evolutie zijn opgetreden, zijn verschillende vormen van ERV's geïdentificeerd, variërend van solitaire *long-terminal repeats* (solo LTR's) tot volledige provirale genomen waarin de meeste of zelfs alle retrovirale elementen aanwezig zijn. De meeste ERV's coderen niet langer voor functionele eiwitten en zijn niet in staat een replicatiecompetent virus te vormen. Maar er zijn ook meerdere ERV's bekend die een rol spelen in zowel fysiologische als pathologische processen van zoogdieren doordat zij de genregulatie en -expressie beïnvloeden.

Gezien de uitgebreide aanwezigheid van ERV's bij gewervelde dieren, is het zeer waarschijnlijk dat veel van het biologisch materiaal dat routinematig in de biotechnologie wordt gebruikt, met inbegrip van materiaal voor genetische modificatie, ERV's bevat. Afhankelijk van de intrinsieke kenmerken van de gastheer (cel), het aanwezige type ERV en de experimentele omstandigheden, kan de ERV-sequentie geactiveerd worden. Activatie zou uiteindelijk kunnen resulteren in de productie van infectieuze virusachtige deeltjes en replicatiecompetent virus. Indien dit laatste zou gebeuren, dan zou een bijkomend risico kunnen zijn dat deze infectieuze virusdeeltjes vrijkomen met mogelijke blootstelling van mensen of het milieu.

Een literatuurstudie werd uitgevoerd volgens de typisch stappen van een systematische analyse. Na het uitvoeren van de eerste uitgebreide literatuurstudie werd bijkomend gezocht naar relevante publicaties. De meeste publicaties gaven informatie over de rol van specifieke ERVs in gezondheid en ziekte of over de evolutie van ERVs. Sommige studies beschreven kenmerken die relevant kunnen zijn voor een risicobeoordeling (bv. vorming van replicatiecompetent virus (RCV), verandering in gastheertropisme), maar geen van de studies had tot doel de bioveiligheid van ERVs als zodanig te bestuderen. Naast de wetenschappelijke literatuur werd bijkomend gezocht naar risico-overwegingen in verschillende adviezen en aanbevelingen eerder uitgevaardigd door COGEM, evenals in de informatie beschikbaar via de websites van (inter)nationale organisaties die betrokken zijn bij (bio)veiligheid.

Uit het literatuuronderzoek is gebleken dat ERV's in veel verschillende vormen aanwezig zijn in biologisch materiaal dat routinematig in de biotechnologie wordt gebruikt. Zo werden ERV's beschreven in ongeveer 100 verschillende celtypen, cellijnen en weefsels afkomstig van verschillende gewervelde dieren, waaronder kippen, muizen, hamsters, katten, varkens, herkauwers, koala's, niet-humane primaten en mensen. Er werd zowel melding gemaakt van spontane activatie van ERVs, als activatie als gevolg van externe factoren zoals chemische en fysische agentia, niet-retroviral infectie van cellen, alsook het langdurig in passage houden van gastheercellen.

De analyse in het merendeel van de studies ging niet verder dan de vroege stadia van de retrovirale levenscyclus als indicator voor activering. In dertig gevallen konden partikels worden aangetoond. Tien van de dertig bleken infectieuze virusachtige partikels te zijn, met andere woorden, partikels die cellen kunnen infecteren. In zeven gevallen was er ook sprake van replicatiecompetentie, met andere woorden, de partikels bleken niet alleen in staat nieuwe

cellen te infecteren maar konden ook repliceren met productie van nieuwe viruspartikels tot gevolg. Activatie van onvolledige provirale sequenties leidde zelden tot de productie van infectieuze of replicatiecompetente virale partikels aangezien belangrijke deleties en/of mutaties in het virale genoom enkel hersteld kunnen worden door complementatie (op eiwitniveau) en/of recombinatie (op genniveau) tussen ERV's of exogenene retrovirussen.

Het omgekeerde is ook mogelijk, waarbij ERV's door complementatie en/of recombinatie de eigenschappen van wildtype retrovirussen kunnen beïnvloeden, zoals bijvoorbeeld hun tropisme. Tot slot kunnen ERV's door complementatie of recombinatie zorgen dat replicatiedeficiënte retrovirale vectoren weer replicatiecompetent worden.

Aangezien van de meeste ERV's niet bekend is dat zij pathogeen zijn voor hun gastheer en onder natuurlijke omstandigheden geen exogene virussen kunnen vormen of andere gastheren kunnen infecteren, kan een pathogeniteitsklasse 1 overwogen worden. Risicobeoordelaars moeten in de praktijk voor een risicobeoordeling doorgaans echter ook andere factoren in aanmerking nemen, zoals

- Replicatiecompetentie
- Volledige vs. gedeeltelijke aanwezigheid van het ERV
- Mutaties waardoor het viruspartikel nieuwe functies verwerft
- Tropisme
- Effect op de (cellulaire) fysiologie van de gastheer
- Insertionele mutagenese
- Immunosuppressief effect geassocieerd met hoge virale lading

Verschillende (wettelijke) richtlijnen beschrijven een op voorzorg en feiten gebaseerde aanpak om de (impact van een) mogelijke aanwezigheid van ERV-deeltjes in medicinale producten (of hun uitgangsmaterialen) te beoordelen, om eventuele maatregelen te implementeren om de deeltjes te verwijderen. Daarnaast zijn in onderzoek en ontwikkeling verschillende studies beschreven waarin onderzocht wordt hoe de expressie en de aanwezigheid van - relatief volledige - ERV's zoveel mogelijk beperkt kan worden. De studies zijn gericht op specifieke ERV's in welbepaalde toepassingen zoals bijvoorbeeld productie van vaccins, waarbij wordt erkend dat voor een groot deel van de ERV's tot nu toe geen functie en/of negatief effect is vastgesteld.

Tot slot kunnen volgende conclusies worden getrokken:

- Endogene retrovirussen (ERV's) zijn alom aanwezig in het dierenrijk en hun aanwezigheid is bijgevolg waarschijnlijk in verschillende vormen van (uitgangs)materiaal gebruikt in de biotechnologie (bv. weefsels, primaire cellen, cellijnen);
- Voor zowel intacte als partiële ERV's die aanwezig zijn in de gastheer(cel) is het onwaarschijnlijk dat deze een gevaar vormen voor de gastheer of de omgeving;
- Door manipulatie (bv. kweek, xenotransplantatie) kan activatie van ERV's plaatsvinden die uiteindelijk zou kunnen resulteren in de productie van infectieuze virusachtige deeltjes en replicatiecompetente virussen, al is het eventuele milieurisico nog onduidelijk;
- Een grondige risicoanalyse (zoals vermeld in het rapport) is noodzakelijk om ERV's en het (uitgangs)materiaal waarin de ERV's aanwezig kunnen zijn in te delen in een pathogeniteitsklasse.

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Abbreviations

ABSA	ABSA International - Association for Biosafety and Biosecurity
ALV	Avian leukosis virus
ASCT	Sodium-dependent neutral amino acid transporter
BaEV	Baboon endogenous virus
BBB	Blood-brain-barrier
bHLH	Basic helix-loop-helix
BoERV	Bovine endogenous retrovirus
c-myb	Animal cellular myoblastosis transcription factor
CA	Capsid
Cas	CRISPR-associated protein
CDC	Centers for Disease Control and Prevention
CEUCO	French Expert Committee for contained use of GMOs Comité d'expertise des utilisations confinées d'OGM
CHMP	Committee for Medicinal Products for Human Use
COGEM	Commissie Genetische Modificatie
CpG	Cytosine-Guanine repeat
CRFK	Crandell-Rees Feline Kidney Cell
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CHMP	Committee for Medicinal Products for Human Use (EMA)
CVMP	Committee for Medicinal Products for Veterinary Use (EMA)
DNA	Deoxyribonucleic acid
EBSA	European Biosafety Association
EM	Electron microscopy
EMA	European Medicines Agency
EMSA	Electrophoretic mobility shift assay
enFeLV	Endogenous feline leukaemia virus
Env	Envelope
ERE	Oestrogen responsive element
ERV	Endogenous retrovirus
ERV-DC	Domestic cat endogenous retrovirus
EST	Expression sequence tag
FACS	Fluorescent activated cell sorting
(Ex)FeLV	(Exogenous) feline leukaemia virus
FISH	Fluorescent in-situ hybridisation
G1/S	Growth-cycle 1 / DNA Synthesis checkpoint
Gag	Group-specific antigen
GFP	Green fluorescent protein
GM	Genetically modified
HERV	Human endogenous retrovirus
HIV	Human immunodeficiency virus
HSV-1	Herpes simplex virus type 1
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IHC	Immunohistochemistry

IFN	Interferon
IL	Interleukin
IN	Integrase
iNOS	Inducible nitric oxide synthase
IRES	Internal ribosome entry site
ISD	Immunosuppressive domain
IVMP	Immunological veterinary medicinal product
LINE	Long interspersed nuclear element
LTR	Long terminal repeat
MA	Matrix
MCB	Master Cell Bank
MCC	Merkel cell carcinoma
MERV	Melanoma-associated endogenous retrovirus
MLV	Murine leukaemia virus
MMTV	Mouse mammary tumour virus
Mn^{++}	Manganese cation
MOG	myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSRV	Multiple sclerosis-associated retrovirus
MuERV	Murine endogenous retrovirus
MuLV	Murine Leukaemia Virus
M-MuLV	Moloney murine leukaemia virus
NC	Nucleocapsid
NCBI	National Centre for Biotechnology Information
Nef	Negative regulatory factor
NGS	Next Generation Sequencing
NIH	National Institutes of Health
NJ	Neighbour joining
NLM	U.S. National Library of Medicine
ORF	Open reading frame
PBS	Primer binding site
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PERT	Product-enhanced reverse transcriptase
PERV	Porcine endogenous retrovirus
Pol	Polymerase
Poly-A	Poly-adenylation
PR	Protease
PRR	Pathogen recognition receptor
qPCR	Quantitative real-time polymerase chain reaction
RT-(q)PCR	Reverse transcription (quantitative real-time) polymerase chain reaction
R&D	Research and development
RCV	Replication-competent virus
RCR	Replication-competent retrovirus
RNA	Ribonucleic acid
RRE	Rev response element

RT	Reverse transcriptase
RV	Retrovirus
RXR-α	Retinoid X receptor alpha
SAG	Scientific Advisory Group (EMA)
SCNT	Somatic cell nuclear transfer
SG-PERT	SYBR-Green PERT
shRNA	Short-hairpin RNA
siRNA	Small interfering RNA
SMIA	Suspension multiplex immunoassay
SRV	Simian retrovirus
STF-PERT	Single tube fluorescent PERT
SU	Soluble
Syn1	Syncytin-1
TALEN	Transcription activator-like effector nucleases
TAR	Trans-activation response element
TASA-TD PCR	TAG-aided sense/antisense detection PCR
Tat	Trans-activator of transcription
TEM	Transmission electron microscopy
TF	Transcription factor
TFBS	Transcription factor binding site
TLR	Toll-like receptor
TM	Transmembrane
TM-PERT	TaqMan fluorescent probe-based PERT
TNF	Tumour necrosis factor
tRNA	Transfer ribonucleic acid
U3	3-prime untranslated region
U5	5-prime untranslated region
UNG RT-qPCR	Uracil-N-glycosylase-coupled RT-qPCR
Vif	Viral infectivity factor
VLP	virus-like particle
VP	Viral particle
Vpr	Viral protein R
Vpu	Viral protein U
WB	Western blot
WHO	World Health Organization
XMRV	Xenotropic murine leukaemia virus-related virus
ZKBS	German Central Committee on Biological Safety Zentrale Kommission für die Biologische Sicherheit

Glossary

Non-infectious virus-like particle	Particles that closely resemble viruses but that are non-infectious (for example, because they contain no/incomplete viral genetic material)
Infectious virus-like particle	Virus particles capable of infecting cells but unable to subsequently produce new virus particles / viral progeny
Replication-competent virus (RCV)	Virus particles capable of infecting of and replicating in cells to produce new virus particles / viral progeny

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Introduction

So-called endogenous retroviruses (ERVs) are present in the genome of vertebrates. It is assumed that these are remnants of retroviruses from infections that took place in the past and for which integration has occurred in the genome of a germ cell. In some animal species, even (virtually) intact ERVs are found, in which most or even all retroviral elements are present. The majority of ERVs no longer code for functional proteins and are unable to form an infectious and/or replication-competent virus. However, quite a few of the ERV sequences have now been attributed important roles in the regulation of gene expression, for example in placental formation and human embryo development, but also in several pathologies such as neurodegenerative conditions.

When using a host (cell or animal) in which ERVs are present, there is a risk that these ERVs are activated during experimental activities (e.g., upon exposure to mutagenic agents, during prolonged cell culture or by reactivation under the influence of other viruses). Moreover, when using a retroviral vector, this vector could recombine with ERV sequences or be complemented by ERV proteins (or vice versa) allowing the formation of replication-competent virus particles (RCVs). For RCVs, a subsequent but so far theoretical risk may be the release in the environment and potential exposure of people. Finally, even if ERV sequences are not activated or expressed during the experimental procedures, they may still pose a potential risk after accidental (e.g., during experimental procedures) or deliberate (e.g., after xenotransplantation or gene therapy) exposure to the material in which they are present. Although such risks have so far only been studied for ERVs intrinsically present in a host (cell) and not upon exposure to ERV containing material.

The above suggests that the presence of ERVs is a potential complicating factor in environmental risk assessments that may impact the risk rating of certain laboratory work.

This project reviewed the available scientific information to gain insights in the presence of ERVs in materials derived from human and animal origin, and on the potential related risks for humans and the environment. In consultation with the Advisory Committee, the research questions were further defined as:

- What is known about the presence of ERVs in biological material routinely used in biotechnology (e.g., cells, animals)?
- Are ERVs capable of independently generating a fully infectious virus and if so, what are the conditions that facilitate or promote such generation?
- What other types of ERV activation can occur and what is the potential risk of such activation?
- Can infection or transduction of ERV containing material with a virus / viral vector result in activation / complementation / recombination to establish an infectious (chimeric) virus?
- What are indications for classifying ERVs to a risk group (pathogenicity class), acknowledging that most ERVs are not pathogenic when they are not expressed?
- What precautionary approaches have been described towards managing potential risks of ERVs?

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1 Methods

1.1 Literature study

Based on the questions formulated in the mandate for this study and further refined with the input of the Advisory Committee, a literature review was undertaken. Search strings were formulated, based on the following keywords:

- Endogenous retrovirus / biosafety / risk / adverse effect / safety / environment
- Heterologous recombination / endogenous retrovirus
- Homologous recombination / endogenous retrovirus

With these keywords, different queries were composed using Boolean operators and these were submitted to the different bibliographical databases and using a time frame from 2012 to 2022. Three electronic bibliographic multi-disciplinary databases were chosen to **search for relevant publications**:

- **Web of Science™** core collection[1] consists of six online databases indexing scholarly books, peer reviewed journals, original research articles, reviews, editorials, chronologies, abstracts, as well as other items. Disciplines included in this index are agriculture, biological sciences, engineering, medical and life sciences, physical and chemical sciences, and many others. The database contains 1.4 billion cited references going back to 1900.
- **Scopus®** [2] by Elsevier is an abstract and citation database of peer-reviewed literature, including scientific journals, books and conference proceedings, covering research topics across all scientific and technical disciplines, ranging from medicine and social sciences to arts and humanities. Scopus® is updated daily and includes over 71 million records and over 1.4 billion cited references after 1970.
- **PubMed®** [3] is a free resource supporting the search and retrieval of biomedical and life sciences literature with the aim of improving health, both globally and personally. The PubMed database contains more than 33 million citations and abstracts of biomedical literature. Available to the public online since 1996, PubMed was developed and is maintained by the National Center for Biotechnology Information (NCBI), at the U.S. National Library of Medicine (NLM), located at the National Institutes of Health (NIH).

Each search was expected to result in the identification of publications in English or, if in another language, having a title, abstract and/ or keywords in English. Initially, only original articles were searched for. In case the number of articles on a specific topic was large, relevant review articles were consulted.

The literature search was concluded on the 16th of December 2022. In total, 1171 scientific publications were identified.

Table 1 contains an overview of the different queries of the systemic literature search and their corresponding hits per database.

In the first stage of **selection**, the title, keywords and abstract of the retrieved references were screened. This resulted in a reduction of the number of potentially relevant publications. Of the selected references an attempt was made to retrieve a full text document, after which the full content was examined. The references of the included studies were manually screened to search for further papers. No language or publication restrictions were applied, and studies were not selected based on quality.

The full text documents were further screened for relevant data and the final set of publications was selected. The **key findings** of the selected, full text papers were then summarized including, but not limited to, information on the occurrence of ERVs, their role in physiology and pathology as well as their potential risks during phenomena such as recombination.

[1] https://clarivate.com/products/web-of-science/databases/
[2] https://www.scopus.com
[3] https://pubmed.ncbi.nlm.nih.gov

Table 1 Overview of query hits per consulted bibliographical database

Query	Scopus®	Web of Science™	NCBI PubMed®	Total	Doubles removed
(endogenous retrovir*) AND (biosafe* OR risk OR adverse effect OR safe* OR environment)	324	644	586	1554	838
((heterol*) AND (recombin*)) AND (endogenous retrovir*)	73	9	7	89	73
(heterologous recombination) AND (endogenous retrovirus)	73	4	73	150	
homol* AND recombin* AND endogenous retrovir*	251	82	76	409	277
((homol*) AND (recombin*)) AND (endogenous retrovir*)	251	82	234	567	
Total					**1171**

Additional publications were retrieved until May 15, 2023, and were based on reference lists of publications identified in the primary literature study, on reference lists in different advices and guidelines, and on internet searches using terms relevant for the current study.

1.2 Survey of advisory documents and recommendations

Further insight in the role and risks of ERVs was obtained through an analysis of advices and recommendations related to (bio)safety issued by COGEM. In addition to COGEM advices, a limited number of documents were retrieved from:

- the European Medicines Agency (EMA),
- the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH),
- the German Central Committee on Biological Safety (Zentrale Kommission für die Biologische Sicherheit, ZKBS), and
- the French Expert Committee for contained use of GMOs (Comité d'expertise des utilisations confinées d'OGM, CEUCO).

2 Retroviruses

Before focusing on the roles and potential risks of ERVs, in particular when performing activities in the context of genetic modification, we would like to provide the reader with some background information on retroviruses in general. Acknowledging that this is a broad and dynamic scientific field of interest beyond the scope of this report, this short overview will point out the topics relevant for this study.

The information in this section is based on what was previously published in report CGM 2023-01 that covered the detection of replication-competent virus formation during production and use of lenti- and gammaretroviral vectors.

2.1 Classification of retroviruses

Retroviruses belong to the family of *Retroviridae*. This family consists of two subfamilies: *Orthoretrovirinae* and *Spumavirinae*. In the context of this review, the focus will be on genera within the subfamily of *Orthoretrovirinae*. Further focusing on humans and mammals, only the alpha-, beta-, delta-, and gammaretroviruses, as well as lentiviruses are described. Epsilonretroviruses are outside the scope of the current study. Although some are known to cause several important diseases in fish, they have not been described in mammals (Brown et al., 2014; Holzschu et al., 2003; J. C. Leong, 2008) . Also, epsilon-like retroviruses are not discussed further as they have been identified in only very few mammalian species (Brown et al., 2014)

2.2 Retroviral structure

All retroviruses share a common virion appearance (Figure 1), consisting of two main compartments, surrounded by a protein coat. In the interior, the virus harbours the single-stranded RNA genome, which is associated with nucleoproteins. The nuclear content is surrounded by the capsid which is, in turn, surrounded by a phospholipid bilayer coat, the envelope, which is derived from the host. On the envelope, viral envelope proteins are present that serve as epitope in docking with a cognate receptor (Coffin, 1992).

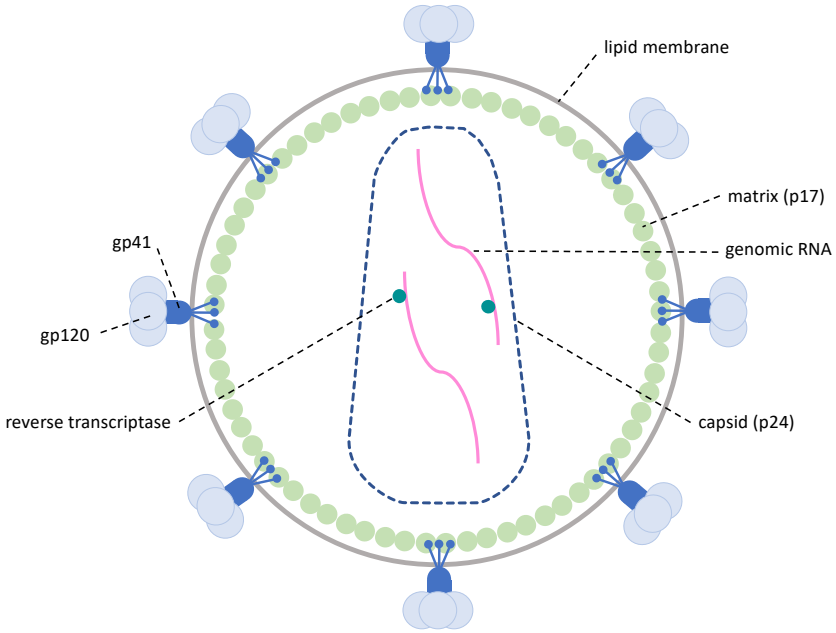

Figure 1. Schematic representation of an HIV particle, a representative member of the *Retroviridae*

Based on: http://microbialcell.com/figure-2-hiv-aids-pandemic/ and copied from report CGM 2023-01

Depending on their genetic structure, retroviruses can be divided into simple and complex retroviruses. **HIV**, a representative member of a complex retrovirus, is shown in Figure 1. It consists of a lipid bilayer membrane or envelope that surrounds the nucleocapsid. Within the capsid, two copies of the genomic RNA are present. During reverse transcription, catalysed by the retroviral polymerase, a double-stranded DNA intermediate is formed, which then stably integrates into the genome of the infected cell (provirus).

The HIV genome is about 10 kb long and can be divided into three major open reading frames: *gag*, *pol* and *env* (Figure 2a). The *gag* gene encodes the group-specific antigen, which is proteolytically processed into four structural proteins: matrix (MA), capsid (CA), nucleocapsid (NC) and p6. The *pol* gene encodes for a polyprotein which is processed during virion maturation into three enzymes, i.e. protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene is translated into the envelope protein(s) composed of a surface and transmembrane domain, which in lentiviruses corresponds to gp120 and gp41 protein, respectively. The genome also contains long terminal repeats (LTR) being repetitive sequences that occur at both termini of the integrated DNA provirus and harbour the enhancer, promoter and poly-adenylation signals.

Apart from the proteins mentioned above, the lentiviral genome encodes additional essential and accessory proteins (Lindemann et al., 2013). For HIV-1, these include the Vif, Vpr, Vpu and Nef proteins, which have an accessory function, and the Tat and Rev proteins, which have an essential regulatory function in viral RNA production.

The genome of the **murine gammaretrovirus**, is representative for the simple retroviruses. It is flanked by LTRs and contains the major open reading frames *gag*, *pol* and *env* (Figure 2b). However, it lacks the genetic sequences coding for the other proteins.

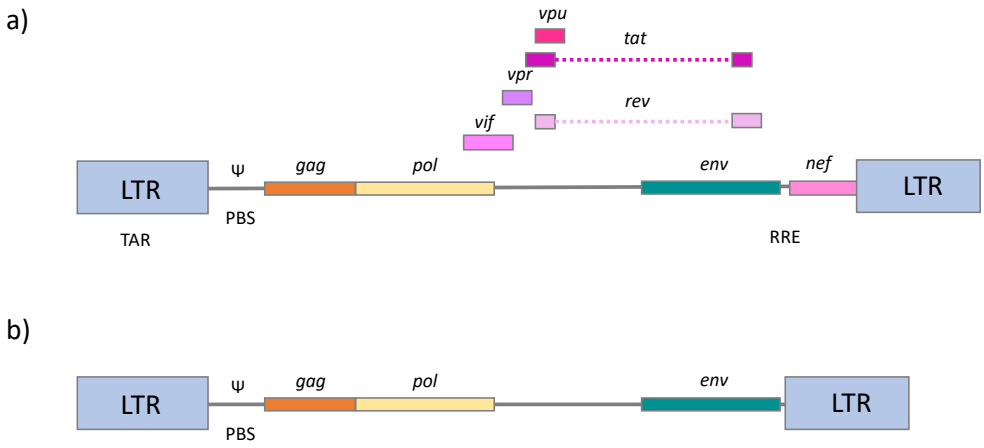

Figure 2. Schematic representation of the integrated retroviral genome

PBS (primer binding site) denotes the binding site for the tRNA primer (needed for reverse-transcription initiation); Ψ marks the packaging signal; gag (group specific antigens, matrix, capsid and nucleocapsid proteins), pol (protease, reverse transcriptase and integrase) and env (envelope proteins) are coding regions; LTR (long terminal repeats) contain repetitive sequences that occur at both termini of the integrated DNA provirus.

a) Complex retrovirus using the example of human immunodeficiency virus 1 (HIV-1). Several regulatory elements, such as the PBS, LTRs, Ψ, trans-activation response element (TAR) and rev response element (RRE), are indicated. In addition to the genes encoding the Gag, Pol and Env proteins, HIV-1 has reading frames for the regulatory proteins Rev and Tat (unspliced variants are marked by the dotted lines), and accessory proteins Vif, Vpr, Vpu and Nef.

b) Murine leukaemia virus (MLV)

Based on: General position statement of the ZKBS on frequently carried out genetic engineering operations based on the criteria of comparability: Stable and transient gene expression using γ-retroviral and lentiviral vectors. (ZKBS, 2011)[4] and copied from report CGM 2023-01.

2.3 Virus replication

A schematic picture of the retroviral replication cycle is shown in Figure 3.

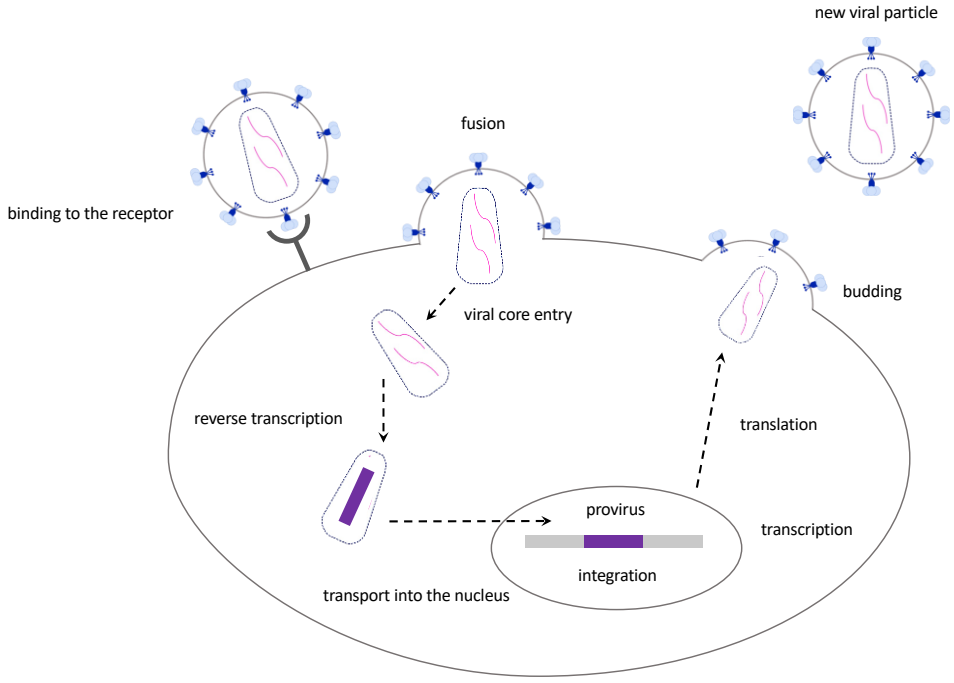

Figure 3. Schematic representation of the retroviral replication cycle

Based on: Shalev et al. (2009) and adapted from report CGM 2023-01

The retroviral replication cycle starts with binding of the virus to the host cell surface. This binding is receptor-mediated and involves the envelope proteins of the retroviral particle on the one hand and host cell receptors on the other hand. The capsid then enters the cell, either by fusion of the viral and cellular lipid membranes or by transition through the endosome. Subsequently, the capsid partially disintegrates, and the retroviral protein called reverse transcriptase transcribes the viral RNA into a double stranded DNA intermediate. The viral DNA is transported into the nucleus, where the retroviral protein integrase integrates the viral DNA into the host's DNA.

The transcription machinery of the host transcribes the viral DNA into multiple copies of new viral RNA. Part of the nascent RNA forms the genome of new viral particles, whereas other copies are used by the cell to make new retroviral proteins. Hereby, gene expression is driven by promoter regions embedded in 5' LTR regions, which include upstream transcription factor binding motifs to enhance or repress viral transcription. Non-canonical translation through internal ribosome entry sites (IRES) has been observed for a variety of retroviruses (Balvay et al., 2009). Other genetic elements (e.g., promoter regions, transcription factor binding sites, regulatory elements, capping signals, or poly-adenylation signal) may also play a role in regulation of gene expression, depending on the type of virus. A detailed description remains outside the scope of this report. Therefore, the reader is referred to e.g., Zhang et al. (2022) and Xiang & Liang (2021). The new viral RNA and proteins assemble to form an immature viral particle

[4] https://www.zkbs-online.de/ZKBS/SharedDocs/Downloads/01_Allgemeine%20Stellungnahmen/10_Viren/Retrovirus_K113_humanes_endogenes_2011.pdf?__blob=publicationFile&v=2

that leaves the cell by budding. The retroviral protease finally cleaves newly synthesized polyproteins to form a mature infectious virus particle.

Lentiviruses are capable of productively infecting both dividing and non-dividing cells. On the other hand, simple retroviruses are either infecting dividing or non-dividing cells: while productive gammaretrovirus infection is limited to dividing cells, alpha-, beta- and deltaretroviruses show a preference to infect non-dividing over dividing cells. Unlike alpha- (Yamashita & Emerman, 2006) and deltaretroviruses (Pluta et al., 2020), betaretroviruses do not require any cell-cycle progression to successfully infect the host (Konstantoulas & Indik, 2014).

3 Endogenous retroviruses

3.1 Introduction

As described above, retroviral infection leads to proviral insertion into a limited number of cells of the infected individual. Production of new infectious virions subsequently results in horizontal transmission of the virus to other host (cells). Occasionally, a retrovirus infects germline cells and becomes endogenized. As a result, both somatic and germline cells will now carry a copy of the ERV in its proviral format, and the viral genome can be directly transmitted during the replication cycle of a cell, without requiring production of virus particles. Such includes vertical (Mendelian) transmission (Figure 4) (reviewed by e.g., Lavialle et al. (2013) between hosts, but will also occur when cells or cell lines containing ERVs are duplicated. ERV loci may subsequently increase their copy number within the host species genome, either by reinfection of germ line cells or by retrotransposition within them, leading to the generation of multicopy ERV lineages.

In humans alone, at least 8% of the genome has been shaped by ERVs over the course of a quarter of a million years of human evolution (Lander et al., 2001). But also in other mammalian and non-mammalian species, ERVs are widespread. For example, in mice it is estimated that up to 10% of the genome contains ERVs (Brent et al., 2002; Stocking & Kozak, 2008).

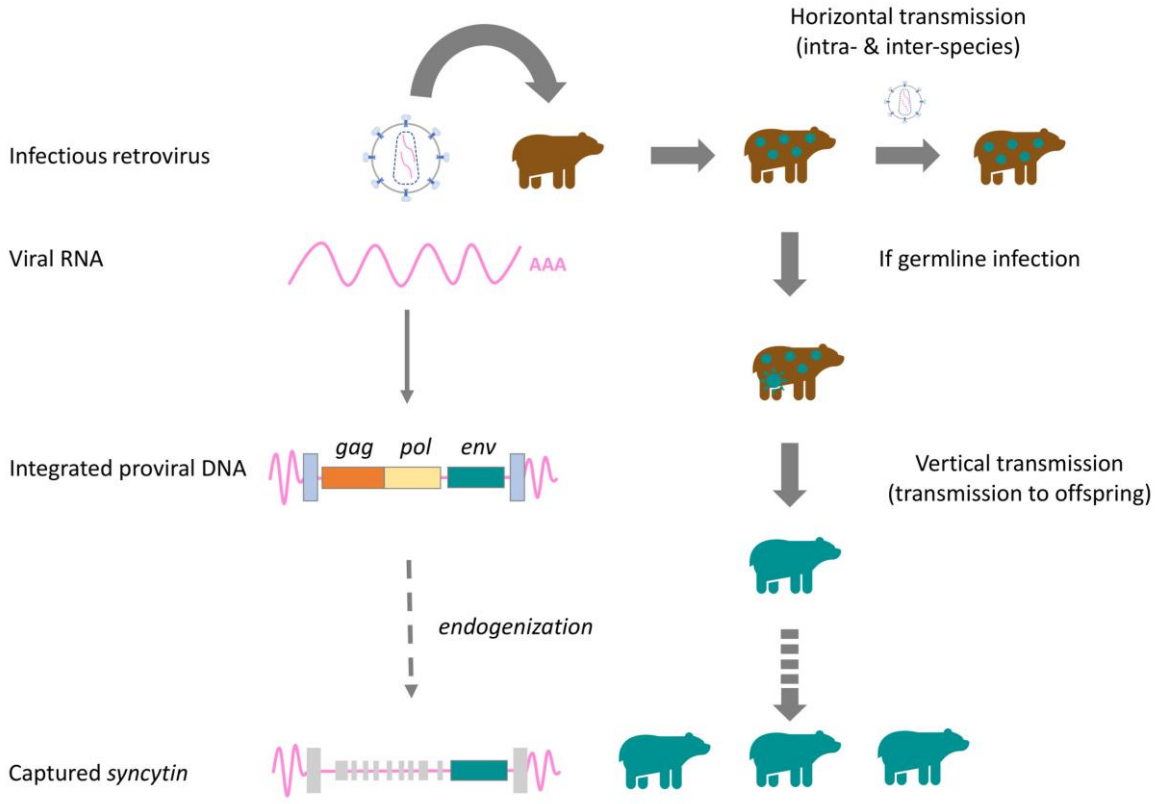

Figure 4. Schematic representation of the integration resulting in an ERV

Infectious retroviruses are required to integrate in the genome of the host to complete their replication cycle. Autosomal integrations lead to intra- and interspecies horizontal transmission. Integration in germ cells however promotes vertical transmission, leading to the offspring carrying a copy in all cells (somatic and germline). Selective pressure on the now endogenous retrovirus may favour the capture of specific retroviral proteins or other retroviral elements, which may give the host a selective advantage. The illustrated example is a capture of the envelope gene, encoding Syncytin. The capture (also referred to as 'domestication') of Syncytin contributed to mammalian development, being crucial in placental development.

Based on: Lavialle et al. (2013)

Throughout evolution, the proviral ERV sequences have accumulated mutations such as inversions, insertions, and deletions thereby preventing viral assembly and horizontal transmission (reviewed by e.g., Gifford et al. (2018). Also, recombination events between the LTR regions flanking proviral sequence have occurred, predominantly leading to the formation of 'solo LTR' elements, whereby the proviral coding sequence has been deleted (Jern & Coffin, 2008; Tsangaras et al., 2015). The host cell has gained control over these retroviral insertions by virtue of nucleotide substitutions (Dörrschuck et al., 2008) and methylation of promoter regions allocated to retroviral LTR elements (Y. D. Jung et al., 2013). While many mutations will compromise the infectivity of the virus, abolish the formation of retroviral particles, and prevent the production of viral proteins, some ERV sequences continue to have an impact on the host (cell), more specifically by affecting the regulation of gene expression and/or viral protein expression. Viral proteins may even become 'domesticated' by the host or, in other words, become a benefit to the host. Key examples are the *env* gene encoded envelope proteins Syncytin-1 and Syncytin-2. Both proteins play a physiological role in the syncytiotrophoblast formation in placental development (reviewed by e.g., Grandi & Tramontano (2018); Lavialle et al. (2013)).

3.2 Classification of ERVs

In contrast to exogenous retroviruses, ERVs are not formally included in the classification system of retroviruses. Still, they can be broadly classified into three classes, based on the relatedness to exogenous genera (Figure 5):

- Class I are most similar to the gamma- and epsilonretroviruses;
- Class II are most similar to the alpha-, beta-, deltaretroviruses and lentiviruses;
- Class III are most similar to the spumaviruses.

The above-mentioned approach of classifying ERVs is mainly based on repeated elements, such as LTRs, which allows association with the exogenous retroviral classes. ERVs are further segregated into families, whereby the species 'signature' is represented by one or two letters (e.g., human: HERV; porcine: PERV; murine: MuERV; domestic cat: ERV-DC; etc.). Each family clusters together specific subtypes of ERVs, often based on sequence similarity (reviewed by e.g., Gifford et al. (2018)).

Additionally, HERV-families are further divided based on the tRNA responsible for priming reverse-transcription at the primer binding site (PBS). Examples are HERV-K (lysine), HERV-W (tryptophan), and HERV-H (histidine). Additional approaches for classification have been based on proviral copy numbers, and/or amino acid motifs to assign ERV-nomenclature.

Still, classification schemes are not complete and often fail to account for the species where the ERV was originally found. For example, the ERV RD-114 in cats has a joint origin of different Old World primates and cats which cannot be deduced from the current naming (reviewed by e.g. Kawasaki & Nishigaki (2018)). Furthermore, chromosomal allocations are often (but not always) neglected, making it more difficult to identify founder and duplication events. The current methods of classification also do not reflect on hybrid ERVs that belong to more than one class (reviewed by e.g., Gifford et al. (2018)).

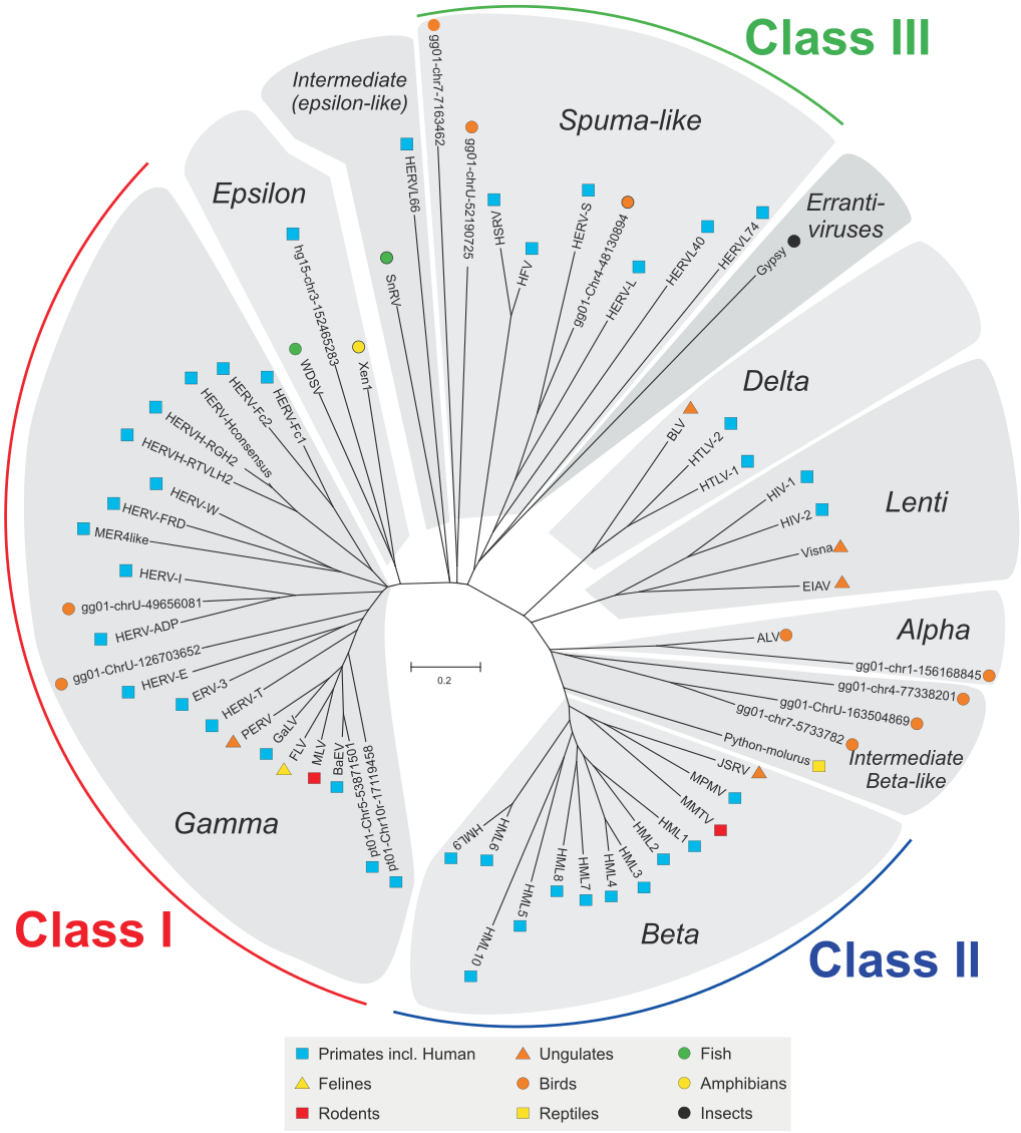

Figure 5. Dendrogram classification of exogenous and endogenous retroviruses

Unrooted Pol neighbour joining (NJ) dendrogram of the seven retroviral genera: alpha-, beta-, gamma-, delta-, epsilon-, lenti- and spuma-like retroviruses. The somewhat more loosely defined (endogenous) retroviral classes are indicated in the periphery. The various host species are indicated with symbols next to each taxonomic unit. Note, this dendrogram is limited to Class I, Class II and Class III ERVs.

Figure taken from Jern et al. (2005)

3.3 Types of ERVs

As mentioned earlier, ERVs acquire mutations throughout the course of evolution following their endogenization. Depending on the extent of mutations, different isoforms of ERVs are described. Figure 6 depicts three main isoforms (solitary long-terminal repeats (solo LTRs), incomplete and complete proviral genomes).

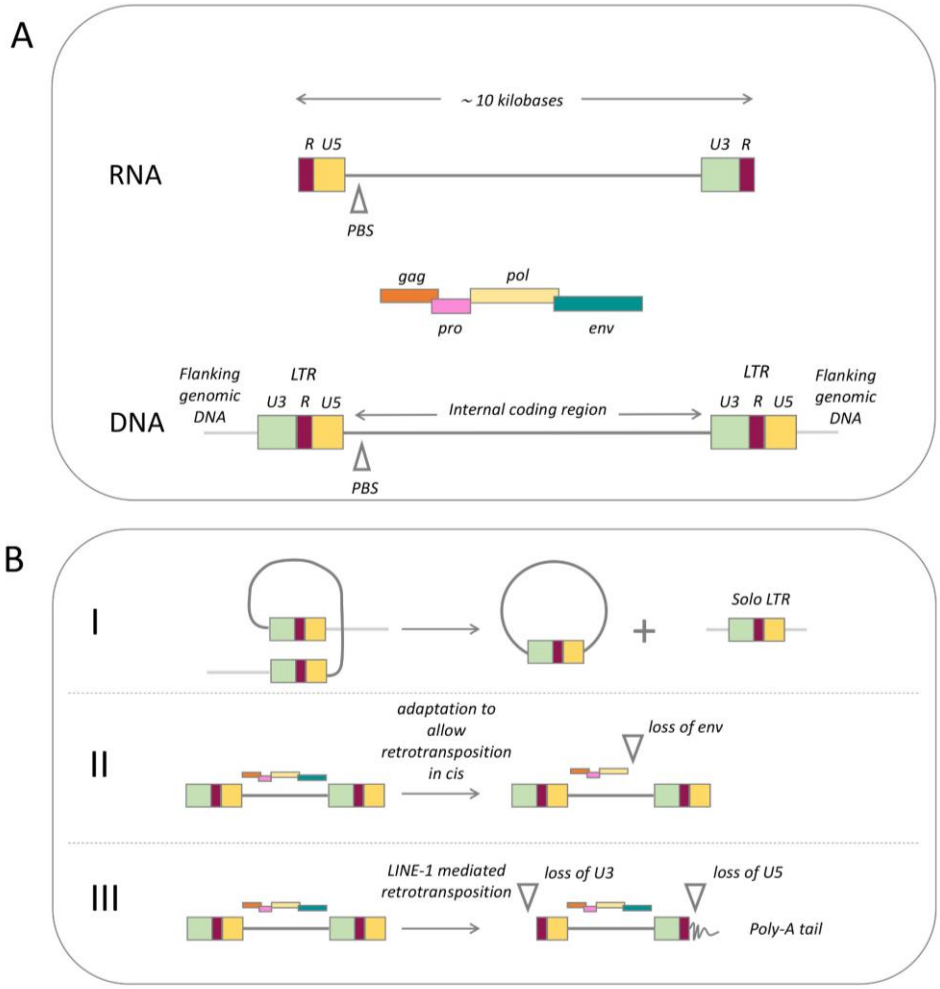

Figure 6. Illustration of different types of ERVs

An overview of the parental retroviral RNA, alongside the encoded proteins is shown in (panel A, top). During the replicative cycle, the RNA genome is integrated in the host DNA (panel A, bottom). Endogenous retroviruses may be integrated in the genome as one of three main forms: solo LTRs (panel B, I), incomplete proviruses with partial coding sequences (panel B, II), or the entire coding sequence can remain preserved within the genome (panel B, III).

PBS: primer binding site; gag (group specific antigens, matrix, capsid and nucleocapsid proteins), pro (protease pol (protease, reverse transcriptase and integrase) and env (envelope proteins) are coding regions; LTR (long terminal repeats) contain repetitive sequences that occur at both termini of the integrated DNA provirus. Each terminus consists out of an U5 (unique 5' region), R (repeat region) and U3 (unique 3' region) element. Other abbreviations include: LINE-1 (long-interspersed nuclear element 1).

Based on: Gifford et al. (2018)

Solo LTRs are the most frequently occurring type of ERVs within vertebrate genomes. As the name implies, only (part of) the LTR sequences of the provirus remain, and no open reading frames are present. Through non-allelic homologous recombination between the 5' LTR and 3' LTR, a single LTR is established, whereby the internal coding sequence from the viral genome is deleted (Figure 6 panel B, I) (reviewed by e.g., Gifford et al. (2018)). This process has occurred so frequently that it vastly outnumbers the pool of full proviral ERV sequences embedded in the host (Belshaw et al., 2007; Henzy et al., 2014; Tsangaras et al., 2015). Solo LTRs can retain their ability to influence transcription of nearby genes through their promoter and transcription factor binding motifs (reviewed by e.g., Rowe & Trono (2011)), which in some cases has led to their domestication in regulating host gene expression (reviewed by e.g., Zhang et al. (2022)). LTRs themselves may acquire mutations resulting in the deletion

of specific repeats within the LTR regions. The latter can limit the interaction with transcription factors, resulting in restrictive gene-expression (Denner, 2021b).

Also, incomplete **proviral coding sequences** have been detected in the host genome. Due to the accumulation of many mutations during evolution, proviruses may be incomplete (Figure 6 panel B, II). They may miss parts of or entire specific coding sequences, so that specific gene products are produced but no infectious and/or replication-competent progeny can be generated. Interestingly, both complete and incomplete open reading frames (ORFs) of the proviral genes can have a beneficial role for the host. Building on the example of *env* genes, as mentioned in section 3.1, complete coding sequences result in the expression of membrane-anchored Env proteins, effectively contributing to cell fusion (key in placenta formation). An example of an incomplete *env* ORFs with a beneficial role for the host has been described in cats (Kawasaki & Nishigaki, 2018; Kuse et al., 2016). Briefly, an ERV variant was shown to encode a truncated version of the Env protein, lacking the transmembrane (TM) domain. The resultant, soluble SU domain was shown to play a role in host-virus defence mechanisms (see 3.4.1).

Finally, ERVs can comprise of the **entire proviral genome** (Figure 6 panel A. DNA & panel B.III). Entire ERV proviral genomes have been observed in e.g., domestic cats (Anai et al., 2012), koalas (Tarlinton et al., 2006), pigs (Mattiuzzo et al., 2012; Prabha & Verghese, 2012), and primates (Jha et al., 2011; Moyes et al., 2005; Turner et al., 2001). Presence of the entire proviral genome may lead to spontaneous production of infectious virus-like particles (reviewed by e.g., Denner (2021a)).

It is accepted that the type of ERV largely depends on how much time has passed since the integration of the ERV in the host. Indeed, ERVs that have integrated earlier in evolution (i.e., the oldest sequences) have been subjected to greater selective or neutral pressure and thus are more likely to gather mutations, as compared to ERVs that integrated more recently (i.e., the youngest sequences). For example, some HERV-K (HML-2) variants which integrated presumably less than 100,000 years ago (Moyes et al., 2007) have shown to be more intact than more ancestral HERV sequences (e.g., HERV-W and HERV-FRD) that integrated in the genome of the Old-World primate ancestor 30 to 35 million years ago (Steinhuber et al., 1995). However, the establishment of solo LTRs appears to be inversely correlated with the time of integration. In brief, younger integrations share more homology among their LTR regions as compared to older viruses. As a result, the younger ERVs are more likely to undergo non-allelic homologous recombination between the 5' LTR and 3' LTR (see above) (Belshaw et al., 2007).

3.4 Role of ERVs in nature

The following sections are intended to provide representative examples of the diversity of ERVs and their involvement in mammalian physiology and ecology, with the aim to illustrate both the benefits and risks associated with their activation on a genetic and protein level. The examples mentioned in this chapter are not exhaustive. For more detailed background and/or additional examples, the reader is referred to relevant reviews on each of the topics.

3.4.1 ERVs in vertebrate physiology

Several ERV elements have been shown to actively contribute to the host cell's physiology. The best studied example are syncytins, Env **proteins expressed by ERVs**. While envelope proteins of native retroviruses play a role in the fusion of the viral particles with the host membrane, ERV encoded syncytins are assigned a role in placental formation in multiple vertebrate species (Aswad & Katzourakis, 2012; Garcia-Etxebarria & Jugo, 2014; Mao et al., 2021; Rowe & Trono, 2011). The differential expression of syncytins and their associated receptors, contributes to directed fusion establishing an interface between maternal and embryonical blood flows (reviewed by e.g., Lavialle et al. (2013)). Some syncytins are equipped with an immunosuppressive domain (ISD) (Eksmond et al., 2017a), and therefore, these syncytins have also been implicated in avoiding immune responses (Lavialle et al., 2013).

Other ERV expressed Env proteins have been assigned a role in the protection against retroviral infection. Hereby, ERV Env glycoproteins are thought to restrict entry of exogenous virus by receptor interference. These so-called co-opted *env* genes with antiviral activity have been described in mice (reviewed by e.g., Kozak, 2014), as well as in cats, chickens and sheep (Kawasaki & Nishigaki, 2018; Kuse et al., 2016), indicating that this is a common antiviral strategy in populations naturally exposed to

infectious retroviruses. Additional antiviral restriction factors have been described. For example, the co-opted murine ERV sequence Fv1 targets the virus capsid of mouse-tropic Murine Leukaemia Viruses (MuLVs) thereby inhibiting virus replication (Y. T. Jung & Kozak, 2000; Kozak & Chakraborti, 1996; Stevens et al., 2004). It can also restrict some non-MuLV retroviruses (Yap et al., 2014).

The above illustrates that ERV Env proteins have been domesticated throughout evolution in a variety of mammalian species and now play a role in developmental biology or innate immunity. Similarly, ERV elements may also impact **host gene regulation and subsequent host protein expression**. Some representative examples:

- ERV sequences can act as transcription factor binding sites (TFBSs) or enhancer elements, which contribute to the regulation of gene transcription by binding either enhancing or repressing factors (Grandi & Tramontano, 2018; Thompson et al., 2016). For example, in silico analysis of the LTR of Bovine ERVs (BoERVs) identified a transcription factor binding site for the hormone and steroid receptor RXR-α (Garcia-Etxebarria & Jugo, 2014). This finding is in line with the finding that BoERV env genes show an elevated expression in bovine endocrine thyroid tissue.

- ERVs may contribute to genome expansion. A representative example has been described by (Garcia-Etxebarria & Jugo, 2014). The authors observed that several interferon (IFN) copies have been integrated into BoERVs. The researchers suggest that BoERVs may have contributed to the expansion of the IFN repertoire in the bovine and ruminant genome through the copy-paste mechanism associated with retro-transposition. The abundance of copies could have resulted from an initial recombination event where an IFN cassette was captured through horizontal gene transfer (Caprari et al., 2015), subsequently leading (following genome integration) to expansion of the host genome.

- A regulatory role of ERVs in host protein expression has also been described. For example, (Samuelson et al., 1990) reported that the inverse orientation of HERV-E near a pancreatic copy of the amylase gene triggers expression of amylase, and subsequently resulting in an increased secretion of amylase into saliva. Medstrand et al. (2001) demonstrated that knocking out the HERV-E LTR resulted in a 40% reduction of apolipoprotein C-I expression in the HepG2 liver cell line, indicating that HERV-E LTR plays a role in enhancing expression of the lipoprotein.

Host factors may, in turn, **impact** the **ERV** gene regulation and protein expression. For example, activation of ERV genes by host factors has been described in case of physiological stresses, such as DNA-damage. In brief, in an animal study in mice, Wu et al. (2012) showed that, upon addition of a well-known genotoxic chemical agent to murine cells, the murine transcription factor p53 triggered the expression of the gene BC005512, a member of the GLN-family of murine ERVs, in a dose-dependent manner in NIH/3T3 cells. The exact role of BC005512 remains to be elucidated but it may contribute to the G1/S-phase checkpoint in cellular proliferation.

An example of silencing of ERVs by host factors is the methylation of ERV sequences. Through methylation of CpG-islands in LTRs, the silencing of proviral sequences is enabled (Y. D. Jung et al., 2013). It is argued that methylation has the sole evolutionary purpose of keeping ERVs at bay (reviewed by e.g., Jern & Coffin, 2008). On the other hand, as aging has been shown to reduce the epigenetic methylation ability, this may result in a less stringent control over ERVs, leading to non-tissue specific activation of ERVs, and subsequent pathological effects (Hurme, 2019).

3.4.2 ERVs in vertebrate pathology

Apart from their physiological role, some ERV genes and proteins have been (tentatively) linked to disease development and progression, either actively or passively, although a causal relationship is difficult to ascertain. Representative examples are described below. It is important to mention, that the same ERV genes or their expression products as previously described to have a physiological role, may also be linked to pathological processes.

In view of **ERV protein expression**, the previously mentioned Syncytin1 protein has been linked to cell-to-cell fusion of cancer cells and development of tumours in humans (reviewed by e.g., Grandi & Tramontano, 2018; Lavialle et al., 2013). Briefly, Syn1 expression profiles of specific HERVs have been found to be drastically upregulated in the context of human endometrial, breast and bladder urothelial carcinomas. Upregulated expression seems to be driven by host factors, such as binding of TF c-myb

at the 3' LTR element of the ERV in urothelial carcinomas, or hypomethylation of an oestrogen responsive element (ERE) in the 5' LTR of the ERV in the case of breast and endometrial cancer (reviewed by e.g., Grandi & Tramontano, 2018).

HERV-W Env proteins have been assigned a role in multiple sclerosis (MS). In brief, the Env proteins encoded by a specific ERV referred to as multiple sclerosis-associated retrovirus (MSRV) have been shown to trigger TLR4 receptors through the production of viral-like particles. Triggering TLR4 receptors resulted in the incapacitation of different neuronal functions such as oligodendrocytes differentiation, myelin production, and maintenance of the blood-brain-barrier (BBB) (reviewed by e.g., Grandi & Tramontano, 2018). Furthermore, MSRV Env proteins have been reported to affect the adaptive immune responses due to the high similarity to the myelin oligodendrocyte glycoprotein (MOG) (Bello-Morales et al., 2021).

In the context of veterinary medicine, Krunic et al. (2015) investigated domestic cats with onset lymphomas to understand the role of endogenous feline leukaemia virus (enFeLV) in the generation of lymphomas. Under healthy conditions, enFeLV has been reported to be non-pathogenic and non-infectious. It was assumed that during infection, the exogenous counterpart (exFeLV) can recombine with enFeLV, resulting in the production of infectious virus-like particles (VLPs). However, this study showed that cats developed lymphomas in the absence of exFeLV, indicating that the presence of exFeLV is not a prerequisite for lymphoma formation. Furthermore, since a significantly reduced expression of *env* genes in intestinal lymphoma tissues was observed when compared to non-malignant lymph nodes (healthy control), the authors speculated that lymphoma development would be a result of **insertional mutagenesis by proviral sequences**, rather than of the immunosuppressive effects of Env proteins.

Keynotes

- ERVs reside in the host genome through host cell division and over generations. They can accumulate and become a major part of the host genome (e.g., at least 8% of the human genome).
- In most cases viral genomes will be present as partial and incomplete sequences, which could even be as limited as a solo LTR. These modifications are seen as an indication of the evolutionary time since their initial integration.
- The intactness of the proviral sequences will largely determine the characteristics of the ERV. Nevertheless, all ERV types have been linked with different physiological and/or pathological functions.

4 Sources of ERVs in research, development and production

Taking the abundant presence and types of ERVs in vertebrates into account, it is highly likely that much of the biological materials routinely used in biotechnology, including those used for genetic modification, carry ERVs. In this section, an overview is provided of commonly used cells and animal species for which the presence of ERVs has been demonstrated. The overview is not intended to be comprehensive, but rather to illustrate the range of biological materials in which ERVs have been identified. Before reviewing this information, we would like to point out that different methods used to detect ERVs each have their limitations in view of what is being detected. This may have an effect on the significance of the finding.

4.1 Methodology for identifying endogenous retroviral elements

An overview of methods described to study ERV elements and/or ERV activity, identified in the context of the current report, is provided in Table 2. Depending on the method used different stages of the retrovirus life cycle can be detected:

- **DNA-based methods (1)** can be used to detect the inserted provirus (ERV), however this would not automatically indicate that any further step in the life cycle will be realized,
- **RNA-based methods (2)** can be used to detect unspliced and spliced viral RNA products generated by the ERV,
- **Methods to detect proteins (3)** can be used to identify ERV-related protein(s) in case the RNA transcript is translated into a (functional) protein,
- **Techniques able to detect particles (4)** can be used to detect intra- and extracellular virus particles. However, additional testing methods are required to differentiate particles according to **infectivity (5)** and **replication competence (6)** (see also Glossary).

When methods 2 and 3 are applied on supernatant harvested upon cultivation of materials containing ERVs, positive results may also be indicative for the production of virus(-like) particles, although specific testing methods under fields 4 to 6 will need to be used to further characterize the virus(-like) particles.

Table 2 Methods used to study elements / activity of ERVs

Method	What type of ERV is detected?					
	1 DNA	2 RNA	3 Protein	4 VLP	5 Infectious VLP (VINF)	6 Replication-competent VLP (VRC)
Coculturing					X	
CpG methylation analysis	X					
CRISPR-Cas9 knock-out	X					
Deep amplicon sequencing	X					
DNA sequencing (Sanger, NGS)	X					
Electron microscopy				X		
Electrophoretic mobility shift assay (EMSA)			X			
Enzyme-linked immune-sorbent assay (ELISA)			X	X		
Expression Sequence Tag mining (EST-mining)		X				
Flow cytometry			X			
Fluorescence-activated cell sorting (FACS)			X			
Fluorescent in-site hybridisation (FISH)	X					
Gene-transfer assay	X	(X)	(X)			
Genome-wide amplification of proviral sequences (GAPS)	X					

Method	What type of ERV is detected?					
	1 DNA	2 RNA	3 Protein	4 VLP	5 Infectious VLP (VINF)	6 Replication-competent VLP (VRC)
Green fluorescent protein (EGFP) reporter gene assay	X					
Immunodepleting assay			X	(X)		
Immunofluorescence			X			
Immunocytochemistry			X			
Immunohistochemistry			X			
Immunoprecipitation			X	(X)		
Infection assay (re-infection, pseudotype virus)					X	X
Inverse PCR	X					
In virion reverse-transcription assay		X*		X		
In vivo inoculation					X	X
Long-range PCR	X					
Long Terminal Repeat (LTR) reporter assay	X					
Manganese (Mn^{++}) dependent reverse transcriptase assay			(X)	X		
Marker rescue assay				(X)	X	X
Microarray		X				
Northern blot		X				
Nested PCR	(X)				X	
PCR-enhanced reverse transcriptase assay (PERT)			(X)	X		
Polymerase chain reaction (PCR)	X					
Quantitative real-time PCR (qPCR)	X					
Reverse transcriptase (activity) assay			(X)	X		
Reverse transcriptase inhibition assay			X			
Reverse transcription quantitative real-time PCR (RT-qPCR)		X		X		
Reverse transcription-PCR (RT-PCR)		X		X		
Ribonuclease protection assay	X	X				
RNA sequencing		X				
RNase T1 protection assay		X				
Single-tube fluorescent PERT (STF-PERT)			(X)	X		
Southern blot	X					
SYBR-Green PCR-enhanced reverse transcriptase assay (SG-PERT)			(X)	X		
Suspension multiplex immunoassay (SMIA)			X			
TAG-aided sense/antisense detection (TASA-TD) PCR		X				
TaqMan fluorescent probe-based PERT (TM-PERT)			(X)	X		
Transmission electron microscopy (TEM)				X		
Uracil-N-glycosylase-coupled RT-qPCR (UNG RT-qPCR)		X		X		
Western blot			X	X		

*: Pre-packaging and packaged virions prior to budding

(X): product can be detected in different forms: gene-transfer assay – test product may be detected as transcript (RNA) or protein; immunodepleting and immunoprecipitation assay – based on antibody-antigen capture, antigen = viral coat protein; marker rescue assay – indirect detection VLP, otherwise no infectious and replication-competent form; nested PCR – detection of integrated provirus (DNA); reverse-transcriptase assays – VLP contains reverse-transcriptase (enzyme)

4.2 Cells and cell lines

Literature was screened for actively expressed ERVs in tissues, primary cells, and established cell lines. Annex 1 comprises 106 entries based on literature references indicating the presence of ERVs and expressed products.

As pointed out in the previous section, the interpretation of the results largely depends on the specific method that was used. Molecular techniques (DNA) on cells can confirm the presence of a specific ERV. Detection of specific (m)RNA and/or specific proteins indicates that an ERV is expressed. Yet it requires demonstration of VLP, infectious VLP and finally replication-competent VLP to have confirmation that the ERV has reverted into a functional virus. However, the latter demonstrations are more tedious and depending on the purpose of the research, determination of earlier stages may be considered sufficient.

In 32 cases VLPs could be identified, of which ten concerned infectious VLPs, including seven that were shown to be replication-competent. In such cases, another technique had been used to also determine an earlier stage in the retroviral life cycle (RNA or proteins). Although some publications only indicate a single testing method (e.g., only a molecular technique or a protein-based technique), in most research a combination of techniques were used.

Whenever tests for RNA and/or protein were negative, also no VLP could be identified. If a technique was used to identify a VLP and the result was positive, other detection methods targeting RNA and/or proteins also gave a positive result. These results seem to indicate that RNA and protein-based testing provide a reliable first indication of the potential for VLP production. Since not all RNA and/or protein positive cases effectively lead to VLP, the result may lead to an overestimation and should be followed with additional testing whenever more robust information is required.

It must be stressed that such research focusses on specific ERVs, in particular those for which it is already known or expected that there is a high likelihood that VLPs are produced. Again, the ubiquitous presence of ERV in many mammal species makes it unlikely that a cell can be found free of ERV. The publications cover a small number of specific ERV in specific cells/cell lines with a focus on the possible activation and if this activation leads to the production of (infectious and replication-competent) VLPs.

The information from the literature cited in Annex 1 was further consolidated by grouping repetitions of the same cells/cell lines. This resulted in a list of 98 unique materials. Most of the references concern established cell lines, identifying a total of 28 unique cases in which VLPs are reported. Ten of which are infectious, including seven that are replication competent. A summary of the results is provided in Table 3.

Table 3 Origin of tissues, primary cells and cell lines in which VLP production of specific ERVs were demonstrated. VLPs are enumerated as particles. Enumeration with a double asterisk (**) identifies infectious VLPs (VINF). Enumeration with a double dagger (‡) identifies replication competent VLPs (VRC).

Animal species	No. of unique entries in which ERV was detected (based on Annex 1)					
	Cell lines		Primary cells		Tissues	
	Total	Particles ** (VINF) ‡ (VRC)	Total	Particles ** (VINF) ‡ (VRC)	Total	Particles ** (VINF) ‡ (VRC)
Cat	**4**	3 3 **; 3 ‡	**1**	0	**0**	0
Chicken	**1**	1 0 **; 0 ‡	**0**	0	**0**	0
Hamster	**1**	1 0 **; 0 ‡	**0**	0	**0**	0
Human	**23**	6 4 **; 1 ‡	**3**	2 0 **; 0 ‡	**14**	0
Koala	**0**	0	**2**	0	**0**	0

Animal species	No. of unique entries in which ERV was detected (based on Annex 1)					
	Cell lines		Primary cells		Tissues	
	Total	Particles ** (VINF) ‡ (VRC)	Total	Particles ** (VINF) ‡ (VRC)	Total	Particles ** (VINF) ‡ (VRC)
Mouse	**16**	7 2 **; 2 ‡	**8**	6 0 **; 0 ‡	**1**	0
Non-human primate	**4**	0	**0**	0	**0**	0
Pig	**1**	1 1 **; 1 ‡	**6**	1 0 **; 0 ‡	**12**	0
Ruminant	**0**	0	**0**	0	**1**	0
Total	**50**	**19** **10 **; 7 ‡**	**20**	**9** **0 **; 0 ‡**	**28**	**0**

4.3 Experimental animals

As part of fundamental or preclinical research, a variety of animal models are used including, but not limited to small rodents, cats and large animal species such as pigs. The chapter below will focus on the most extensively studied animal species either in the context of R&D, or in the context of xenotransplantation.

4.3.1 Laboratory mice

The genomes of virtually all strains of **laboratory mice** have been shown to contain ERV sequences (Eksmond et al., 2017b; Kozak, 2015). In many cases, these sequences are fragments of retrovirus genomes originating from different murine retroviruses such as mouse mammary tumour virus and murine gammaretroviruses. In several mouse strains, such as BALB/c, C57BL/6, DBA/2, and AKR, complete endogenous ecotropic proviruses have been identified (Bedigian et al., 1983; Copeland et al., 1988; Jenkins et al., 1982; O'Brien et al., 1982). Even though complete proviruses are present, production of infectious VLPs does not necessarily occur due to relatively minor defects in viral sequences. Bartman et al. (1995) described that recombination events amongst different ERVs present in the mouse genome can lead to repair of the defects and subsequent production of infectious VLP, whereby recombination is particularly relevant in aged mice. Here, aging is believed to contribute to a reduction in epigenetic control, such that previously silenced ERVs may become activated (reviewed by e.g., Hurme (2019)). Recombination events are not solely known to occur between ERVs. Golovkina et al. (1994a) reported recombination between an endogenous and an exogenous betaretroviral mouse mammary tumour virus (MMTV). Briefly, the co-packaging of endogenous viral RNA into MMTV particles was reported in lactating C3H/NeH mice, with the exogenous virus inducing high transcription of its endogenous counterpart. In mammary tumours from the mother, the presence of both MMTV and recombinant viral particles was identified. Recombinant viral particles, exogenous virus particles and co-packed recombinant and exogenous particles were found to be secreted in milk and transferred to susceptible progeny. The recombination was found to be restricted to the *env* encoding region of the exogenous virus, more specifically the region encoding a so-called 'superantigen domain'. This domain is essential in enabling retroviral infection of susceptible T-cells. By recombining with (part of) the ERV *env* sequence, a heteromeric viral RNA genome is established, resulting in an expansion of the viral host tropism.

Production of infectious VLPs may also occur irrespective of recombination. Indeed, mice strains have been described that constitutively produce infectious VLPs from birth due to carrying non-defective proviruses (reviewed by e.g., Kozak(2015)).

Finally, murine experiments have revealed that defective betaretrovirus-like ERV proviruses can trans-complement one another on the protein level, establishing 'new' virus like particles (Evans et al., 2009; Young et al., 2012).

Interesting to note is that although many common inbred mouse strains may not produce infectious VLPs, cultured cells of these strains can do this following e.g., chemical induction (Lieber et al., 1974) or stimulation by bacterial lipopolysaccharide (Greenberger et al., 1975; Sherr et al., 1974) (see also Chapter 5).

As proviruses are often found to be mutated, the likelihood that they give rise to infectious VLPs depends on different factors, mainly age of the animal, and exogenous influencing factors (viruses or chemical agents). It is therefore important to consider information on the source of the cells, as primary cells derived from aged mice may be more prone to spontaneous ERV activation and recombination. Furthermore, chemical inducers or retroviruses (and retroviral vectors) could reactivate dormant ERVs, potentially leading to the induction of virus-like particles.

4.3.2 Animals as source of tissues and organs for xenotransplantation

Even though this not in the scope of the current study, it is worthwhile mentioning that many studies on ERVs have been performed in view of xenotransplantation. The presence of ERVs and their potential activation in the human recipient has raised concerns in view of safety of the approach. Below, representative studies addressing ERVs in xenotransplantation are briefly summarized. For a more extended overview, the reader is referred to review articles such as Denner & Tönjes (2012) and Denner (2021b).

So far, more than 200 humans have received a xenotransplantation product comprising pig cells, or tissues including *ex vivo* perfusion of pig organs or pig cell-based bioreactors (Denner & Tönjes, 2012; Morozov et al., 2017). For a number of reasons, including size, anatomical, and physiological similarities with humans, nearly unlimited availability, short breeding time, and large litters, the **pig** is proposed as the preferred donor species for humans. However, the presence of PERVs poses a risk when considering xenotransplantation. *In vitro* research has shown that PERV-A and -B are polytropic and able to infect susceptible human HEK293 cells (Takeuchi et al., 1998). PERV-C was identified in some but not all pigs (Denner & Tönjes, 2012). While PERV-C in itself is ecotropic within members of porcine species, autosomal recombination between PERV-A and -C was found to occur spontaneously (Karlas et al., 2010), generating a recombinant isoform (PERV-A/C) which has the capacity to produce high titers in susceptible human cells (Kaulitz et al., 2013; Mattiuzzo et al., 2012). Even though the information above may imply that PERVs thus can be transmitted to human cells upon transplantation, so far none of the preclinical and clinical trials performed up until the date of writing this report have reported such transmission.

Apart from pigs, also **other animal species** have been studied for their potential as organ donor. The potential risk of ERVs was addressed in a study by Allan et al. (1998). The author described two patients suffering from advanced liver cirrhosis, who had received a baboon liver as a xenotransplant. Following xenotransplantation, proviral BaEV sequences were detected in patient tissues, more specifically bone marrow, liver, kidney, and lymph nodes. However, the simultaneous detection of mitochondrial baboon DNA in the BaEV-positive patient samples, indicated that the BaEV DNA originated from baboon lymphocytes transferred to the human with the xenograft and not from the infection of human cells (Allan et al., 1998).

Keynotes

- While molecular techniques enable the detection of ERV sequences in a host genome, it is more difficult to ascertain that the presence of these sequences (potentially) leads to VLPs, infectious VLPs or RCVs.
- Many publications report the presence and activation of ERVs. No truly negative results were found (e.g., a systematic screening identifying that ERV activation didn't occur). This most likely illustrates a bias in literature and hence leads to an overestimation of the importance of ERV activation.

- Similarly, many studies do not go beyond certain early stages of the retroviral life cycle as indicators for activation. Although the presence of specific RNA and/or proteins can be seen as a relevant indication, additional testing is needed whenever more reliable information on the potential for VLP production is required.
- Taking these limitations into consideration, it can nevertheless be concluded that ERV expression can be detected in a wide range of mammalian cells and tissues and some cases illustrate that this can lead to the production of infectious VLPs or even RCVs.

5 Potential risks of ERVs in research, development and production

As mentioned above, the ERV provirus can be present in part or in its entirety in the vertebrate host (cell). Depending on the intrinsic characteristics of the vertebrate host (cell), the type of ERV present and the experimental conditions, the ERV sequence may become activated. Such activation could ultimately result in the production of infectious VLPs. If such would occur, a subsequent but so far theoretical risk may be the release of the particles and potential exposure of people or the environment.

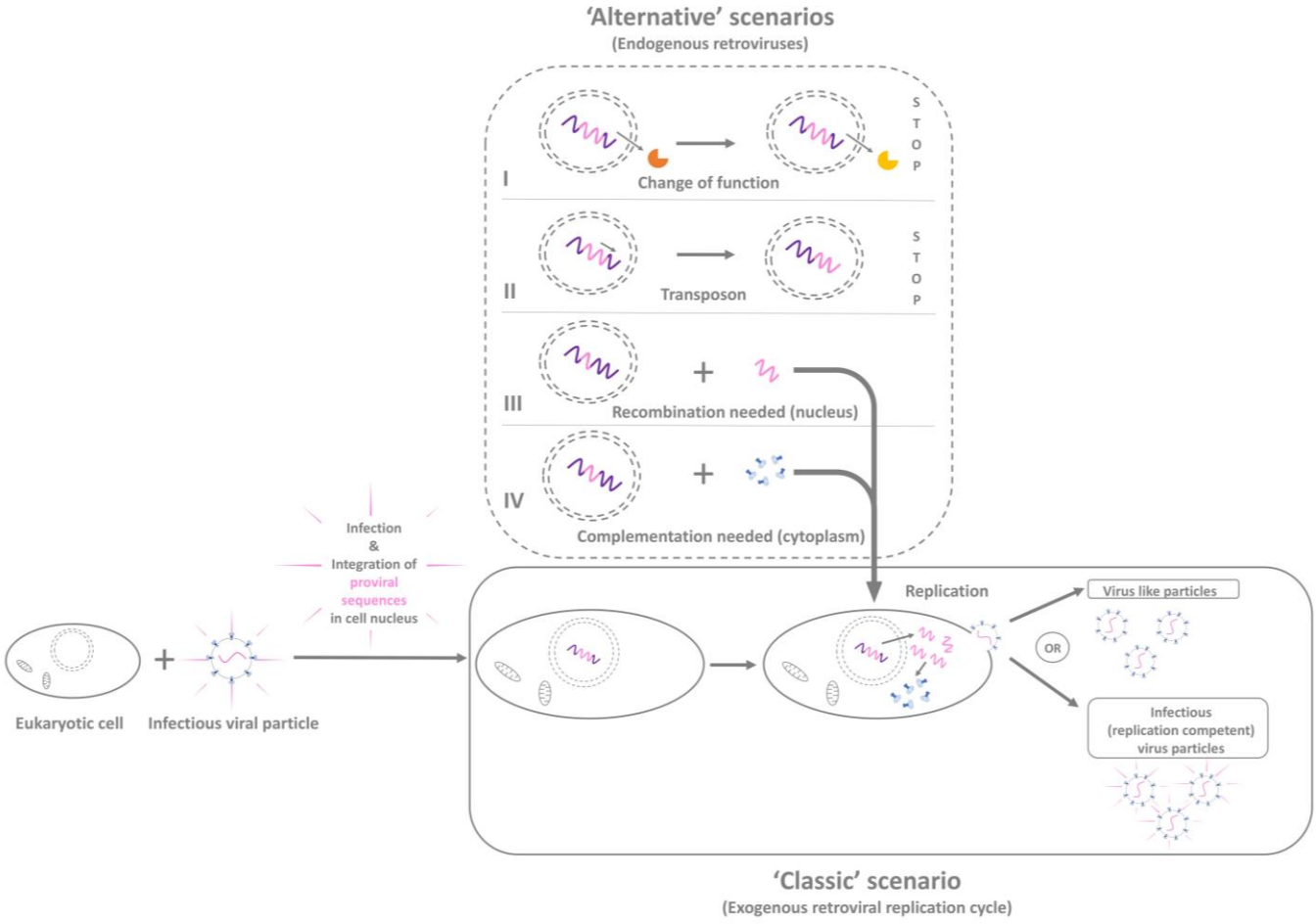

Figure 7. The ERV cycle: from inserted genetic material to released replication-competent virus particles

Figure 7 compares the 'classic' scenario of an exogenous retroviral replication cycle with 'alternative' scenarios that could occur upon activation of an ERV and that may be of relevance for biotechnology applications:

I. ERVs that are only partially present as solo LTR or as domesticated gene - they can induce a change of function, but cannot revert to replication (unless recombination occurs).
II. Transposon - Transposition is possible if the provirus is flanked by LTR sequences and actively encodes reverse-transcriptase and integrase[5].

[5] Transposon activity of ERVs, i.e. their change in position within the genome of a host cell with potential mutations within the host cell genome, is not discussed in the context of the risk assessment as it concerns an intrinsic change in the host cell rather than a specific risk for the user of such host cells.

III. Recombination - The presence of functional retroviral genes and/or preservation of homologous sequences may allow for genomic recombination during infection with an exogenous retrovirus.
IV. Complementation - The formation of functional proteins by ERVs may complement exogenous viruses during infection, which may result in chimeric viruses and virus-like particles. Vice versa, infections by exogenous viral agents may aid ERV genomes and/or encoded proteins to establish ERV-derived virus-like particles.

Some ERVs may, alike wild-type viruses, remain active and continue to encode for VLPs with infectious (1) and possibly, replication-competent (2) abilities (classic scenario).

The section below focuses on the different (retro)viral and non-viral factors that could lead to such activation. Hereby, the formation of (infectious) VLPs is deemed most relevant for the risk assessment and is thus discussed most extensively. However, as mentioned in Section 4, data of the scientific literature are to be interpreted carefully, since the type of ERV product that is identified in a paper is directly related to the method used for the screening. If not looked for, other ERV products cannot be excluded and, therefore, infectious VLPs may be induced more frequently than can be concluded from the scientific literature. Furthermore, whereas the publications elaborated on the presence of different types of ERV and their activation, the potential risk of the ERV activation for health and/or environment was in most cases not addressed.

5.1 Activation of ERV

5.1.1 Spontaneous production of (infectious) viral-like particles

Several cells, cell lines and tissues have been described to spontaneously release infectious VLPs (see also Section 4.1 and Annex 1). A few representative examples are described below.

- **Porcine** cells such as *ex vivo* cultured hepatocytes (Han et al., 2011) and porcine peripheral blood mononuclear cells (PBMCs) (Mourad et al., 2017), and cell lines such as PK-15 cells (Godehardt et al., 2020; Kaulitz et al., 2013) are very well known to release PERVs particles in culture supernatant. PERVs are also released from a variety of tissues (Y. D. Jung et al., 2013). Released PERV particles have the ability to infect human cells as was demonstrated in several studies.

- **Feline** tissues and cells are known to actively secrete one particular ERV, RD-114. This infectious and replication-competent ERV is found to be secreted by feline kidney (Crandell-Rees Feline Kidney Cell, CRFK), feline embryonic fibroblast and granular lymphoma (Merkel cell carcinoma, MCC) cell lines used in the development of live-attenuated vaccines for both cats and dogs (Okada et al., 2011). Interestingly, live-attenuated canine vaccines produced in non-feline cell-lines (variety of origins: avian, canine, mink, porcine, simian) were found to be contaminated with RD-114. This is presumably caused by the contamination of seed stock viruses with infectious RD-114 virus. When RD-114 susceptible, non-feline cell lines are used in subsequent vaccine production, the RD-114 virus is maintained as a contaminant in both these cell lines and resultant vaccines (Yoshikawa et al., 2014).

- **Human** cells and tissues are found to rarely release infectious VLPs. Still, for the youngest and most active human retrovirus, HERV-K (HML-2), which can code for all viral proteins, production of virus-like particles was demonstrated (Morozov & Morozov, 2021). Moreover, virus-like particles as produced by some teratocarcinoma and breast cancer cell lines were able to pack and transmit retroviral sequences after which the retroviral transcripts could undergo reverse transcription (Contreras-Galindo et al., 2015). However, the retroviral sequences did not integrate into the host cell genome but rather formed episomes. No evidence was found of a sustained and spreading infection. Also, Boller et al. (1993) and Dube et al. (2014) reported production of non-infectious HERV-K retroviral-like particles in human teratocarcinoma cells as well as in their derived cell lines NCCIT and Tera-1.

5.1.2 Production of infectious retrovirus-like particles by deliberately introducing ERV sequences into cells

Whereas the cases described above refer to the activation of ERVs as present in a host cell, some scientific papers focused on the experimental introduction of known ERV sequences in host cells. Anai et al. (2012) aimed to investigate whether ERVs of the domestic cat, which have intact *gag/pol/env* reading frames, could be infectious for feline and other host cells. One approach to test this hypothesis was to amplify full length ERV-DC10 and ERV-DC18 proviral DNA from primary feline PBMC by PCR and to subsequently transfect this PCR DNA product, without cloning of the proviruses, into the human cell line HEK293T. The transfection resulted in the production of type C infectious VLPs after 2 to 3 weeks post inoculation. These findings show that deliberate introduction (through transfection) of specific and foreign ERV sequences in a different cell types and species may result in the production of infectious VLPs. Furthermore, virus particles resulting from the transfection of HEK cells with ERV-DC10 and -18 proviral plasmid DNA were able to infect a number of human, dog, and monkey cells, suggestive of a broad tropism of potentially generated virus particles. On the other hand, when coculturing PBMC of ERV-DC10 positive cats with HEK293T cells, no infectious VLPs were formed, which in turn may indicate that infectious VLP production from full proviral ERVs in the PBMC is regulated in a tissue-specific manner, for example by silencing or by inducing a specific methylation status.

In 2013, Xiang et al. generated an infectious replication-competent clone of PERV and characterized its infectivity and replication competency in HEK293 cells. Upon transfection of the cells, expression of Gag proteins was detected by the indirect immunofluorescence assay and western blot. Following transfection, the released VLPs were found to be infectious and replication-competent when infecting naïve HEK-293 cells. Subsequent passage cycles revealed that PERV had stably integrated and expressed in the HEK-293 genome, as identified by PCR, qPCR, and western Blot.

5.1.3 Activation of ERVs by viruses not belonging to the Retroviridae

Apart from retroviruses, also human viruses not belonging to the family *Retroviridae* have been described to affect ERV expression in human cells. A non-exhaustive overview is provided in Annex 2 and summarized in

Table 4.

Table 4 Viruses (other than retroviruses) affecting ERV expression (non-exhaustive)
(more details, including references are provided in Annex 2)

Virus family	Virus species	ERV element detected	Detection method
Alphaviridae	Chikungunya virus	RNA	RNA sequencing
	Mayaro virus	RNA	RNA sequencing
Coronaviridae	Severe Acute Respiratory Syndrome Coronavirus 2	RNA	RNA sequencing
Flaviviridae	Denguevirus 2	RNA	RNA sequencing
	Zikavirus	RNA	RNA sequencing
Herpesviridae	Epstein Barr virus	RNA	RNA sequencing
		Protein	EMSA, IHC, WB, RT-qPCR
	Human herpesvirus 1	Protein	ELISA, IHC, WB, qPCR
		VLP	RT-qPCR, EM
	Human herpesvirus 6	RNA	RT-qPCR
	Kaposi's Sarcoma-associated herpesvirus	RNA	RT-qPCR
		Protein	RT-qPCR, WB
Peribunyaviridae	Oropouche virus	RNA	RNA sequencing
Pneumoviridae	Respiratory syncytial virus	RNA	RT-qPCR

Virus family	Virus species	ERV element detected	Detection method
Orthomyxoviridae	Influenza A virus	RNA	RNA sequencing

VLP: virus-like particle; EMSA: electrophoretic mobility shift assay; IHC: immunohistochemistry; WB: Western blot; RT-qPCR: quantitative reverse-transcriptase polymerase chain reaction; EM: electron microscopy

Most of the identified studies referred to the detection of **RNA** as an indication of ERV activation. Depending on the virus family responsible for the ERV activation, a range of ERV sequences were shown to be upregulated as detected by RNA sequencing. For example, up to 15 different ERVs were activated for Chikungunya virus and Mayaro virus, both members of the *Alphaviridae*.

ERV **protein** expression was reported e.g., upon infection of primary oral mucosa cells with severe acute respiratory syndrome virus 2 (Apostolou et al., 2022), and upon infection of different human and porcine cells infected with members of the *Herpesviridae* (Dai et al., 2018; Gross et al., 2011; Kim et al., 2015; Ruprecht et al., 2006). (Kim et al., 2015) subsequently speculated that the observed expression of PERV Gag antigens upon infection of porcine PBMC with human herpes virus 1 could potentially mount a strong antibody response towards Gag antigens, although the study itself did not provide evidence that this increased PERV Gag expression would be sufficient to produce a host antibody response.

Production of **(infectious) virus-like particles** was only reported for herpes simplex virus type 1 (HSV-1) (reviewed by Bello-Morales et al. (2021)). In brief, HSV-1 infection of leptomeningeal cells from a MS patient strongly enhanced the expression of retrovirus-like particles which would be later identified as a novel HERV, belonging to the HERV-W family, and named MS-associated retrovirus, MSRV. The viral particles are able to infect naïve leptomeningeal cells *in vitro*.

Interestingly, whereas most viral infections described in

Table 4 resulted in an upregulation of ERVs, infection with respiratory syncytial virus was shown to downregulate HERV-H, HERV-W, HERV-FRD, and HERV-K mRNA expression profiles (Tovo et al., 2023), and Epstein Barr virus was shown to downregulate HERV-W1 (Wieland et al., 2022) but upregulate several other HERVs (Gross et al., 2011; Wieland et al., 2022).

5.1.4 Activation by exposure to chemical agents

Several scientific publications address the activation of endogenous retrovirus by chemical or physical agents, as outlined in Annex 3 and summarized in Table 5.

Table 5 Chemical factors affecting ERV expression (non-exhaustive)
(more details, including references are provided in Annex 3)

Agent	ERV element detected	Detection method
5-iodo-2'-deoxyuridine (IUdR)	VLP	PERT, RT-PCR, TEM, infectivity assay
	RCV	RT assay
Azacytidine (Aza-C)	RNA	RT-qPCR, RNA sequencing
	VLP	STF-PERT, RT-PCR, TEM
Calcium ionophore A23187	RCV	RT assay, infectivity assay, TEM, Sanger sequencing
Dimethylbenzanthracene (DMBA)	Protein	ELISA, flow cytometry
	VLP	TEM
Interleukins (IL) 4 and 13	RNA	qPCR
	Protein	Western Blot, fluorescence microscopy
Lipopolysaccharide (LPS)	VLP	RT-PCR, WB, cDNA sequencing
Mitogens (e.g. PHA, PMA)	RCV	Co-culture, RT assay, infectivity assay, TEM, nested PCR, cDNA sequencing

Polybrene	None	Marker rescue assay, re-infection assay
Silver nanoparticles	RNA	RT-PCR
	Protein	Western Blot
Sodium butyrate (NaBut)	RNA	RT-PCR, STF-PERT
TNF-alpha	Protein	Fluorescence microscopy, western blot, RT-qPCR

VLP: virus-like particle; RCV: replication-competent virus particle; ELISA: Enzyme-Linked Immune Sorbent Assay; STF-PERT: Single-Tube Fluorescent PCR-Enhanced Reverse Transcriptase assay; WB: western blot; RT-qPCR: reverse-transcription quantitative real-time polymerase chain reaction; TEM: transmission electron microscopy

Treatment with various chemical agents, routinely used in research and development, was shown to activate ERVs as demonstrated by upregulated mRNA, protein expression, and/or viral particles. When treating porcine peripheral blood mononuclear cells with the calcium ionophore A23187 or with mitogens such as phorbol myristate acetate and phytohemagglutinin, infectious and replication-competent PERV viral particles were found to be produced.

5.1.5 Activation by exposure to physical agents

Also, exposure to physical agents has been shown to activate ERVs. In 2020, Lee and colleagues reported the induction of HERV-transcripts (MER21C, MER57B1, MLT1C49) in lung adenocarcinoma epithelial cell lines A549, following the exposure to X-ray radiation at a dose of 8 Gray (Gy). Leong et al. (1988) showed the budding of type-C VLPs from the UV-induced C3H murine cell line K1735 by using immunoelectron microscopy.

5.1.6 Activation by long term passage of cells

In murine primary cells and established cell lines, it has been observed that the number of cell passages contributes to the potential release of endogenous virus-like particles. Lieber and colleagues have shown that a subpopulation of the BALB/3T3 cell line (termed S16) release high titers of endogenous type C RNA virus particles past 100 generations (Lieber & Todaro, 1973). Whereas Rasheed and colleagues (1976) identified that rat embryonal cell lines produce type C virus-like particles in considerable less passages than in murine cell lines.

One possible explanation for this phenomenon is that of the loss of epigenetic control, in particular CpG methylation (as discussed in 3.4.1). Endicott et al. (2022) provide experimental evidence that the loss of CpG methylation could be attributed to the exposure to oxygen and reactive oxygen species during culturing procedures. In turn, this promotes cellular aging, whereas aging is known to be associated with a more active ERV profile (for a review, see Cardelli (2018) and Mao et al., (2021)).

5.2 Production of (infectious) virus-like particles as a result of complementation by retroviral proteins

Apart from complementing vector particles, envelope proteins can also support the formation of vesicles in which genetic information is packaged and that can spread from cell to cell, so-called extracellular membrane vesicles. For example, Uygur and colleagues (2019) demonstrated that treatment of cells with specific anti- and proinflammatory cytokines stimulated Syn1 (ligand) and ASCT2 (alanine, serine, cysteine-preferring transporter 2 (receptor)) production. This resulted in an increase of the fusogenic potential of ERV Env Syn1 present in the human cells. Subsequently, the increased fusogenic potential facilitated the production of Syn1-coated extracellular membrane which could transmit to other cells. If such extracellular membrane vesicles contain genetic material, transmission of these sequences to cells previously devoid of these sequences, may occur. The authors additionally infected human prostate carcinoma (PC3) cells with replication-competent gamma-retroviral particles derived from the murine stem cell virus (MSCV), which also resulted in an increase of ERV Env Syn1 and the formation of extracellular membrane vesicles. The vesicles carried the target gene (green fluorescent protein, GFP), which could now be transmitted to other (non-target) cells without the need for an infectious VLP.

The above example illustrates that prudence is in order when using cell lines known to express functional ERV Env proteins with viral vectors that could be complemented with these proteins. This implies that artificial conditions in the context of R&D may inadvertently activate ERVs and influence result interpretations, resulting from potential off-target induction. In turn, this feature may represent a (bio)safety hazard.

5.3 Production of (infectious) virus-like particles as a result of recombination with retroviral sequences

5.3.1 Recombination with (homologous) retrovirus sequences

When cells containing ERVs are infected with an exogenous retrovirus, the virus and ERV sequences may recombine, ultimately leading to the production of infectious VLPs. This has been extensively described for rodents.

Martinelli & Goff (1990) showed that defective exogenous retroviruses carrying deletions in essential functions can recombine with endogenous retroviral sequences to form viable, replication-competent viruses. They demonstrated the reversion of a mutant Moloney murine leukaemia virus (M-MuLV) with a deletion in the protease domain of the *pol* gene after infection of NIH/3T3 cells. Two weeks after infection of the cells, revertants arose that were derived by recombination of the defective exogenous virus with a long sequence of *gag* and *pol* of a murine ERV. One such recombinant was fully infectious, indicating the repertoire of viral sequence in the NIH/3T3 genome must include substantial stretches of functional viral genes. Examination of the viral DNAs very early in the infection revealed the presence of defective genomes, formed by nonhomologous crossovers between the two parental sequences. The authors suggested that these may serve as intermediates in the eventual formation of the viable revertant genomes.

Similarly, Villanueva et al. (2003) reported that a defective M-MuLV stably transfected into Rat1 cells activated an endogenous, defective rat leukaemia virus (RaLV). This resulted in the production of an infectious, replication-competent hybrid virus. The hybrid, RaLV flanked with M-MuLV LTRs, was believed to be generated through recombinational patch-repair during DNA-synthesis. This effectively allowed for the packaging of RaLV viral RNA into M-MuLV particles.

Evans et al. (2009) studied the recombination between exogenous and endogenous retroviruses in more detail in mice. In brief, they showed that recombination occurred during reverse transcription of a virion RNA heterodimer comprised of an RNA transcript from an endogenous polytropic virus and an RNA transcript from an exogenous ecotropic MuLV RNA. In the resulting recombinant polytropic murine leukaemia virus, the *env* gene sequences of the exogenous ecotropic MuLV was replaced with *env* gene sequences from the endogenous polytropic retrovirus. It is possible that homodimers corresponding to two full-length endogenous RNA genomes are also packaged. Therefore, the authors concluded that infection by an exogenous virus may not only result in recombination with endogenous sequences, but also in the mobilization of complete ERV genomes without recombination within exogenous retroviral virions. Although the endogenous virus as such did not become replication-competent, the recombinant viruses were shown to be capable of infecting cells, integrate into the cell genomes and subsequently replicate and spread as pseudotyped viruses. It was observed that uninfected cells were permissive to ecotropic infection, whereas cells chronically infected with the exogenous pathogen were resistant to ecotropic infection (Evans et al., 2009). Ottina et al. (2018) also reported the production of infectious pseudotyped VLPs in murine melanoma cell lines containing defective ERVs upon exposure to exogenous murine leukaemia virus.

5.3.2 Recombination with (homologous) retroviral vector sequences

In case cells containing ERVs are used to produce viral vectors, recombination between the introduced exogenous retroviral vector sequences and the ERV sequences may give rise to (replication-competent) virus particles. Several examples from literature are described below. It is highlighted that such recombination events were only described for gammaretroviral vectors, but not for vectors based on lentivirus.

In 1998, Chong et al. detected a replication-competent retrovirus in supernatant from a murine vector producing line. Contamination by an exogenous retrovirus from the laboratory was excluded. Rather, it

was suggested that the replication-competent virus was a result of a recombination event at sites of partial homology, between sequences in the vector, one of the packaging constructs, and endogenous retroviral elements. Interestingly, the recombination was not yet present in stocks of the packaging cell line or in an initial stock of the vector-producing line. This indicates that the recombination event likely occurred while the vector-producing line was undergoing passaging for harvest of supernatant stocks.

Purcell et al. (1996) reported the development of lymphomas and death of monkeys after exposure to a gammaretroviral vector contaminated with replication-competent retroviral particles (RCRs). The RCRs were formed by the recombination between ERV sequences present in the NIH 3T3-derived production cells and the exogenous vector sequences. Murine cells used for production of gammaretroviral vectors also frequently contain ERV sequences that show similarity to the murine-based gammaretroviral vectors (summarized in e.g., Commissie Genetische Modificatie (COGEM), 2021a; van der Meulen & Rüdelsheim, 2023 and associated offering letter). For gammaretroviral vectors, using vector producing cells of a non-murine species will help to reduce the likelihood of this type of recombination (Cosset et al., 1995; Forestell et al., 1997; Ghani et al., 2007). Importantly, in human cell lines, (nearly) intact endogenous gammaretroviruses are not present and RCR formation has never been reported for human cells upon introduction of retroviral vectors, even when they contained endogenous gammaretroviral sequences (summarized in Commissie Genetische Modificatie (COGEM), 2021).

The presence of more intact gammaretroviral ERVs in mice than in humans might be an important factor influencing the likelihood of generating replication-competent viruses when using gammaretroviral vectors. The potential for complementation and recombination may be higher in murine cell lines, whereas the likelihood of complementation and recombination in human cell lines is low given the absence of complete gammaretroviral ERVs.

5.3.3 Recombination between different ERVs within the host cells

Not only can recombination occur when exogenous viral sequences are introduced in an ERV-containing host cell (see above), also different ERVs present in a host cell have the ability to recombine. Such has been extensively described for murine cells. For example, Curriu et al. (2012) demonstrated that a xenotropic murine leukaemia virus-related virus (XMRV) was generated after a recombination event between two endogenous murine leukaemia viruses during the production of a human prostate cancer cell line. The pathogenicity of the recombinant virus remains to be determined, but it was shown that it was able to replicate in human cells *in vitro* and in human tonsillar tissue *ex vivo*. The infection did not result in changes of T or B-cells, immune activation, nor inflammatory chemokines. Infectious VLPs could be recovered from supernatant of infected tonsils by reinfecting an indicator cell line. Reinfecting fresh tonsil culture did not result in infection indicating that the viral replication was controlled by innate antiviral restriction factors. These studies indicate that recombination can occur leading to infectious VLPs with a host tropism that includes humans.

Keynotes

- Several cells, cell lines and tissues have been described to spontaneously release infectious virus-like particles from ERV families. This activation can also be triggered by factors such as infection by non-related viruses, chemical agents, physical agents, or long-term passaging.
- The formation of functional proteins by ERVs may **complement** homologous viruses or retroviral vectors during infection, which may result in chimeric viruses and virus-like particles. Vice versa, infections with homologous viruses may aid ERV genomes and/or encoded proteins to establish ERV-derived virus-like particles.
- The presence of functional retroviral genes or preservation of homologous sequences may allow for genomic **recombination** during infection with homologous viruses or retroviral vectors.
- Demonstration of complementation and recombination resulting in infectious virus-like particles illustrates that such scenario's cannot be ruled out. Nevertheless, the cases are limited and context-specific, not allowing to generalize the likelihood of such outcome.

6 Risk classification of ERVs

International risk classifications of ERVs are scarce. Only in a few instances, advisory bodies have considered a risk group classification of ERVs. In this study, different cases were evaluated to gain an insight in how different elements contributed to the determination of a risk group for ERVs. An overview is presented in Table 6.

6.1 Risk classification of the feline endogenous retrovirus RD-114

The domestic cat retains both replication-competent and incompetent ERV-DCs in its genome. Those that are infectious and replication-competent could be of concern, i.e. ERV-DC10 and ERV-DC18 of group III (Anai et al., 2012), as well as ERV-DC14 of group I (Kuse et al., 2016). Indeed, infectious and replication-competent ERVs have been shown to be actively passed between cats engaged in social activities, such as grooming (Anai et al., 2012; Kawasaki & Nishigaki, 2018). It is also known that cats harbour ERV-DC-like elements, which have been identified in the genomes of both primates and rodents. This suggests that ERV-DCs have been transmitted between cats, rodents, and primates alike. Furthermore, this illustrates that ERV-DCs may act as platforms for homologous and heterologous recombination events in cross-species transmission. This was confirmed through phylogenetic analysis by (Anai et al., 2012), who identified strong similarities between ERV-DCs, RD-114 and primate ERVs on the level of the *pol* gene, whereas the *env*/TM gene of ERV-DCs showed higher similarities with rodent ERVs, murine and feline virus (MLV, FeLV respectively). Furthermore, the same researchers identified a new FeLV variant, FeLV-D resulting from the transduction of a group I ERV-DC *env* gene into FeLV.

COGEM states that **RD-114** should be considered a strictly animal pathogenic virus to be classified as pathogenicity class 2. This is in line with Brigulla & Zentrale Kommission für die Biologische Sicherheit (ZKBS) (2020) that also classifies RD-114 in its list of donor and recipient organisms for genetic modification as Risk Group 2.

6.2 Risk classification of porcine endogenous retroviruses A, B and C

Three subtypes of PERV have been described based on cell tropism, sequence variation, and receptor specificities. PERV-A and PERV-B are present in the genome of all pigs, while PERV-C is present in many, but not all pigs. PERV-A and PERV-B infect human cells and therefore pose a risk for xenotransplantation, while PERV-C infects pig cells only. However, in some pigs, PERV-A/C recombinants were found, which were able to infect human cells and were characterized by a high replication rate (Kaulitz et al., 2013; Mattiuzzo et al., 2012). PERV-A/B recombinants have not been described so far.

So far, no diseases have been described in pigs or humans that are causally associated with activation of PERV. In the case of a high viral load, PERV can also have an immunosuppressive effect, since at high concentrations the envelope protein has a similar inhibitory effect on the proliferation of human mitogen-stimulated lymphocytes as the envelope proteins of human immunodeficiency virus 1 and baboon endogenous virus.

Taking the above-mentioned characteristics of PERV into account, ZKBS (2018) has issued a position on the classification of **PK-15** cells, a cell line exhibiting epithelial morphology that was isolated from the kidney of an adult pig. This cell line was found to spontaneously express porcine type-C gammaretroviruses, which were initially reported as defective and non-infectious for a variety of mammalian (tumour) cell lines (Todaro et al., 1974). Subsequent analysis of a subset of porcine type-C gammaretroviruses, porcine endogenous retroviruses (PERVs), revealed however the transmission of polytropic subtypes PERV-A and PERV-B to a broader variety of hosts, including human cells. Furthermore, when PERVs produced by PK-15 cells were subsequently passaged in human cells, the viruses acquired resistance to inactivation by the human complement system, thus providing it with an immune-evasive strategy. Based on this knowledge and provided that exposure to PERVs could additionally result in insertional mutagenesis, the ZKBS (2018) assigned the polytropic subtypes PERV-

A and PERV-B as well as recombinant PERV-A/C to a risk group 2 as donor and recipient organisms for genetic engineering work.

For similar reasons, also other porcine materials which contain PERV-B or PERV-A/C can be considered risk group 2.

As PERV-C is an ecotropic virus that can only infect pigs, ZKBS has assigned this virus to risk group 1.

6.3 Risk classification of baboon endogenous retrovirus

Baboon endogenous virus (BaEV) is an endogenous type-C retrovirus present in multiple copies in many Old-World monkey species. The limited level of variation between different BaEV isolates indicates a relative recent integration. Furthermore, BaEV proviruses may contain open reading frames for all major genes. BaEV is mainly defective (and therefore inactive) in the genome but can form infectious VLPs under specific conditions as is indicated by transfection experiments into permissive cells (Cohen et al., 1981; van der Kuyl et al., 1995). To date it is unclear to what extent the exogenous virus particles occur *in vivo* or contribute to infection and horizontal transmission.

The ZKBS (2017) assigned BaEV to risk group 2 as a donor and recipient organism in the context of genetic engineering. Although it is acknowledged that no causal relationship between infection with BaEV and disease has been established, this classification is based on the following considerations:

- BaEV is replication-competent;
- it has a broad cell tropism and a wide host range in vitro, including various human cells;
- in the course of infection, the viral genome integrates into the genome of the host cell and it is not known whether insertional mutagenesis can lead to an altered transcription rate of proto-oncogenes or to the inactivation of tumour suppressor genes;
- BaEV can have an immunosuppressive effect.

Similarly, COGEM (2021a) recommends classifying BaEV in pathogenicity class 2 as a strictly animal pathogenic virus.

6.4 Risk classification of human endogenous retrovirus MERV/HERV-K and HERV-K113

Melanoma-associated endogenous retrovirus (MERV) / Human endogenous retrovirus K (HERV-K) particles are formed in melanoma cell lines or tumour tissues. However, MERV/HERV-K particles are defective and non-infectious. On this basis, ZKBS (2010) assigned MERV/HERV-K to risk group 1 as a donor and recipient organism for genetic engineering work. Whether or not MERV/HERV-K plays a role in the development of melanoma or the progression of the disease is being discussed. Proof of an etiological connection has not yet been provided.

Also, **HERV-K113**, a human ERV that is integrated into human chromosome 19 does not form infectious VLPs nor replication-competent particles, despite of its full-length presence. The lack of infectivity is the result of a defective reverse transcriptase and of an Env protein that is not efficiently incorporated in the retroviral particle (Beimforde et al., 2008; Dewannieux et al., 2005). ZKBS (2011) thus justified that no risk group should be assigned to HERV-K113, however indicating that case-by-case assessments are required when considering genetic modification that complements the defective functions.

6.5 Other risk classifications

ZKBS[6] includes in its list of donor- and receptor organisms for genetic modification a general indication of risk group 1 for **endogenous animal retroviruses** that have entered the genome naturally and are apathogenic.

[6] https://www.zkbs-online.de/ZKBS/SharedDocs/Downloads/Organismenliste.html

An overview of the different risk classifications for the ERVs can be found in table 6.

Keynotes

- Taking into account that most ERVs are not known to be pathogenic to their hosts and, under natural conditions, cannot form exogenous viruses or infect other hosts, a pathogenicity class 1 may be considered.
- However, for a risk assessment risk assessors should usually consider additional factors, such as:
    - Replication competence
    - Full length vs partial presence
    - Effect of mutations
    - Tropism
    - Impact on (cellular) physiology of the host
    - Insertional mutagenesis
    - Immunosuppressive effect associated with a high viral load

Table 6 Summary of the risk group classification of different ERVs in cats, baboons, pigs and humans

	RD-114	BaEV	PERV-A	PERV-B	PERV-C	PERV-A/C	MERV/HERV-K	HERV-K113
Replication competence	Yes	Yes, but mostly inactive Exogeneous virus particles formed under specific conditions					Yes Exogeneous virus particles formed under specific conditions	No
Host range *in vivo*	Likely limited to housecats, not found in big cats such as lion or puma (some indications that dogs might be infected)	Baboon and related species, not in apes or humans (broad host range *in vitro*)	Pigs	Pigs	Pigs	Pigs	Humans	Humans
Host range *in vitro*		Broad mammalian host range, including humans	Broad mammalian host range, including humans	Broad mammalian host range, including humans	Ecotropic	Broad mammalian host range, including humans		
Disease in host	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	No causal relation between MEV/ERV-L & melanoma origin	
Disease in other animals	Not known to infect or cause disease	Not known to infect or cause disease	Not known to infect or cause disease	Not known to infect or cause disease	Not known to infect or cause disease	Not known to infect or cause disease	Not known to infect or cause disease	Not known to infect or cause disease
Disease in humans	Not known to infect or create disease	Not known to infect or create disease	Not known to infect or create disease	Not known to infect or create disease	Not known to infect or create disease	Not known to infect or create disease	Not known to infect or create disease	Not known to infect or create disease
Risk Group	**2**	**2**	**2**	**2**	**1**	**2**	**1**	**No risk**
Reference	COGEM CGM/150529-01 (2015)	COGEM CGM/210316-02 (2021) ZKBS Az. 45242.0147 (2017)	ZKBS Az. 6790-10-65 (2018)	ZKBS Az. 6790-10-65 (2018)	ZKBS Az. 6790-10-65 (2018)	ZKBS Az. 6790-10-65 (2018)	ZKBS Az. 6790-05-02-0060 (2010)	ZKBS Az.6790-05-02-0073 (2011)

7 Precautionary approaches towards managing the potential risks of ERVs

Because of the many unknowns on the potential risks of certain ERV elements, several approaches have been described to test for the absence of ERV (non-)infectious virus-like particles in biological material and/or to clear such ERVs from the biological material. Most information is included in guidance documents prepared for industry, in particular for biological material to be used in the production of medicinal products. Below, a summary is provided of the guidance documents which refer to (endogenous) retrovirus. Additionally, some representative examples are described aiming at new technologies to inactivate or remove ERVs from biological material.

7.1 Guidance for industry on viral safety

Various guidelines address the microbial or viral safety of biotechnology products, focussing on screening methods as well as clearance methods. A summary of the guidelines that refer to (endogenous) retroviruses is provided in Table 7 and in the text below.

Obviously, the focus of the guidelines is broader than (endogenous) retroviruses and includes many other exogenous and endogenous adventitious agents. However, testing for exogenous viruses may also be important in view of ERVs, as ERV expression profiles may be altered due to the presence of an exogenous virus. For example, it has been demonstrated that ALV infection in chicken fibroblasts induces ERV-encoded long, non-coding RNA molecules which interfere with antiviral pathways and subsequently favour ALV propagation (Chen et al., 2021). Therefore, by ensuring that exogenous viral pathogens are absent, potential complicating factors attributed to ERV expression may also be avoided.

Table 7: Overview of international guidelines on ERVs present in biotechnologically relevant material.

Source	Focussing on	Recommendations on	Reference
ICH Q5A(R1)	Viral safety in biotechnology products derived from cell lines	Control of source material Testing methods Clearance methods [7] Risk evaluation	EMEA CPMP/ICH, 1997
WHO Technical Report 941	Microbial safety of rabies vaccine	Control of source material Testing methods Clearance methods	Bourhy et al., 2007
FDA Guidance for Industry	Characterisation and qualification of cell substrates and other biological material for vaccine production	Control of source material Testing methods Clearance methods Risk evaluation	FDA, 2010
EMEA/CHMP/BWP/398498/2005	Viral safety in biotechnology products	Control of source material Testing methods Risk evaluation	EMEA CHMP/BWP, 2008
EMA/CHMP/267815/2011	Metagenomics approach for vaccine testing	/	EMEA CHMP, 2010
CVMP/EMA opinion	RD-114	/	EMEA CVMP, 2010
EMA/CVMP/IWP/592652/2014	RD-114	Risk evaluation Risk management	EMEA CVMP, 2017

[7] Process steps that can be considered to be effective in inactivating/removing viruses during the production process.

Source	Focussing on	Recommendations on	Reference
EMA/CVMP/IWP /37924/2014	Live immunological veterinary medicinal products	Heat treatment to inactivate endogenous retroviruses	EMEA CVMP, 2015
European Pharmacopoeia	Viral safety for cell cultures used for veterinary vaccine production	Testing methods Risk evaluation	European Directorate for the Quality of Medicines, 2023

7.1.1 International Council for Harmonisation (ICH)

In 1997, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) issued the guideline called **Q5A "Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin"** (**CPMP/ICH/295/95, Q5A(R1)**)[8]. This guideline for industry extensively describes screening methods of cell lines for endogenous viruses, including (endogenous) retroviruses. Recommended tests for (endogenous) retroviruses include infectivity assays by direct inoculation or co-cultivation, assays for RT activity, and evaluation of particles by transmission electron microscopy. Induction studies can be used for a new cell substrate to evaluate the presence of so far unknown ERVs. Finally, recommendations are included on how to determine the capacity of the manufacturing process on removing and/or inactivating rodent retroviruses from products obtained from cells containing ERVs or (infectious) VLPs. As fluctuations may occur in the levels of ERV over time in the production, the timepoint(s) of assessment should be carefully considered to allow a reliable calculation of the so-called dose risk factor.

Importantly, the guideline highlights that cell lines expressing endogenous retrovirus particles may be acceptable for use in manufacturing based on a risk evaluation for which the guideline provides some examples, including the assessment of viral clearance during production.

The ICH Expert Working Group is currently working on a revision of the guideline Q5A(R1). In view of (endogenous) retroviruses, the revision mainly includes additional clarifications.

7.1.2 World Health Organisation (WHO)

In 2007, the **WHO** released a **Technical Report** providing recommendations for inactivated rabies vaccine for human use produced in cell substrates or embryonated eggs (Bourhy et al., 2007). The report describes specific testing methods for endogenous (retro)viruses for animal colonies from which cells are harvested, including antibody testing, reverse transcriptase PCR and nucleic acid amplification tests. The choice of tests and testing procedures for monitoring as well as the appropriate number of animals should be approved by the national regulatory authority. It is highlighted that results of the reverse transcriptase PCR need to be interpreted with caution as reverse transcriptase activity is not unique to retroviruses.

When embryonated chicken or duck eggs are used for vaccine production, they should be derived from SPF-flocks, which are regularly monitored for the absence of avian pathogens, including avian retroviruses using test methods as described for the animal colonies. Vaccination of the flocks is not allowed (chickens) or should be carefully documented (ducks) to allow a thorough risk assessment.

Also, raw materials used in the production process, such as animal serum, trypsin or cell culture media, should be tested to demonstrate the absence of microbiological agents. Finally, high-level recommendations are provided for the inactivation of potential contaminant viruses. Only those cells shown to be free from contamination should be used.

7.1.3 European Medicines Agency (EMA)

Several documents were published by the Committee for Medicinal Products for Human Use (CHMP) and the Biologics Working Party (BWP) of the European Medicines Agency (EMA) (see Table 7).

In 2008, a general recommendation "**Guideline on virus safety evaluation of biotechnological investigational medicinal products"** (**EMEA/CHMP/BWP/398498/2005**)[9] was issued aiming to ensure the

[8] https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-5-r1-viral-safety-evaluation-biotechnology-products-derived-cell-lines-human-animal-origin_en.pdf
[9] https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-virus-safety-evaluation-biotechnological-investigational-medicinal-products_en.pdf

virus safety of biotechnological products, such as cell lines, raw materials of biological origin and unprocessed bulk. Similar as for the documents issued by ICH and FDA, focus is on ensuring viral safety by providing appropriate documentation, using screening methods and performing thorough risk evaluations. Testing methods for specific types of cells are outlined in detail. Also, clear instructions are provided on the validation of virus reduction. In brief, the objective of the validation is two-fold; firstly, to characterise and evaluate process steps that can be considered to be effective in inactivating/removing viruses and secondly, to estimate quantitatively the overall level of reduction of any virus/viral particle, including ERV particles. With the precautionary principle in mind, the guideline states that even when no raw materials of biological origin have been used and a cell line is fully tested, the downstream process for all investigational medicinal products (IMPs) should be evaluated for virus inactivation/removal due to limitations in viral detection assays.

In 2010, EMA/CHMP published an additional report (**EMA/CHMP/267815/2011**) in response to a publication by (Victoria et al., 2010) which presented new information regarding the possible presence of nucleic acid sequences from endogenous avian leukosis virus (ALV) or endogenous simian retrovirus (SRV) in a few batches of different live attenuated viral vaccines tested by a new analytical metagenomics approach (i.e. polymerase chain reaction (PCR) combined with pyrosequencing). EMA/CHMP acknowledged the value of the new technology, however, it is to be understood as a supplement to current standards of testing which cannot be replaced. The CHMP has started a dialogue with other authorities, including the US Food and Drug Administration, the World Health Organization and the European Directorate for the Quality of Medicines, to start working towards a common approach for the use of metagenomic testing in biological medicines.

Apart from the general recommendations, EMA also issued several documents on feline replication-competent RD-114 in veterinary vaccines in response to a publication by Miyazawa et al. (2010) which described the detection of RD-114 in a number of commercially available live attenuated feline and canine vaccines in the UK and Japan. Even though there have not been any reports of an association of RD-114 with disease in dogs or cats, a first **opinion paper** published by the **Committee for Medicinal Products for Veterinary Use (CVMP) of EMA** in 2010 highlighted that it is not considered acceptable to have vaccine batches on the market containing unwanted live virus particles, without trying to investigate and correct this issue. Therefore, considerations for improvement of the vaccines are needed and appropriate actions might include replacement of cell lines, introduction of manufacturing steps to allow clearance of the virus, and inactivation of retrovirus RD-114.

In a follow up report published in 2017 (**EMA/CVMP/IWP/592652/2014**), a risk management strategy is described in view of the potential presence of replication-competent endogenous retrovirus RD-114 in starting materials and final products of feline and canine vaccines. The risk management should be based on a thorough risk assessment starting with determining whether replication-competent RD-114 is present or absent in starting materials. In case RD-114 is present, either starting material should be replaced or downstream processes should be implemented to remove or inactivate replication-competent RD-114. For example, material could be treated with heat as explained in detail in the reflection paper by the CVMP (**EMA/CVMP/IWP/37924/2014**)[10].

Where starting materials have been identified as positive for replicative retroviral RD-114 qualitative and/or quantitative tests should be performed for the presence of replication-competent RD-114 retrovirus in final vaccines or in active ingredients derived from the concerned starting material. If there is no feasible alternative, then the risk assessment should demonstrate that the risk associated with the presence of RD-114 is negligible.

7.1.4 European Pharmacopoeia

Chapter 5.2.4 of the **European Pharmacopoeia** on cell cultures to produce veterinary vaccines requires cells to be examined for the presence of retroviruses. If the presence of retrovirus is known or established by testing, such as an endpoint product-enhanced reverse transcriptase (PERT) assay, then infectivity assays should be carried out to investigate the presence of replication-competent virus. Cell seeds that show the presence of infectious retroviruses are not acceptable to produce vaccines. In exceptional cases of positive or equivocal result in the infectivity assay, it may be justified and authorised to use such cells, provided that a justification is provided based on a risk assessment including all available data and any downstream processing steps until the final product stage. The results of the risk assessment must

[10] https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-use-heat-treatment-inactivate-endogenous-retroviruses-live-immunological-veterinary_en.pdf

demonstrate that the risk associated with the presence of infectious retroviruses is negligible in the final product.

In summary, the recommendations made for industry in view of assuring the viral safety of biological materials, including those relevant for (endogenous) retrovirus, include:

- Appropriate documentation should be provided to demonstrate the microbiological/virological (safety) status of the materials
- Testing should be performed to demonstrate the absence of contaminating viruses;
- Specific care is required for novel cell lines as the presence of contaminating viruses, including ERVs, has not yet been determined;
- An assessment is to be made to characterize and evaluate process steps that can be considered to be effective in inactivating/removing viruses, irrespective of whether viral testing has a positive or negative outcome;
- A quantitative assessment will help to determine the overall level of reduction of virus / virus particles;
- If, in exceptional cases, positive cells are to be used in the production process, a justification is to be provided based on a risk assessment that shows a negligible risk associated with the presence of infectious retroviruses in the final product.

7.2 New technologies to suppress, inactivate, or remove ERVs

In this section, a few representative examples are described on new technologies that have been explored to suppress, inactivate or remove ERVs from biological material. It remains outside the scope of the current study to provide a complete overview of all new technologies.

Fukuma et al. (2013) established a feline cell line stably expressing human Tetherin (Teth-CRFK cells). The presence of human Tetherin **suppressed** the release of infectious ERVs to undetectable levels, without affecting the production of non-enveloped viruses (such as parvoviruses). This is effectuated by the dimerized form of Tetherin, which establishes a tether or 'hook' the moment enveloped viruses bud from the cell, thereby limiting viral spread (Andrew et al., 2009).

To **prevent** the **production** of humanotropic PERVs (especially the recombinant PERV-A/C) as a precautionary measure in xenotransplantation, CRISPR-Cas technology proved effective. In 2015, Yang et al. demonstrated that genome-wide inactivation of PERVs was made possible in PK15 cells by targeting the catalytic core of the *pol* gene with CRISPR, effectively compromising PERV production. Later, Godehardt et al. (2020) identified by means of electron microscopy that the compromised production could be linked to a defective morphology of the PERV particles.

Noteworthy, Scobie et al. (2017) utilized the same CRISPR-Cas to engineer transgenic PERV-inactivated pigs through somatic cell nuclear transfer (SCNT) of primary fetal fibroblast cells and obtained a 100% inactivation efficiency. The high efficiency is attributed to a combinatorial approach of CRISPR-Cas, p53 apoptosis inhibitor (pFTα) and growth factor (bFGF). Other than CRISPR-Cas, transgenic pigs may also be established through transduction with viral vectors carrying small RNAs, such as small interfering RNA (siRNA) and short-hairpin RNA (shRNA), actively downregulating PERV expression in donor-tissue (Denner & Tönjes, 2012).

Transcription activator-like effector nucleases (TALEN) have proven successful in establishing a **knock-out** CRFK producer cell line devoid of RD-114 for live-attenuated vaccine production (Shimode et al., 2022). In brief, by applying the TALEN technique they disabled the *env* gene of RD-114-related proviruses present in the CRFK producer cells, so that the generation of infectious RD-114 was prevented, while maintaining high titer (10^7 $TCID_{50}$/mL) production of live-attenuated viruses.

Keynotes

- As a precautionary measure, approaches have been described for medicinal applications of biological materials to reduce the likelihood of ERV virus(-like) particles being present and, where relevant, to clear the material from adventitious agents (including (endogenous) retroviruses).

- These efforts are focused on specific ERV of particular concern e.g., in vaccine production, acknowledging that for a major part of ERVs, no function and/or negative effect has been identified.
- New technologies have been explored in R&D to suppress, inactivate or remove ERVs from biological material.

8 Conclusion

In this study of ERVs, we reviewed different aspects of a wide array of scientific research and attempted to extract the key findings that can assist risk assessment and management, in particular when considering applications of biotechnological techniques. The **main challenge** in this field remains the limited knowledge available towards biosafety implications of ERVs. Specific cases have been identified, which only present a fraction of the huge diversity and amount of published data and scientific studies about ERVs in general. Care should therefore be taken that findings in specific cases are not generically extrapolated to all ERVs. Notwithstanding these limitations, we would like to formulate some conclusions based on our findings and the research questions as outlined at the beginning of this report.

What is known about the presence of ERVs in biological material routinely used in biotechnology (e.g. cells, animals)?

In humans alone, at least 8% of the genome has been shaped by ERVs over time. Also, in other mammalian and non-mammalian species ERVs are widespread. It was therefore no surprise to see that a screening of literature resulted in many papers identifying ERVs in cell lines, primary cells, tissues and animals regularly used in research. While in some cases complete proviral genomes are present, in most cases ERVs seemed to be limited to partial and incomplete sequences. However, these results need to be interpreted with great care, as in most studies only RNA detection methods were used. Consequently, no conclusion can be drawn on the presence of other ERV elements. Other approaches are required to ascertain whether VLPs, infectious virus particles or replication-competent virus particles are formed.

Of the different ERVs present within biological material, the majority is argued to consist of non-coding, regulatory elements that correspond to the promoters and transcription factor binding sites present in long-terminal repeats of these ancient retroviruses, termed solitary LTRs (solo-LTRs). Within the host (cell), solo LTRs contribute to both physiological and pathological processes, though their harmful application appears to be limited in the context of biotechnological activities. In contrast to the deliberate introduction of ERV sequences into cells as part of a research approach, exposure to integrated ERV sequences seems highly unlikely.

Are ERVs capable of independently generating a fully infectious virus and if so, what are the conditions that facilitate or promote such generation? What other types of ERV activation can occur and what is the potential risk of such activation?

Depending on the intrinsic characteristics of the vertebrate host (cell), the type of ERV present and the experimental conditions, ERV sequences may become activated. Cases were presented of spontaneous activation as well activation by infection of non-related viruses, chemical agents, physical agents or long-term passaging.

The activation of incomplete proviral sequences will rarely lead to the production of infectious, replication-competent viruses since deletions and /or mutations of vital survival functions will require complementation (protein level) and recombination (genetic sequences) to result in an infectious viral particle.

Still, in several cases the activation was reported to result in the assembly of VLPs, infectious virus particles or even RCVs. Whether or not the production of infectious viral particles creates a risk in the context of biotechnology activities, depends on the effects upon infection of the host cell (e.g., integration, gene activation, productive infection). For example, even though porcine tissues naturally produce infectious and replication-competent PERVs, preclinical and clinical studies on bioartificial livers and pancreatic island xenotransplantation have not shown an active infection of the human recipient by PERV.

Can infection or transduction of ERV containing material with a virus / viral vector result in activation / complementation / recombination to establish an infectious (chimeric) virus?

Some cases of infection with non-retroviral viruses have been demonstrated to have an effect on ERV transcription in the infected cells. While the production of proteins and VLPs following such viral infections has been demonstrated, no generation of infectious or replication-competent particles have been reported.

On the other hand, when using homologous retroviruses, ERVs may recombine with genetic material of, or be complemented by proteins of the homologous virus. Such events could ultimately result in the production of infectious virus particles. If such would occur, a subsequent, but so far theoretical, risk may be the release of the particles and potential exposure of people or the environment.

What are indications for classifying ERVs to a risk group (pathogenicity class), acknowledging that most ERVs are not pathogenic when they are not expressed?

Whereas the publications elaborated on the presence of different types of ERV and their activation, the potential risk of the ERV activation for health and/or environment was in most cases not addressed. To quantify the risks, one could consider assigning a **pathogenicity class or risk group** to ERVs. So far, risk classifications for ERVs have been scarce. COGEM has classified the evaluated ERVs (RD-114 and BaEV) in pathogenicity class 2. ZKBS follows the same rationale yet has also indicated that PERV-C and endogenous animal retroviruses that have entered the genome naturally and are apathogenic, are assigned to risk group 1.

Interestingly, the risk appreciation appears to be different in the context of ERVs reported as contamination in medicinal products. As an example, RD-114 is classified by COGEM and ZKBS for application in genetic engineering as a pathogenicity class 2 organism. On the other hand, the experts of the Committee for Medicinal Products for Veterinary Use of the EMA concluded in a detailed risk assessment that replication-competent RD-114 present in vaccines for cats and dogs was highly unlikely to have a safety impact. Still, risk management guidelines were developed to ensure that there is negligible risk for animals administered dog and cat vaccines and for users of the products by ensuring that the vaccines comply with current quality requirements.

What precautionary approaches have been described towards managing potential risks of ERVs?

Because of the many unknowns about the potential risks of certain ERV elements, several precautionary approaches have been described in R&D as well as in clinical settings to either test for the absence of ERV (non-) infectious virus(like) particles in biological material or to try to remove ERVs present in biological material. In particular for industry, regulatory frameworks require consideration of the likelihood of ERVs being present in the source material. Different guidelines stipulate a cautionary, fact-based testing approach towards the assessment and clearance of ERV particles in vaccine formulations. Additional guidelines on ERV clearance are being developed (e.g., by the ICH).

On the experimental level, both cell lines and animals of interest in vaccine production and xenotransplantation have been genetically modified to minimize the shedding of particles and/or the expression of ERV sequences. Approaches include, but are not limited to expression of human Tetherin, TALEN-technology, small RNAs (siRNA, shRNA) and CRISPR-Cas.

It can be concluded that ERVs are present in most lifeforms, both in mammals and non-mammalian species. The vast amount of ERV sequences, segregated or intact, contained within an organism are unlikely to cause any harm to the environment by themselves. It is only through the exposure to related viruses, viral vectors, and deliberate induction of ERV sequences that virus-like particles and potentially (novel) recombinant viruses may be shed into the environment. A cautionary approach should therefore be employed, based on a thorough risk assessment (as provided in the report), to assign the appropriate pathogenicity group to the ERV and its originating source material.

9 Annex information

The annexes to this review can be considered as a guideline for researchers working with cell lines or source material which have been reported in the literature to either actively express ERVs (Annex 1); express ERVs as a result of viral (Annex 2) or artificial (chemical, physical) induction (Annex 3), ranging from RNA to RCVs. The annexes stipulate the form(s) of ERV(s) that have been found for a biological resource, including the technique(s) used, which may help the researcher to identify the reported and potentially other ERVs in their respective substrates.

These annexes were summarized in Sections 4 and 5. Underneath, both legend and annex description can be found per respective annex header. The different literary references which were consulted to assemble the annex information, are outlined underneath. Their full description can be found in the reference list (Section 10).

Annex 1: ERVs in cells and tissues

This table provides a non-limitative overview of different ERVs, the biological material in which they were identified, and the methods used to screen for their presence and activity. The table consists of four main tabs: 'cells or tissues', 'ERV detected', 'detection methods', and 'references'.

The tab 'cells and tissues' provides information on the type of biological material (tissue, primary cells (outlined as 'primary') and established cell lines (outlined as with the respective identifier) and its vertebrate origin (mice: *Mus musculus*, *Mus dunni*; domestic cat: *Felis silvestris catus*; human: *Homo sapiens*; pig: *Sus scrofa*; hamster: *Cricetulus griseus*; cow: *Bos taurus*; green monkey (non-human primate): *Chlorocebus sabeus*; koala: *Phascolarctos cinereus*; chicken: *Gallus gallus*).

The tab 'ERV detected' provides information on the type/format of ERV (including nomenclature, ERV lacking nomenclature is denoted as 'NR') that was detected/not detected using a method aimed at detecting different ERV stages (i.e. (genomic)DNA: (g)DNA; messenger or total RNA: RNA; protein; virus-like particle: VLP; infectious VLP: VINF; infectious and replication-competent VLP: VRC). Per type-tab, a division in detection strategy is outlined as detected in cells (C) or detected in supernatant (SN). A positive correlation between a used method and detected ERV is illustrated with a number one (1)). If the ERV was not reported as detected and no detection method was used, a minus (-) is assigned. If a detection method was used and the ERV was reported as not detected, a minus (-) on an orange background is assigned to the respective entry.

The tab 'detection methods' outlines the methods used to detect ERVs in a variety of isoforms. Between brackets per entry, the biological resources tested is presented (cells: primary or established, depending on the resource; supernatant; target cells: relevant in case of co-culturing and infection assays; tissue; vaccine: supernatant (in case supernatant-derived vaccines were tested)). One method may have been used to test for different ERV isoforms, which results in more than one term between brackets, often: (cells, supernatant).

At the bottom of the table, a summary of detection hits is provided per different ERV stages. An overview on the number of entries that successfully detected a parameter is also provided, grouping cell and supernatant positive hits per entry, per ERV format.

The reference tab contains the literature references (which can also be found in the reference section of the literature review report.

- **Literature** (Anai et al., 2012; Bartman et al., 1995; Bhardwaj et al., 2015; Bittmann et al., 2012; Bjerregaard et al., 2006; C.-P. Chen et al., 2008; Chiappinelli et al., 2015; Chuong et al., 2016; Contreras-Galindo et al., 2015; Curriu et al., 2012; Dube et al., 2014; Duroy et al., 2020; Fei et al., 2014; Garcia-Etxebarria & Jugo, 2014; Godehardt et al., 2020; Golovkina et al., 1994; Han et al., 2015; Kim et al., 2015; Knouf et al., 2009; Kuse et al., 2016; S. P. L. Leong et al., 1988; C. Liu et al., 2019; Macfarlane & Badge, 2015; Machnik et al., 2014; Miller et al., 1996; Mourad et al., 2017; Okada et al., 2011; Ottina et al., 2018; Paprotka et al., 2011; Patzke et al., 2002; Ruprecht et al., 2008; Sakuma et al., 2018; Shimode et al., 2022; Simpson et al., 1996; Tarlinton et al., 2006; Uygur, Melikov, et al., 2019; Victoria et al., 2010; Wang et al., 2020; Wieland et al., 2022; Wu et al., 2012).

Annex 2: Non-retroviral ERV activation

A non-limitative overview of viruses (belonging to different genera than retroviruses) that can influence the expression profile of ERVs. The table consists of five tabs: 'virus affecting ERV', 'ERV affected', 'impact of viral infection on ERV', 'experimental conditions', and 'references'.

The 'virus affecting ERV' tab contains both genus and species of virus per affected ERV.

The tab 'ERV affected' displays the nomenclature of the identified ERV (including the affected locus in some), the ERV isotype that was detected during viral infection/exposure (i.e. (genomic)DNA: (g)DNA; messenger or total RNA: RNA; protein; virus-like particle: VLP; infectious VLP: VINF; infectious and replication-competent VLP: VRC), as well as the origin of the biological resource that was tested. A positive correlation between a used method and detected ERV is illustrated with a number one (1). If the ERV was not reported as detected and no detection method was used, a minus (-) is assigned.

The column 'impact of viral infection on ERV' displays whether the virus caused increased (upregulation) or decreased (downregulation) activation and expression of the ERV.

The tab 'experimental conditions' outlines which 'cell / tissue' was used (reported as primary cells (mention of 'primary') or established cell line (identifier is provided)), and the 'detection method' used to screen for ERVs of a particular isoform.

At the bottom of the table, a summary of detection hits is provided per different ERV stages.

References are included in the 'reference' tab.

- **Literature** (Apostolou et al., 2022; Castro et al., 2022; Dai et al., 2018; Gross et al., 2011; Kim et al., 2015; Kitsou et al., 2021; H. Liu et al., 2022; Perron et al., 1989; Ruprecht et al., 2006; Schmidt et al., 2019; Tovo et al., 2023; Turcanova et al., 2009; Wang et al., 2020; Wieland et al., 2022).

Annex 3: Chemical and physical induction of ERVs

The table contains a non-exhaustive list of information on the type of agent (chemical or physical) and its respective concentration that was used to elicit the activation of ERVs in different biological resources. The table is composed of four tabs: 'agent', 'ERV affected', 'experimental conditions', and 'references'.

The tab 'agent' contains information on the type of agent: CHEM (chemical) or PHYS (physical), its respective nomenclature and concentration used in the experiment.

The tab 'ERV affected' outlines information on the type/format of ERV (including nomenclature) that was detected/not detected using a method aimed at detecting different ERV stages (i.e. (genomic)DNA: (g)DNA; messenger or total RNA: RNA; protein; virus-like particle: VLP; infectious VLP: VINF; infectious and replication-competent VLP: VRC), as well as the origin of the biological resources that was tested. Per type-tab, a division in for detection strategy is outlined as detected in cells (C) or detected in supernatant (SN). A positive correlation between a used method and detected ERV is illustrated with a number one (1). Some positive hits have a grey background, which identifies that of multiple materials screened in a reference, only one (or a set of) specific resource(s) was screened. If the ERV was not reported as detected or no detection method was used, a minus (-) is assigned. If a detection method was used and the ERV was reported as not detected, a minus (-) on an orange background is assigned to the respective entry.

The tab 'experimental conditions' outlines which 'cell / tissue' was used (reported as primary cells (mention of 'primary') or established cell line (identifier is provided)), and the 'detection method' used to screen for ERVs of a particular isoform.

At the bottom of the table, a summary of detection hits is provided per different ERV stages. An overview on the number of entries that successfully detected a parameter is also provided, grouping cell and supernatant positive hits per entry, per ERV format.

References are included in the 'reference' tab.

- **Literature** (Alqahtani et al., 2016; Chiappinelli et al., 2015; Khan et al., 2001; Kwon et al., 2011; Lee et al., 2020; S. P. L. Leong et al., 1988; Ma et al., 2011; Rodrigues Costa et al., 2014; Sakuma et al., 2018; Uygur, Leikina, et al., 2019; Wilson et al., 1998; Yan et al., 2017).

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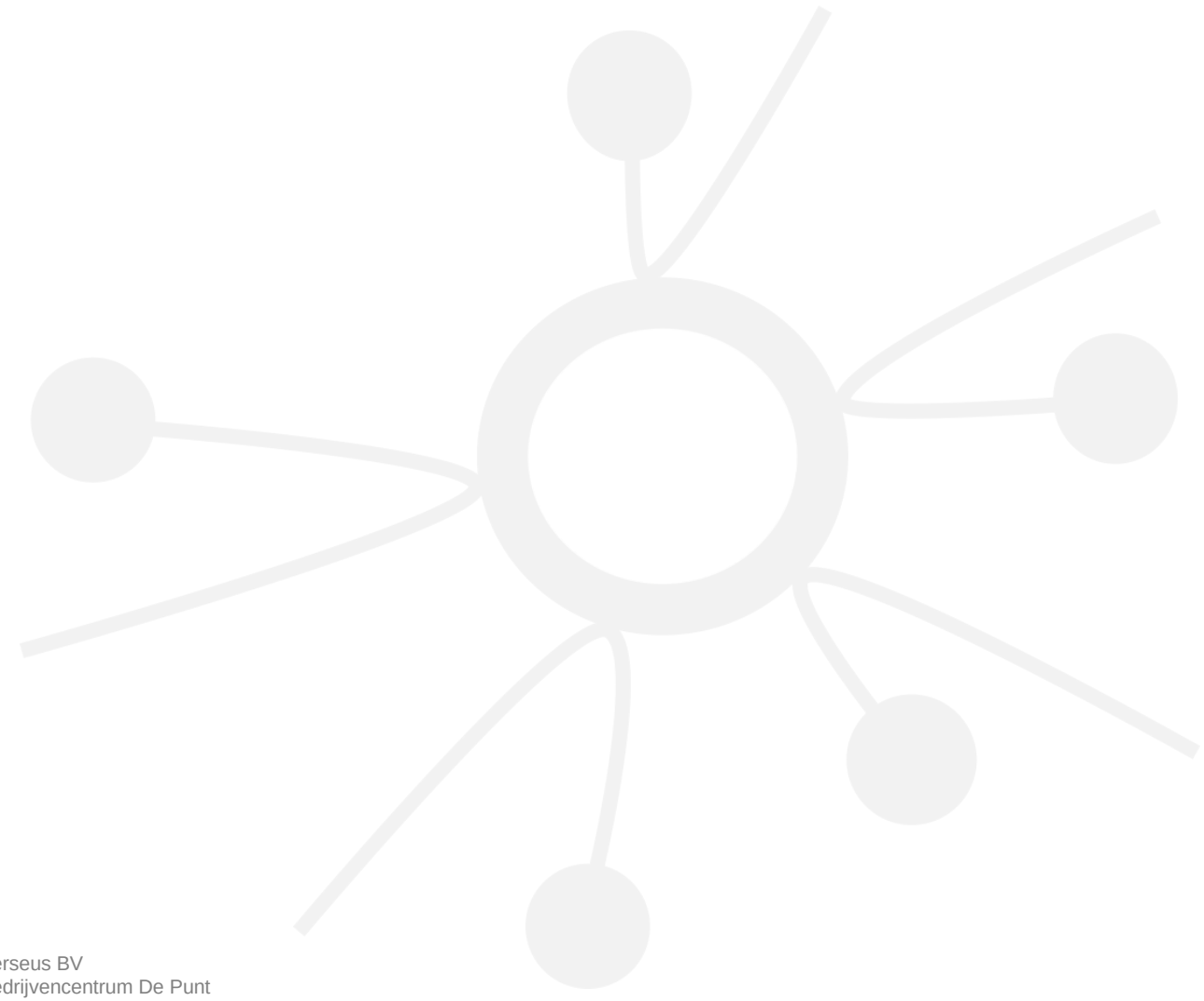

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