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CICLO XXXV

Functional Characterization of Bovine herpesvirus 4 ORF45

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Abstract

Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus, belonging to Rhadinovirus genus, with no strict clear association with disease, even if increasing evidence of its secondary pathogenic role in cases of post-partum metritis in cattle are reported. BoHV-4 potential as a gene delivery vector for immuno-prophylaxis and gene therapy has been already well documented, thanks to its favorable molecular and biological characteristics, such as little or no pathogenicity, absence of oncogenicity; capability to accommodate large amounts of foreign genetic material and the possibility to be manipulated using infectious BoHV-4-derived bacterial artificial chromosome (BAC) genomes. Molecular studies on its ORFs and gene products are on-going, to better clarify the interaction mechanisms between the viral particles and the host cells and also to deeper understand its application as a viral vector. Genome and genes structure are well conserved in Gammaherpesvirus, and ORF45 gene and its product, orf45, is one of those.

BoHV-4 Open Reading Frame 45 (ORF45) codifies for a protein (orf45) of unknown function.

Although preserved the homologues of ORF45 differ greatly in the length of the protein product and most likely perform different biological activities in various viruses.

In addition to differing in the length of the protein, the overall homology of the sequences between homologues is very low. In fact, only a few brief and discrete regions can be aligned with each other. Between these regions, the carboxy-terminal (C-terminal) end shows the highest homology, implying the possibility that this region has a very important biological role.

Similarly, another highly preserved region is the one where the nuclear location sequence (NLS) is present.

For example, in Kaposi's Sarcoma-Associated Herpesvirus (KSHV) ORF45 presents the longest protein product among the various gammaherpesviruses, with its 407aa, while in BoHV-4 is only 241aa and in Murine Gammaherpesvirus-68 (MHV-68) is 353aa.

In recent years the research is focusing a lot on ORF45 and its protein product, in fact in literature there are numerous studies describing different biological activities attributed to the protein product of the ORF45 gene, in particular its belonging to the early-expression protein, expressed at the stage of infection crucial for viral avoidance from the host's immune surveillance.

In particular, ORF45 is a gene not yet investigated in BoHV-4 and we thought to deepen its biological and molecular characteristics.

First we wanted to confirm that also in BoHV-4 the protein product of ORF45 is a nuclear protein and for this we generated a construct with the expression cassette for ORF45 fused with GFP, the green fluorescent protein, placed under the transcriptional control of the heterologous promoter of Human Cytomegalovirus (CMV); following transient transfection in HEK293 of the construct p-CMV-ORF45/EFGP we observed through the acquisition of the images by high resolution confocal microscope that the protein of our interest, that is, ORF45 fused with GFP, was abundantly present in the nuclear compartment of the cells compared to our control given by the transfection of a mock construct expressing only the gene for GFP that unlike was more localized in the cytoplasmic compartment.

These data confirm that reported in the literature where ORF45 is defined as a nuclear protein.

Moreover, we went deeper into the various biological characteristics and activities of ORF45 in BoHV-4, such as being an essential protein for virus replication and its localization in the viral integument area.

To demonstrate our thinkings we have generated and characterized a BoHV-4 mutant ORF45-null exploiting the BAC homologous recombination proces.

To generate a recombinant virus, a BoHV-4 genome clone was isolated from the milk cell fraction from a healthy cow and cloned as BAC and propagated within the bacterial strain E.coli, SW102.
Furthermore, in the first phase of this process called TARGETING we replaced ORF45 in its entirety within the BoHV-4 genome, with a selectable expression cassette for kanamycin resistance and the Galaktokinase gene, This allowed us to discriminate positive clones (that is, where homologous recombination has been successfully carried out and therefore the ORF has been replaced with our DNA of interest) with negative ones, by positive selection on so lid plates with kanamycin.
We then carried out a second growth screening in liquid medium, positive for kanamycin and chloramphenicol.
After extracting the BAC DNA, we performed an enzymatic digestion analysis with the restriction enzyme HindIII. The resultant recombinant pBAC-BoHV-4-AΔORF45-KanaGalK genome of BoHV-4 was electroporated into BEK bovine permissive cells and BEKcre cells.
We could observe that it was not completely able to replenish vital and infectious viral particles (IRVP) and replicate, confirming that ORF45 is crucial for BoHV-4 replication.
To give further confirmation of the fundamental importance of ORF45 for BoHV-4 replication, also by homologous recombination we generated a revertant clone pBAC-BoHV-4-ΔORF45-Revertant, where the expression cassette of ORF45, driven by a CMV heterologous promoter, it was positioned in the opposite direction to natural ORF45 in the BoHV-4 genome.
Also, in this case after extracting the DNA of the BAC we carried out an analysis through enzymatic digestion with the restriction enzyme HindIII and through Southern blotting using a specific probe for ORF45 we confirmed the successful insertion of o the revertant DNA sequence.
In this case, after electroporation in permissive BEK cells, , the reconstitution of the vital and infectious viral particles was successfully achieved, confirming the data.
We then electroporated the DNA of the recombinant clone also in the BEKcre cells, thanks to the enzyme cre-recombinase the BAC floxed cassette is excised and therefore the green fluorescence appears no longer visible, since the GFP gene, being present in the BAC cassette, in turn inserted between two sites LoxP, will be excised along with the BAC cassette.
We then evaluated and compared the growth kinetics of the recombinant virus pBAC-BoHV- 4-ΔORF45-Revertant with a parental virus and a slight decrease in growth kinetics of the recombinant virus is observed.
Since ORF45 was also tagged with an HA epitope (hemoagglutinin), we were able to demonstrate that the product of the ORF45 gene is associated with the virion particles by the western blotting technique, confirming that the protein belongs to the tegument of the virus.
Moreover, the impact of BoHV-4 ORF45 on cellular transcriptome was investigated; many cellular transcriptional pathways were found to be alterated, mainly those involving p90 ribosomal S6 kinase (RSK) and signal-regulated kinase (ERK) complex (RSK/ERK).
This work demonstrates that BoHV-4 replicating cycle is dependent on ORF45 gene product and provides direct evidence that ORF45 gene product is necessary for BoHV-4 lytic replication and thus highlighting the authentic character of BoHV-4 ORF45 and paving the way to further investigations.

Riassunto.

Bovine herpesvirus 4 (BoHV-4) è un Gammaherpesvirus, appartenente al genere Rhadinovirus.
Il potenziale di BoHV-4 come vettore virale per l'immunoprofilassi a scopo vaccinale e nella terapia genica è stato ben documentato in diverse ricerche scientifiche già pubblicate.
In particolare, BoHV-4 presenta numerose caratteristiche sia biologiche che molecolari che lo rendono un'ottima piattaforma vettoriale, come: scarsa o nulla patogenicità, assenza di oncogenicità, capacità di ospitare grandi quantità di materiale genetico estraneo e la possibilità di essere manipolato come Bacterial Artificial Chromosome (BAC) derivati da cloni virali infettivi.
Per migliorare la sua applicabilità ed utilizzo come vettore virale, sfruttando al meglio le sue caratteristiche biologiche e genetiche abbiamo intrapreso alcuni studi per approfondire le funzionalità delle varie Open Reading Frame (ORF) e sui prodotti genici al fine di chiarire al meglio i meccanismi di interazione tra le particelle virali e le cellule ospiti.
Questi studi ci hanno portato a concentrarci in particolare su ORF45.
ORF45 è una proteina tegumentale multifunzionale che si trova solo nei gammaherpesvirus, non è infatti presente negli alfa o nei betaherpesvirus e non esiste un omologo cellulare per ORF45.
In particolare, tra i vari gammaherpesvirus esiste co-linearità tra i vari geni, compreso ORF45, che si trova posizionato sul filamento complementare del genoma. Sebbene conservati gli omologhi di ORF45 differiscono notevolmente nella lunghezza del prodotto proteico e molto probabilmente svolgono diverse attività biologiche nei vari virus.
Oltre a differire nella lunghezza della proteina, l'omologia complessiva della sequenza tra gli omologhi è molto bassa. Infatti, solo poche regioni brevi e discrete possono essere allineate tra loro, tra queste regioni, l'estremità carbossi-terminale (C-terminale) mostra l'omologia più alta, implicando la possibilità che questa regione abbia un ruolo biologico molto importante.
Allo stesso modo, un'altra area altamente conservata è la regione dov'è presente la sequenza di localizzazione nucleare (NLS).
Ad esempio, in Kaposi's Sarcoma-Associated Herpesvirus (KSHV) ORF45 presenta il prodotto proteico più lungo tra i vari gammaherpesvirus, con i suoi 407aa, mentre in BoHV-4 è solo di 241aa e in Murine Gammaherpesvirus-68 (MHV-68) è di 353aa.
In questi ultimi anni la ricerca si sta soffermando molto su ORF45 e il suo prodotto proteico, in letteratura infatti troviamo numerosi studi che descrivono diverse attività biologiche attribuite al prodotto proteico del gene ORF45, in particolare come possa essere una proteina ad espressione precoce in fase d'infezione fondamentale per l'elusione virale dalla sorveglianza immunitaria dell'ospite.
Essendo ORF45 un gene non ancora indagato in BoHV-4 abbiamo pensato di approfondire le sue caratteristiche biologiche e molecolari.
Come prima cosa abbiamo voluto confermare che anche in BoHV-4 il prodotto proteico di ORF45 sia una proteina nucleare e per questo abbiamo generato un costrutto con la cassetta di espressione per ORF45 fusa con la cassetta di espressione per la Green Fluorescent Protein (EGFP), posto sotto il controllo trascrizionale di un promotore eterologo di Citomegalovirus Umano (CMV); in seguito a trasfezione transiente in HEK293 del costrutto p-CMV-ORF45/EFGP abbiamo osservato mediante l'acquisizione delle immagini da microscopio confocale ad alta risoluzione che la proteina di nostro interesse, cioè ORF45 fusa con la GFP, era abbondantemente presente nel comparto nucleare delle cellule rispetto al nostro controllo dato dalla trasfezione di un costrutto esprimente solo il gene per la EGFP che, a differenza, era maggiormente localizzata nel compartimento citoplasmatico.
Questi dati confermano quello riportato in letteratura dove ORF45 viene definita appunto come una proteina nucleare.

In seguito, abbiamo approfondito quelle che sono le varie caratteristiche e attività biologiche di ORF45 in BoHV-4, come ad esempio essere una proteina essenziale per la replicazione del virus a localizzazione nella zona del tegumento virale.
Per dimostrare ciò abbiamo generato e caratterizzato un BoHV-4 mutante ORF45-nullo sfruttando il processo di ricombinazione omologa.
Per generare un virus ricombinante è stato isolato un clone del genoma di BoHV-4 dalla frazione di cellule del latte da una vacca sana ed è stato clonato come BAC e propagato nel ceppo batterico E.coli, SW102.
Successivamente, nella prima fase di questo processo definito TARGETING abbiamo sostituito ORF45 per intero all'interno del genoma di BoHV-4, con una cassetta di espressione selezionabile per la resistenza alla kanamicina e il gene Galattochinasi, questo ci ha permesso di discriminare i cloni positivi (cioè dove è avvenuta con successo la ricombinazione omologa e quindi l'avvenuta sostituzione dell'ORF con la nostra cassetta di interesse) con quelli negativi, mediante selezione positiva su piastre solide con kanamicina.
In seguito, abbiamo effettuato anche un secondo screening di crescita in terreno liquido, positiva per kanamicina e per cloramfenicolo.
Dopo aver estratto il DNA dei BAC abbiamo effettuato un'analisi tramite digestione enzimatica con l'enzima di restrizione HindIII. Il risultante genoma di BoHV-4 ricombinante pBAC-BoHV-4-AΔORF45-KanaGalK è stato elettroporato in cellule permissive di bovino BEK e in cellule BEKcre. Dove, abbiamo potuto osservare che non era completamente in grado di ricostituire le particelle virali vitali ed infettive (IRVP) e di replicarsi, confermando che ORF45 è fondamentale per la replicazione di BoHV-4.
Per dare un'ulteriore conferma della fondamentale importanza di ORF45 per la replicazione di BoHV-4, sempre mediante ricombinazione omologa abbiamo generato un clone revertante pBAC-BoHV- 4-ΔORF45-Revertant, dove la cassetta di espressione di ORF45, guidata da un promotore eterologo CMV, è stata posizionata in direzione opposta rispetto all'ORF45 naturale nel genoma di BoHV-4.
Anche in questo caso dopo aver estratto il DNA dei BAC abbiamo effettuato un'analisi tramite digestione enzimatica con l'enzima di restrizione HindIII e tramite Southern blotting utilizzando una sonda specifica per ORF45 abbiamo confermato il successo dell'avvenuta inserzione della cassetta di espressione.
In questo caso dopo elettroporazione in cellule permissive, la ricostituzione delle particelle virali vitali ed infettive è avvenuta con successo confermando il dato.
Abbiamo elettroporato in seguito il DNA del clone ricombinante anche nelle cellule BEKcre, che grazie all'enzima cre-ricombinasi, la cassetta BAC floxata viene escissa e quindi non appare più visibile la fluorescenza verde, poiché il gene della GFP, essendo presente nella cassetta del BAC, a sua volta inserita tra due siti LoxP, verrà escisso insieme alla cassetta del BAC.
In seguito, abbiamo valutato e messo a confronto le cinetiche di crescita del virus ricombinante pBAC-BoHV- 4-ΔORF45-Revertant con un virus parentale, osservando una lieve flessione nella cinetica di crescita del virus revertante ricombinante.
Poiché ORF45 è stato anche taggato con un epitopo HA (emoagglutinina), siamo stati in grado di dimostrare che il prodotto del gene ORF45 è associato alle particelle del virione mediante la tecnica di western blotting confermando che la proteina è appartenente al tegumento del virus.
Infine, è stato studiato l'impatto di BoHV-4 ORF45 sul trascrittoma cellulare dove molte vie trascrizionali cellulari sono risultate alterate, principalmente quelle che coinvolgono la p90 ribosomiale S6 chinasi (RSK) e il complesso della chinasi regolata dal segnale (ERK) (RSK/ERK).

Questo lavoro ci permette di dimostrare che il ciclo di replicazione di BoHV-4 dipende dal prodotto del gene ORF45 e fornisce prove dirette che il prodotto del gene ORF45 è necessario per la replicazione litica di BoHV-4, evidenziando così il carattere autentico di BoHV-4 ORF45 e aprendo la strada a ulteriori indagini.

1. Introduction

Herpesvirus.

Herpesvirus are viruses endogenous to humans and for many animal species (Bernard Roizman and Philip E.Pellett 2001).The ability of these viruses to induce lifelong infection in their hosts, by entering a dormant state after primary infection, earns them the name "herpes", which is derived from the Greek word "herpein", with the meaning of "to creep" (Baskin e Hedlund 2007).
The Herpesviridae family contains over 100 double-stranded DNA (DNAs) viruses.
Based on their properties and ability to remain latent in their hosts they are divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae (Roizman e Baines 1991).
All herpesviruses share a common virion structure, formed by an external Envelope, a Tegument, an Icosahedral Capsid and a Core in which a linear double-stranded DNA is enclosed.
Herpesvirus are widespread, nine viruses are infact pathogenic to humans: Human herpesviruses 6A, 6B, 7 and 8 (HHV-6A, HHV-6B, HHV-7, HHV-8), Epstein-Barr virus (EBV), Human Cytomegalovirus (HCMV), Herpes simplex virus 1 (HSV-1), Herpes simplex virus 2 (HSV-2) and Varicella zoster virus (VZV) (Bernard Roizman and Philip E.Pellett 2001).
The members belonging to this viral family share four biological properties: all can remain latent in their natural hosts (i); need to enter in the nucleus for the synthesis of viral DNAs and assembly of capsids (ii); possess enzymes involved in the processing of proteins and nucleic acid metabolism (iii); for some, the production of progeny virus is accompanied by the destruction of the infected cell (iv).
Their genomes show significant genetical homology, with 40 genes conserved that are expressed in their reproductive cycle, called “Core’s gene’’ (Mettenleiter, Klupp, e Granzow 2009).
All herpesviruses own a large biologic variability, such as the replication rate.
Indeed, in some viruses it appears to be very slow while in others is extremely quick, additionally, also varies the capability to remain latent in a specific set of cells or in several cell types.
These viruses differ also in the plurality of disease and clinical signs they can cause, ranging from epithelia localized vesicular eruptions to necrosis of the liver or fetal death (Roizman e Baines 1991).

Classification of Herpesviruses.

Based on their biological properties, such as ability to infect and destroy or remain dormant in infected cells and host specificity, all Herpesvirus can be classified into three subfamilies: (i)Alphaherpesvirinae, (ii)Betaherpesvirinae and(iii)Gammaherpesvirinae (Roizman e Baines 1991).

Alphaherpesvinae

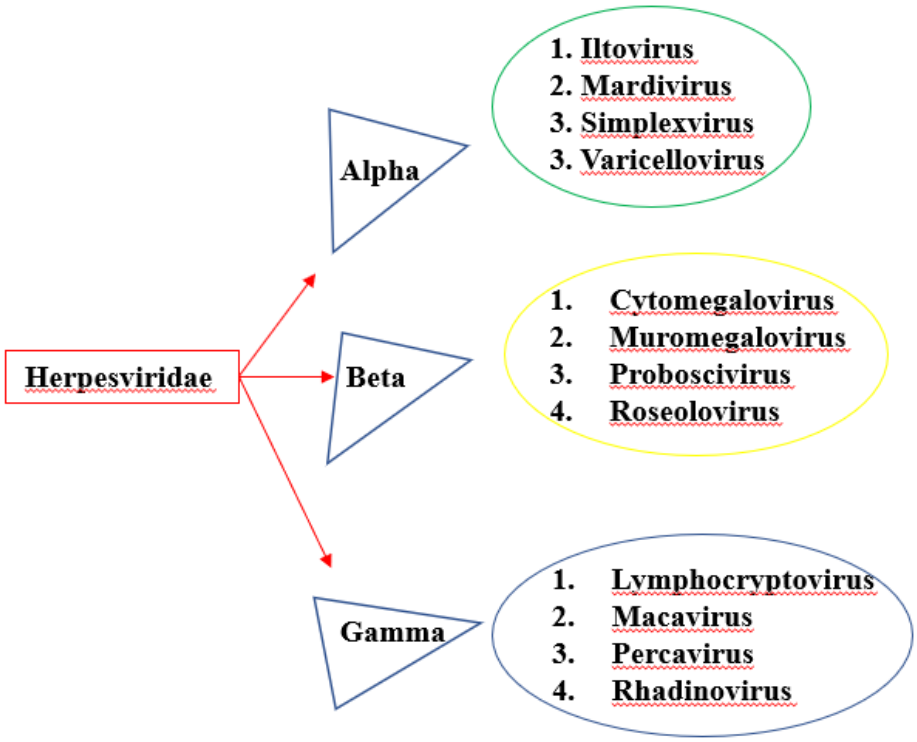
Simplexvirus genus belongs to this subfamily, such as Human herpesvirus-1 or -2 (HSV-1, HSV-2) and bovine mammillitis virus (BoHV-2); also, varicellavirus genus, comprising Varicella-zoster virus (VZV), pseudorabies virus (Suid alphaherpesvirus 1) and Bovine Herpesvirus-1 (BoHV-1), is included in this subfamily.
They all share a few common features such as a variable host range, rapid spread in culture and the subsequent host cell destruction, the capability to remain latent in sensorial ganglia even if in not an exclusive way and a short reproductive cycle.

Betaherpesvirinae

To this class belong five genera, the most known are cytomegalovirus, that includes for example Human Cytomegalovirus (HCMV) and Human Herpesvirus-5 (HHV-5) and muromegalovirus as murine cytomegalovirus.
They all share a few common features, such as the capability to remain latent in secretory glands, kidneys, monocytes and a slow reproductive cycle and a restricted host range.
In vitro infection is usually slow, and the infected cells frequently show cytomegalia.

Gammaherpesvirinae

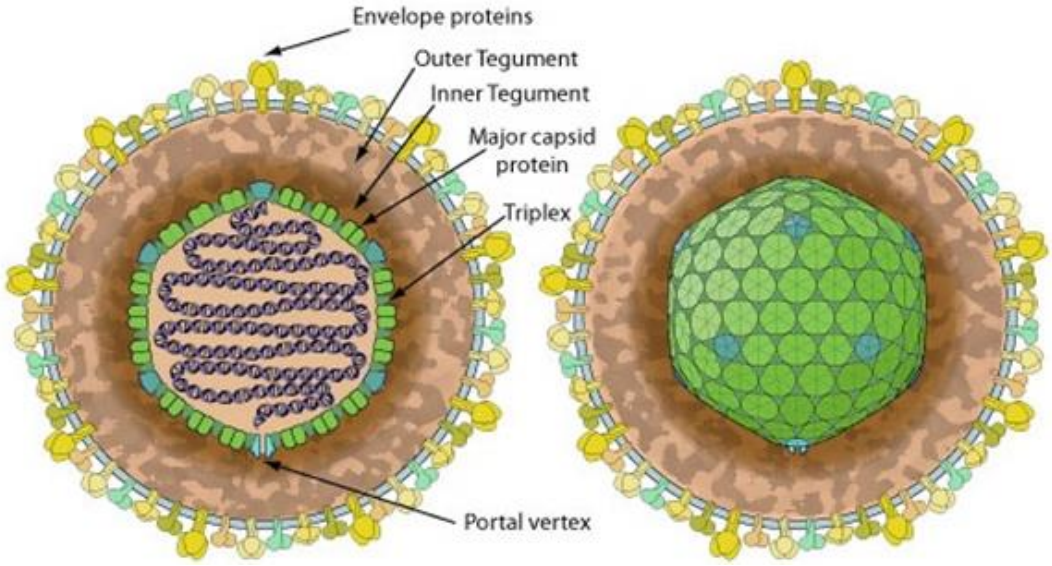
The etiologic agents of mononucleosis and Burkitt's lymphoma, lymphocryptovirus as Epstein-Barr virus (EBV), rhadinovirus as Human Herpesvirus-8 (HHV-8) or Kaposi Sarcoma associated Herpesvirus (KSHV) and Bovine Herpesvirus-4 (BoHV-4) belong to this class.
Their main features are the latency in lymphocytes or in lymphoid tissues and the oncogenic potential, a restricted host range and the ability to *in vivo* infect B and T Lymphocytes.
Another important characteristic is a pre-lytic or lytic stage frequently without production of infectious progeny while *in vitro* infection usually develops within lymphoblastoid cells.



Classification of Herpesviridae (only the most representative genera are shown).

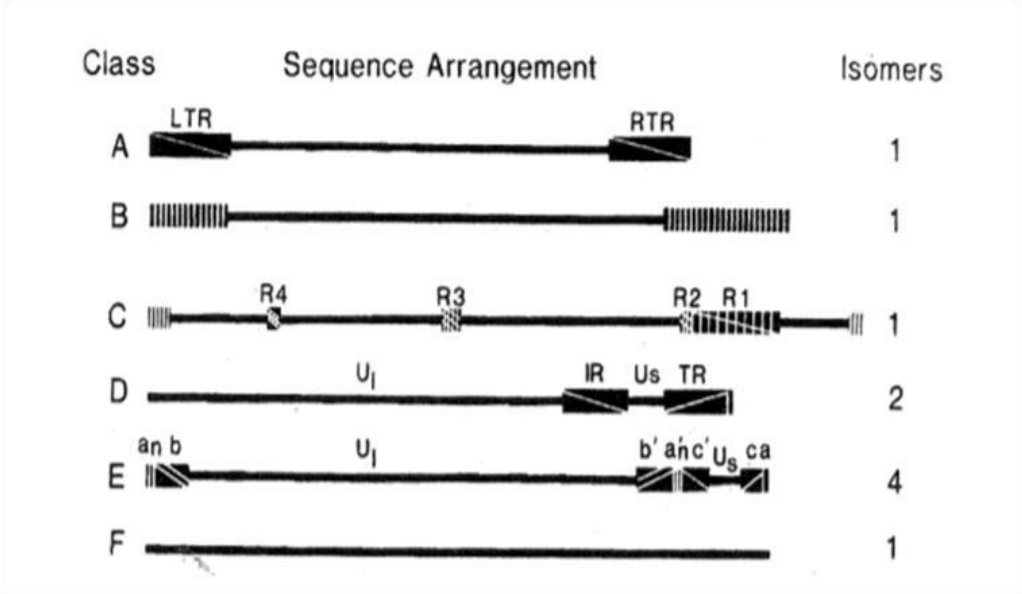
Herpesvirus: Biological structure and DNAs.

The Herpersviridae family is characterized by a well-defined biological structure.
Externally the particles have an envelope composed of lipid elements. The envelope has a lipidic bilayer structure and is strictly connected with the outer surface of the tegument, these two layers together show a typical tri-laminar structure with numerous glycoprotein spikes protruding on its surface (Asher, Heller, e Becker 1969) (Wildy e Watson 1962).
A tidy tegument is characterized as a fibrous poorly defined layer and it is situated between the capsid and envelope, its thickness could be variable, for example in some classes of virus is smaller in virion accumulating in perinuclear space, compared to those that accumulate in cytoplasmic vacuoles (Morgan et al. 1959) (Falke 1997).
An icosahedral capsid, is then present, with a diameter between 100 to 110 nm and a 162 capsomeres composition, within them 150 are hexameric capsomeres in a longitudinal section; in the surface of the long axis shall run a channel that is 4nm in diameter, 12 are the pentameric capsomeres at the vertices which have not been yet characterized (Wildy e Watson 1962).
The core comprising the large double-stranded linear DNA is arranged in a toroid form, that appears to be maintained by proteinaceous fibrils located under the capsid and passing through the hole of the torus (Nazerian 1974) (Furlong, Swift, e Roizman 1972).
Finally, the structure of the virion is formed by a variable number of polypeptides ranging from 35 to 45 which can differ from one virus to another and show a size of approximately 300 nm.
A characteristic which influences the diameter of the virion is the thickness of tegument and variability of the envelope state (Bernard Roizman and Philip E.Pellett 2001) (Roizman B, Furlong D: Fraenkel-Conrat H, Wagner RR, eds s.d.).



Herpesvirus structure showing:
the Envelope, Tegument, Capsid and the Core containing the double-stranded DNA
(https://www.vectorstock.com/royalty-free-vector/structure-of-herpes-virus-vector-1043059)

The genome of the Herpesviruses is characterized by a single linear double stranded DNA.
After the infection, it is capable of circulate into the nucleus of the infected cells thanks to short sequences of bases that are repeated at its extremes, these terminal repeated sequences can increase the genome size also up to 10 kbps and are useful to divide herpesvirus genomes into six classes, designated by the letters from A to F relating to their different numbers and positions.
The molecular weight varies from 80 to 150 million kDa or from 120 kbp (e.g. VZV) to 250 kbp (e.g. HMCV) and has a G+C content varying from 32 to 75%; while the number of their Open Reading Frames (ORFs) is in a range comprised between 70 to > 200 (Whitley 1996).



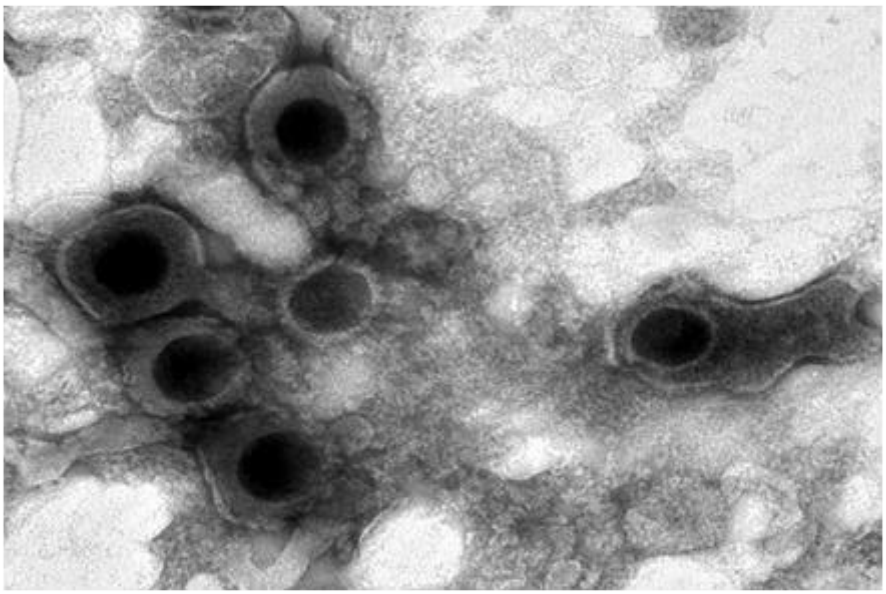
A schematic diagram of the sequence of the six genome classes of viruses belonging to Herpesviridae family (Bernard Roizman and Philip E.Pellett 2001).

Let's elaborate the matter: Gammaherpesvirinae.

The subfamily of gammaherpesvirinae is divided in Lymphocryptovirus and Rhadinovirus, these two viral genera have a basically different host range.
Rhadinoviruses have been identified in a wide range of mammalian species while the lymphocryptovirus almost only in the higher primates. To these genera belong Epstein-Barr (EBV) (lymphocryptovirus) and Human herpes virus 8 (KSHV) (rhadinovirus), two viruses that can host human species.
The Gammaherpesvirinae can modify host gene expression to optimize the cellular environment in favor of the viral replication and immune response evasion.
All these changes do not support cell death but interest protein stability and different signaling pathways, induce secretion of paracrine factors which stimulate survival and cellular proliferation and angiogenesis (Clyde e Glaunsinger 2010).
These viruses can also induce neoplastic and lymphoproliferative disorders (Bublot et al. 1992).
To this class of viruses belongs Bovine Herpesvirus-4 (BoHV-4), this is a virus of particular interest for some its biological characteristics that identify it as a good candidate to be used as a gene delivery vector (Donofrio et al. 2002a).

Bovine Herpesvirus 4 (BoHV-4).

Bovine herpesvirus 4 (BoHV-4) belongs to the Herpesviridae family, Gammaherpesvirus sub-family and Rhadinovirus genus. Its dsDNA genome is around 144 ± 6 kb. BoHV-4 is extremely common in cattle population and is frequently associated with cattle post-partum metritis (Thiry et al. 1992).
The virus was isolated firstly from Bartha et al. in 1963 from a cattle showing conjunctival and respiratory signs (Bartha, Juhász, e Liebermann 1966).
BoHV-4 establishes persistent infection in its natural host, the bovine, but even in the rabbits, used as experimental hosts because they present several different sites of latency (Osorio, Rock, e Reed 1985).
After its discovery, BoHV-4 was known with different words, such as "Orphan Herpesvirus" and "Movar-type Herpesvirus" or "Bovid Herpesvirus-4" and "Bovid cytomegalovirus".
In the 80's Bartha et al. to have only one designation sharing equal characteristics, proposed the name of BHV-4 (Bartha et al. 1987).
Only then, in 2000, the international committee of viral Taxonomy assigned to it the official name of BoHV-4 (Fauquet, C. M., et al 2005).



BoHV-4 particles analyzed through transmission electron microscope

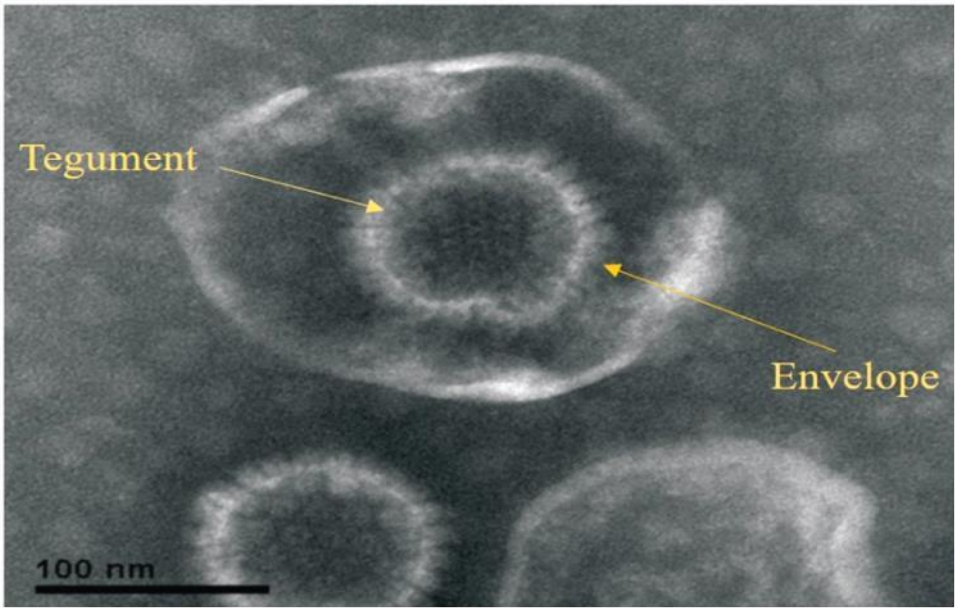
(https://www.microscopie-electronique-fmv.com/photos)

BoHV-4 can be easily detected from nasal and vaginal secretions, also from both primary and immortalized bovine cell lines obtained from tissue homogenates.
Several cell lines can be used to amplify *in vitro* that virus, as for example, bovine, sheep, goat, cat, pig, rabbit and chicken derived cell lines.
Moreover, its replication is associated with the synthetic (S) cellular phase and cell cultures must be sub-confluently cultured in order to optimize BoHV-4 isolation (Vanderplasschen et al. 1995).

The gold technique for BoHV-4 identification is polymerase chain reaction (PCR) for the detection of conserved genes as thymidine kinase (TK) (Egyed e Bartha 1998) or for the analysis of glycoprotein B gene (Gb) (Egyed, Kluge, e Bartha 1997).

In the same manner, PCR can be used to identify BoHV-4 DNA (Egyed e Bartha 1998), indicating only the presence of infected mononuclear blood cells without knowing if BoHV-4 is responsible for the lesion.
In particular, the serum-neutralization assay is not performed for virus identification because BoHV-4 induce low or not detectable levels of neutralizing antibodies titers in infected cows.
Several studies show that the weak host humoral immune response against that virus is due to virus capacity to minimally expose its antigens to the antibodies of the immune system.
Furthermore has been observed that BoHV-4 envelope glycoprotein gp180 is responsible for gH-gL and gB epitopes protection from antibodies neutralization (Machiels, Lété, Guillaume, et al. 2011).
In fact, the serum neutralization can be very effective on recombinant gp180-deficient BoHV-4 immunized animals. Over the years many indirect tests have been used as: ELISA assay, Immuno-peroxidase monolayer assay (IPMA) and immunofluorescence test (IFAT), that have been standardized to detect BoHV-4 seroconversion 14 to 20 days post primary infection (Czaplicki e Thiry 1998) (Osorio e Reed 1983).
No vaccines against BoHV-4 are present in Europe and its control and prevention is only achieved through hygienic measures (Lemaire et al. 2000).

Let's deepen: Biological structure of BoHV-4.



BoHV-4 visualized by electron microscopy (Jeol, Tokyo, Japan) ***at 300 x 10^3 magnification***
(Kruger et al. 2015)

BoHV-4 envelope is characterized by the presence of gB, gH, gL, gM, gN, five highly conserved glycoproteins in all herpesviruses.
Additionally, twenty-nine structural proteins have also been described in BoHV-4, ten are glycosylated (Dubuisson, Bublot, et al. 1991) and four are of particular interest, more in detail:

- gp1, gp6/gp10/gp17: the first is a glycoprotein which has the higher molecular weight (> 300 kDa) and shows N-linked glycans; while the remaining proteins are a complex of 3 glycopolypeptides with N-linked glycans with a molecular weight of 150 kDa/ 120 kDa/ 51 kDa (Dubuisson, Bublot, et al. 1991).

- gB: is essential for viral life and replication. From the proteolytic cleavage of gB protein derives the complex gp10-gp17 which is fundamental for host cell penetration (Dubuisson, Bublot, et al. 1991) (Dubuisson, Danyi, et al. 1991).
  Subsequently, other studies have shown that gB protein cannot be replaced even with an homologous or with an heterologous protein from other Herpesviruses (Franceschi et al. 2013).

- gp8 (135 kDa) and gp11 (115-120 kDa): the first is a late protein and is poorly glycosylated , it is present both into the virion and as a secreted form while gp11 contains N-linked glycans bounds trough non-covalent bindings (Thiry et al. 1992) (Dubuisson, Danyi, et al. 1991).

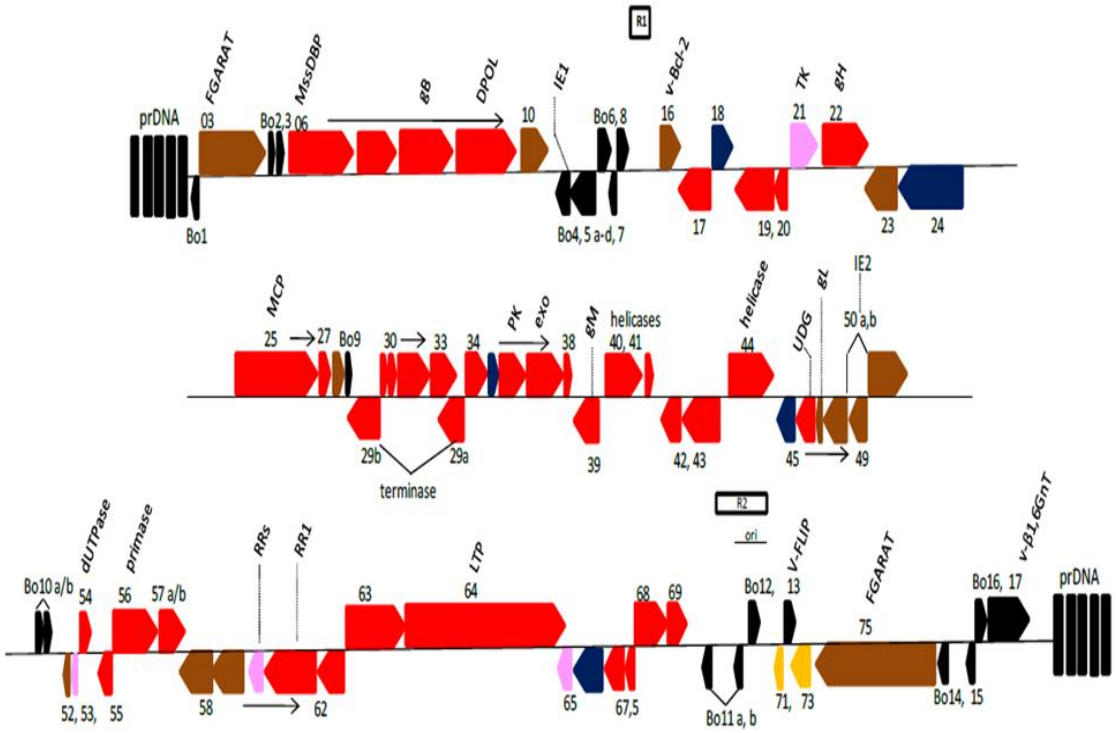
- Through Western Blotting analysis of purified viral envelopes were identified six neutralizing epitopes, three domains in gp6/gp10/gp17, two domains in gp11/VP24 and another one in gp21 (26-27 kDa) (Dubuisson et al. 1989).

Other glycoproteins present in BoHV-4 envelopes are: "gH" or "gp110" that exercises the role of fusing viral envelope with the host membrane and "gL" or "gp31-35 or gp45-65" , that are closely linked and cooperate with gH (Lété, Machiels, et al. 2012).

- gM and gN: have not been studied so far, their functions have not been determined yet.

The tegument of BoHV-4 is characterized by an icosahedrical capsid composed of 12 pentamers and 150 hexamers. Inside the tegument is contained the nucleocapsid of 90-100 nm, although the virion particle is bigger, in fact can reach about 200 nm.
The tegument is composed of about 15 proteins, the most produced is a small protein of about 20 kDa encoded by ORF52 (Lété, Machiels, et al. 2012).
Other proteins that complete the tegument architecture are encoded by genes ORF6, ORF10, ORF16, ORF36, ORF46, ORF54, ORF57, ORF59, ORF60, ORF45 and Bo5 (Lété, Palmeira, et al. 2012).

BoHV-4 genome.



Map of BoHV-4 genome adapted from (Zimmermann et al. 2001) ***In black are BoHV-4 unique genes ; in red ORFs common to all Herpesviruses while in blue those one common only to β and γ; in brown those peculiar to γ herpesviruses; in yellow ORFs of γ2 herpesviruses and in pink those in common to α and γ.***

Through the years, two different BoHV-4 genomes have been isolated and completely sequenced, demonstrating that it has a herpesvirus type B genomic organization.
The genome of BoHV-4 is constituted by a linear double stranded DNA of about 144kb: identified by the name V-test indicating the European strain (Palmeira et al. 2011) while the North American one is called 66-p-347 strain (Broll et al. 1999).
These strains show 99,1% of identity although a wide variability at genome level is highlighted (Palmeira et al. 2011). In particular, BoHV-4 has a central long unique region of about 110 kb with low G+C content and is flanked by two stretches of tandem repeats with high G+C content, named poly-repetitive DNA (prDNA).
Furthermore, prDNA regions are important during rolling cycle replication for the cleavage of produced concatamers and for viral incapsidation; the subseguent packaging happens through the presence of the two sites: pac1 and pac2 (Ehlers, Buhk, e Ludwig 1985).
Another function carried out from prDNA is the anchorage of viral episomes during latency thanks to a latency-associated origin of replication, called *oriP* (Fejér et al. 2003).
BoHV-4 genes are arranged in five blocks from 1 to 5 in 5'➡3' direction, these include different genes with various functions (Bublot et al. 1992) (Bublot et al. 1990). The "core" genes (blocks 1, 2 and 4) are conserved in all Herpesviridae and are involved in viral replication, capsid formation, DNA incapsidation and capsid release from the nucleus.

As regarding to remaining ORFs; blocks 3 and 5 are exclusive only of gammaherpesvirus; there are also particular regions which encode for individual genes, denominated “inter-block”.
In particular, within the “LUR” have been identified two regions called R1, R2, that are constituted by multiple direct repeats (Bublot et al. 1990).
In particular, the two regions have different structures: in fact, R1, is characterized by complete and incomplete direct repeats of 23 and 25, 65 bp in length that are different between the strains while R2, is composed of R2a and R2b.
R2a has 3 imperfect and 28 perfect direct repeats of 22-23 bp in length.
R2b has one inverted repeat with an hairpin-loop and different repeats from 8 to 68 bp in length, where an Ori Lyt, overlapping Bo11 and Bo12 gene sequences, was found, and is related to the other Herpesvirus (Lomonte et al. 1995) (Bublot et al. 1991) (Vanderplasschen et al. 2000).
In its genome have been described 17 ORFs specific to BoHV-4 and 62 ORFs homologous to the other rhadinovirus.
Based on their position into the genome, from 5’➡ 3’, these ORFs are named from Bo1 to Bo17 and in some of them also splicing can occur.
After viral infection one of the first genes transcribed is IE1 (Major Immediate early I Transcript) overlapped to its sequence there are Bo4 and Bo5 ORFs, that are not essential for viral replication (Franceschi et al. 2015).
An important gene is thymidine kinase (TK), encoded by ORF 21. This gene is involved in the pyrimidine metabolism (Lomonte et al. 1992) and its gene product is not necessary for the viral replication. For this reason this gene can be used for insertion of foreign DNA in a BoHV-4 based vector system (Donofrio et al. 2002a).
BoHV-4 has moreover some particular and specific characteristics, such as: lack for gene encoding for thymidylate synthase or dihydrofolate reductase and for complement regulatory protein and cyclins and no genes coding for interleukin receptors and for cytokines, for chemokines and also for cytokines receptors and for viral macrophage inflammatory proteins (Vanderplasschen et al. 2000).
Based on few criteria, which are:

1. 180 bp as minimal size;
2. presence of start and stop codons;
3. less than 50% overlapped sequences with other ORFs;

the scientists have identified 79 Open Reading Frames (ORFs) in BoHV-4. As previously reported, 17 ORFs specifically belong to BoHV-4 and was named as "Bo" followed by an increasing number, ranging from 1 to 17, within the LUR sequence in 5’➡3’ direction: Bo1-Bo17 (Zimmermann et al. 2001).
These genes are conserved and organized in common blocks among γ-herpesviruses genomes, while differ for position, number and orientation (Neipel, Albrecht, e Fleckenstein 1998) (Pedro Simas e Efstathiou 1998).
Furthermore, BoHV-4 gene arrangement is similar to HVS, in fact into the central part of the LUR sequence it is present a stretch of equally oriented ORFs (from ORFs 16 to 69) and miss long non-coding stretches.
Specifically, ORF50 encodes for Immediate Early 2 (IE2) protein; that is a protein highly conserved among Rhadinoviruses and plays a key role in γ-herpesviruses lytic cycle induction (Donofrio, Cavirani, et al. 2004).
BoHV-4 is able to encode for ORF16, a viral B cell lymphoma (v-Bcl-2)-like gene sequence.
At the same time encodes also for ORF71, viral FLICE, an Inhibitory Protein (v-FLIP), involved into host cell cycle regulation.

It is known that v-Bcl-2 and v-FLIP play a role in the early stage of cellular transformation, preventing apoptosis.
BoHV-4 is not related neither to lympho-proliferative disorders or transforming ability (generally attributed to other γ-herpesviruses), even if it has never been officially demonstrated (Staskus et al. 1997). BoHV-4 encodes also for genes with antiapoptotic potentialities.
However, it has been show that some herpesviruses tend to lose anti-apoptotic activities by escaping caspase-mediated conversion into pro-apoptotic proteins (Bellows et al. 2000).
In fact, despite the presence of anti-apoptotic genes in BoHV-4 genome, the productive infection is associated with apoptosis instead with a lytic phase. This feature is common by other gamma2-herpesviruses (Sciortino et al. 2000).
Numerous studies demonstrate that viral genes capable of increasing nucleotides amount into host-infected cells are able to affect not only viral replication but also host cell proliferation in itself (Neipel et al. 1998).
ORF21 is a 1335 nucleotides long sequence, encoding for Thymidine kinase (TK) and is involved into nucleotides metabolism necessary for nucleic acids synthesis and pyrimidine metabolism.
BoHV-4 TK gene promoter regulatory region is specifically trans-activated by IE2 protein (Zhang e van Santen s.d.).
Other genes involved into nucleotides metabolism, favoring both viral replication and cell proliferation are ORF3 and 75 located in opposite directions at LUR ends (Zimmermann et al. 2001).
BoHV-4 presents a unique gene called Bo17, expressed during the early replication, that is indispensable for *in vitro* viral productive infection, is located at LUR right end and encodes for β-1,6-N-acetylglucosaminyltransferase (β-1,6GnT/C2Gnt) and no other known viruses possess it.
Its product shares 81.1% similarity with the human β-1,6GnT homologous (Morán et al. 2015).
This gene is involved in viral replication, particularly in mononuclear blood cells replication (Vanderplasschen et al. 2000).
Since β-1,6GnT family genes encode for enzymes involved in pathological processes, like immunodeficiency and oncogenesis, BoHV-4 could potentially trigger lymphoproliferative or transforming events (Vanderplasschen et al. 2000).
In addition, its contribute to *in vivo* biology is still not clear, it has been suggested that the addition of glycans to viral protein could influence viral tropism or its susceptibility to antibodies neutralization, complement lysis and cell-mediated cytotoxicity in order to favor viral escape from the host immune system defenses (Markine-Goriaynoff et al. 2004).
Bo4 and spliced Bo5 ORFs encode for part of the major Immediate-Early 1 (IE1) transcript (Bermudez-Cruz, Zhang, e van Santen 1997).Bo5-ORF protein shares 27,85% homology with KSHV K5 ORF (Haque et al. s.d.).
Another important gene is ORF29, its product is a terminase involved into viral DNA cleavage and encapsidation processes (Broll et al. 1999).
ORF50, encoded by the spliced IE2 gene, has in its product a putative R transactivator (van Santen 1993).
Other genes, like Bo10, Bo11, ORF29, ORF50 and ORF57 undergo differential splicing processes.
The various ORF of BoHV-4 are involved in several functions, es. ORF57 product could play a key role in viral mRNAs processing and seems to be involved into spliceosome complex redistribution in infected cells (Cooper et al. s.d.).
Furthermore, gp350 gene encodes for a gp180 protein, during viral replication. gp180 is a non-essential BoHV-4 envelope protein, of 180 KDa, involved into Glycosaminoglycans (GAGs) dependent viral attachment.

It has been observed that gp180 deficient virions show growth deficit compared with Wild Type parental ones (WT) (Machiels, Lété, de Fays, et al. 2011).
As described by Bermudez-Cruz et al. Bo11, is shorter than 180bp in length and is encoded by an Immediate Early 2 (IE2) transactivated, spliced 1.1Kbp late RNA (Bermudez-Cruz et al. 1997).

Interblock regions:

The Interblocks regions are the herpesviral genome most variable part.
In fact, within them are located virus-specific genes, involved into several biological viral functions (Lomonte et al. 1996).
BoHV-4 possesses five interblock regions, named A-F. After numerous studies it was sequenced in order to identify its important genes and each region counts an amount of 23000 nucleotides with a 43% G-C content (Bublot et al. 1990).
Each ORFs has been named the BORF- prefix followed by the interblock region letter and the positioning number, if more than one ORF belongs to the same interblock. (Bublot et al. 1990; Egyed 2000; Lomonte et al. 1996).
Some of the ORFs of Herpes Simplex Virus (HSV), es. ORF3,10,16,51,71,73 seem to be homologous to BoHV-4, es. BORFA1, BORFB1-2, BORFD1, BORFE2,BORFF4; while the others are specific to BoHV-4 (Lomonte et al. 1995, 1996).
To date, these are the only established difference between the two viruses (Lomonte et al. 1995, 1996).

Property of BoHV-4.

BoHV-4 has unique characteristics; one of these is the capability to infect a variety of cell lines derived from different animals species and also from human, these characteristics can be useful for vaccinal, oncolytic purposes or gene therapy and this justifies its use as a viral vector (Redaelli et al. 2010).
In particular, both primary and immortalized bovine cell lines derived from different tissues as kidney and lungs, B-T lymphocytes, mammary gland and fetal bovine bone marrow and macrophage-derived cells (Donofrio et al. 2007).
Several *in vitro* studies of BoHV-4 replication cycle, have demonstrated that it is strictly correlated to host cell cycle S phase with a slow growth due, probably, to the low rate of thymidine kinase induction in infected cells (V+S+K) (Donofrio et al. 2007).
BoHV-4 replication cycle can be divided in four phases: (i) an *attachment phase*, where gB envelope protein interacts with heparan sulfate-like structures of the host cellular surface; (ii) a *fusion phase* where the virus thanks to viral envelope interacts with the plasma membrane; (iii) a *transport phases* of the envelope proteins and nucleocapsid, from cytoplasm to nucleus through microtubules and (iiii) *release phase* into the nucleus of viral DNA through nuclear pore (Thiry et al. 1992) (Dubuisson et al. 1989).
After the infection BoHV-4 produces three different kinds of proteins in host cells, named: *IE* or *alpha* (immediate early), *E* or *beta* (early) and *L* or *gamma* (late) all of them implicated in different processes.
Additionally, BoHV-4 replication takes place through rolling circle mechanism starting from the Ori (Origin of replication) with production of concatamers which are subsequently cleaved and packaged inside new capsids (Thiry et al. 1992) (Roizman B, Furlong D: Fraenkel-Conrat H, Wagner RR, eds s.d.).,
BoHV-4 is able to lead two specific types of infection a lytic and latent one. The lytic infection differs to latent one for the expression of alpha and beta genes.
Some researchers have demonstrated that viral DNA can exist or in episomal shape or integrated in random sites of the cellular DNA, as occurs in the latent infection. This certifies that BoHV-4 infection is compatible with replication and cell survival (Donofrio e van Santen 2001).
The presence of a lytic cycle allows to understand which cell lines are susceptible to the infection or which are also permissive.
The susceptible cell lines show receptors in its surface for attachment and entry of the virus, while the cell lines that are susceptible and permissive are able to completely support cell cycle infection.
A study conducted by Prof. Donofrio et al., has demonstrated that over expression of IE2 protein can induce virus replication also in cell lines that are not permissive to viral infection, as demonstrated in RD4 (human rhabdomiosarcoma) cells, normally not permissive for BoHV-4 infection (Donofrio, Cavirani, et al. 2004) (Donofrio et al. 2000).
The worldwide distribution of BoHV-4 in cattle populations supported that the bovine species is the viral natural host, but some studies showed instead that the original host species for BoHV-4 was instead African buffalo (Syncerus caffer). This virus was isolated in several others animals as American bison (Bison bison), zebus (Bos indicus), sheep and goat (Moreno-Lopez et al. 1989) or in some felines as lions and cats (Fabricant et al. 1971).
In other animals, as a in healthy primates, BoHV-4 was isolated from the kidney (Bublot et al. 1991) and in the monkeys behaves as a not oncogenic pathogen. While, the rabbit is the considered the major model to study *in vivo* the biology of BoHV-4 for his capability to support the persistence and replication of the virus (Egyed 2000) (Lin et al. 2000) (Egyed e Baska 2003).

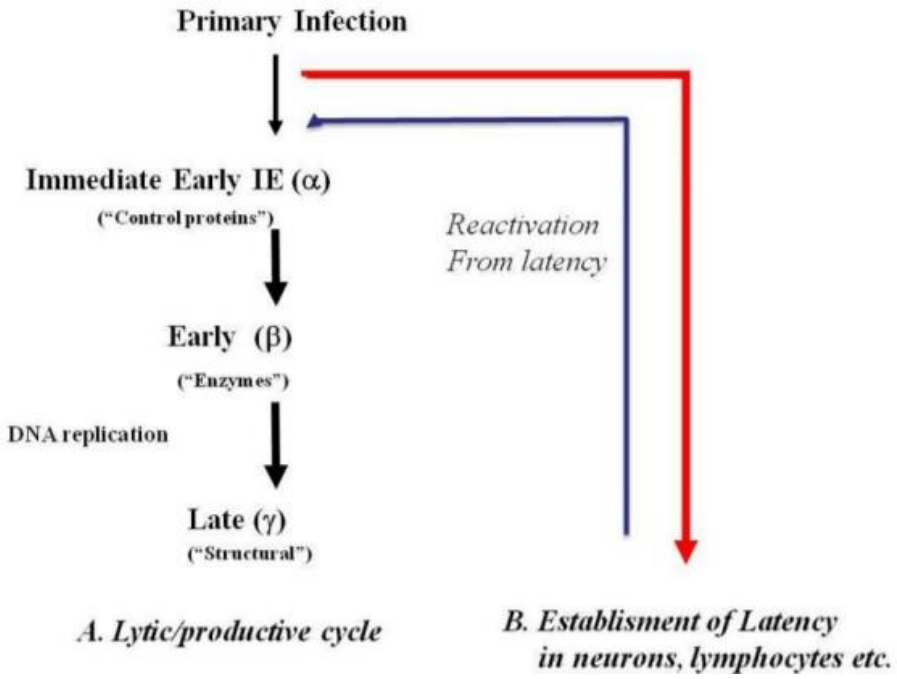
Furthermore, BoHV-4 infection is associated with different clinical signs as: pulmonary lesions, dyspnea, cough and nasal discharge at respiratory level (Bartha et al. 1966); metritis, endometritis, vaginitis or abortion at genital level (Reed, Langpap, e Bergeland 1979).
Important studies have demonstrated that in mastitis BoHV-4 can be isolated in combination with other pathogens, as well as in cellular fraction of milk of healthy cows (Donofrio et al. 2007), also in mammary pustular dermatitis, enteric diseases and neoplastic disease (Kaminjolo, Mugera, e Rosted 1972).
Unfortunately, BoHV-4 pathogenesis is still unclear since experimental *in vivo* studies were not able to reproduce clinical signs.
The transmission of BoHV-4 can happen via alimentary, respiratory, and reproductive routes. Several studies have hypothesized that also the vertical transmission was possible and then the transmission of virus from the mother to the fetus (Deim, Szeredi, e Egyed 2007).
BoHV-4 replicates in the entry site and disseminates in other sites, as epithelial cells of trachea or intestines through monocyte/macrophage lineage cells.
Like all other herpesviruses, even BoHV-4 can lead to latency and lymphoid organs are the possible site but also can be mononuclear blood cells.
Confirming that hypothesis several studies, suggested that macrophages could be latency main site (Lopez, Galeota, e Osorio 1996) and has been shown that BoHV-4 can be reactivated through external or experimental stimuli (Donofrio et al. 2007).
are

In vitro host range:

Since discovery of BoHV-4, infectivity and *in vitro* tropism on several cell types have been studied and different primary and continuous cell lines susceptible to BoHV-4 infection have been identified. Numerous studies show that its broad spectrum might be due to its primary interaction with the cellular heparan sulfate receptors, located on almost all the cell lines (Vanderplasschen et al. 1993).
BoHV-4 can replicate in bovine primary cell lines such as: spleen, skin, kidney, testes, lungs, and thyroid bovine; also, lymphosarcoma calf thymus and fetal bovine bone marrow and Embryo Bovine Tracheal cells (EBTr). Among immortalized cells worth of note are: Georgia Bovine Kidney (GBK) cells, Bovine Embryonic Kidney (BEK) cells, Embryonic Bovine Lung (EBL) cells and Madin Darby Bovine Kidney (MDBK) cells (Nikolin et al., s.d.) (Nguyen et al. 2003).
Furthermore, several studies have demonstrated that BoHV-4 is able to infect different cell lines (tracheal and nasal turbinate cells, endothelial, mammary gland) and also bovine immune system cells (thymocytes, macrophage, histiocytes, T- and B-lymphocytes)(Donofrio e van Santen 2001).
BoHV-4 is not a neurotropic virus but is it has been isolated also from bovine nervous tissues (Yamamoto et al. 2000; Asano et al. 2003) for these reason Donofrio et al. have conducted different studies with recombinant BoHV-4-based vectors in the Neuroblastoma cell line (N2a). This study has demonstrated that BoHV-4 is able to persistently infect N2a cell line and produce viable viral particles, but without interfering with cellular differentiation (Donofrio, Grandi, et al. 2004).
Over the years has been tested the capability of BoHV-4 to *in vitro* infect numerous human cell lines; demonstrating that the virus is able to infect adenocarcinoma cells and carcinoma from various organs but poorly permissive to BoHV-4 replication. While the human cell lines from myeloid and lymphoid origins are resistant to infection (Gillet et al. 2004).

In vivo Host range:

BoHV-4 has an extended worldwide distribution, in fact, was identified from many different animal species, such as: American bison (Bison bison), sheep (Ovis aries), zebu (Bos indicus), goat (Capra hircus), African buffalo (Syncerus caffer) (Moreno-Lopez et al. 1989) (Gillet et al. 2004) (Todd e Storz s.d.); the virus has been isolated also from non-ruminant species such as: cat, lion, black rhinoceros (Diceros bicornis), Asian elephants (Elephas maximus) and owl monkey (Aotus trivirgatus) (Metzler et al. 1990).
Some data, suggest that the African Buffalo might represent BoHV-4 original host specie, as suggested by a study that analyzed 400 sera collected from different geographical areas, that were tested positive for BoHV-4 presence with 70% of serum prevalence, much higher if compared to cattle (Dewals et al. 2005).
Moreover, the rabbit is considered a good experimental model for BoHV-4 pathogenesis studies (Egyed et al. 1997) (Bublot et al. 1992) (Egyed e Baska 2003).
All these data show that BoHV-4 represents an exception to other gamma herpesviruses whose life cycle is restricted only to their natural host species (Gillet et al. 2005) (Toussaint et al. 2005).



Schematic representation of herpesvirus cycle of life

(Riaz, Kifayatullah, e Akhtar 2017).

Applications: BoHV-4 as a vector.

Several studies have demonstrated the great efficiency of BoHV-4 as a viral vector for vaccinal and therapeutic purpose in different species of animals as sheep, goat, rabbit, swine (Donofrio et al. 2002a), chicken (Donofrio et al. 2008), mice (Franceschi et al. 2015).
Thanks to the possibility to manipulate its genome as a Bacterial Artificial Chromosome (BAC) and for other characteristics such as : capacity to manipulate its genome deleting even the 30% of the viral genome but saving its viral replication and the capability to transport exogenous DNA (5-10 kb), complete genome sequencing and its presence in gene bank online facilitates its use as a vector and possibility to manipulate its genome as a BAC; capacity to propagate *in vitro* and *in vivo* in a variety of cells and cell lines and ability to induce a latent infection maintaining the viral genome into the cells as an episome; absence of documented oncogenicity and absence of a clear correlation with disease (considering its role as a secondary pathogen in the uterine diseases in cattle).
Several studies have demonstrated the great efficiency of BoHV-4 as a viral vector in rabbit, swine, goat and sheep (Donofrio et al. 2002a) and chicken (Donofrio et al. 2008) (Gaetano Donofrio et al. 2008) and in mice (Franceschi et al. 2015).
BoHV-4 can be used as a viral vector with the intent to generate different recombinant viruses.
For example, it was used as a potential oncolytic virus for the glioma treatment, exploiting its ability to infect only glioma cells in absence of clinical signs (Redaelli et al. 2010).
Monkeypox virus (MPXV)is a lethal zoonotic virus and virus belonging to the Orthopoxvirus in the family Poxviridae. It is the etiological agent of human MPX and is endemic in the Congo-basin and sporadic in West Africa.
Considering that MPXV is a dangerous virus and could be used as a bioterrorist agent, new therapeutics and new studies are of great interest both for the governments and scientific community.
For these reasons Donofrio et al. have conducted a study for a new vaccination strategy approach against MPXV in STAT1 knockout mice.
In this study were tested three recombinant BoHV-4 vectors able to express different MPXV glycoproteins: M1R, A29L and B6R, as a basis for vaccination (Franceschi et al. 2015).
By BAC recombineering system have been generated three recombinant viruses: BoHV-4-A-EF1α-M1RgD106ΔTK, BoHV-4-A-CMV-A29LgD106ΔTK and BoHV-4-A-EF1α-B6RgD106ΔTK, that resulted safe without evident adverse events.
One of these recombinants was able to protect 100% alone and 80% in combination STAT1 knockout mice against monkeypox virus morbidity and mortality (Franceschi et al. 2015)(Shizuya et al. 1992).

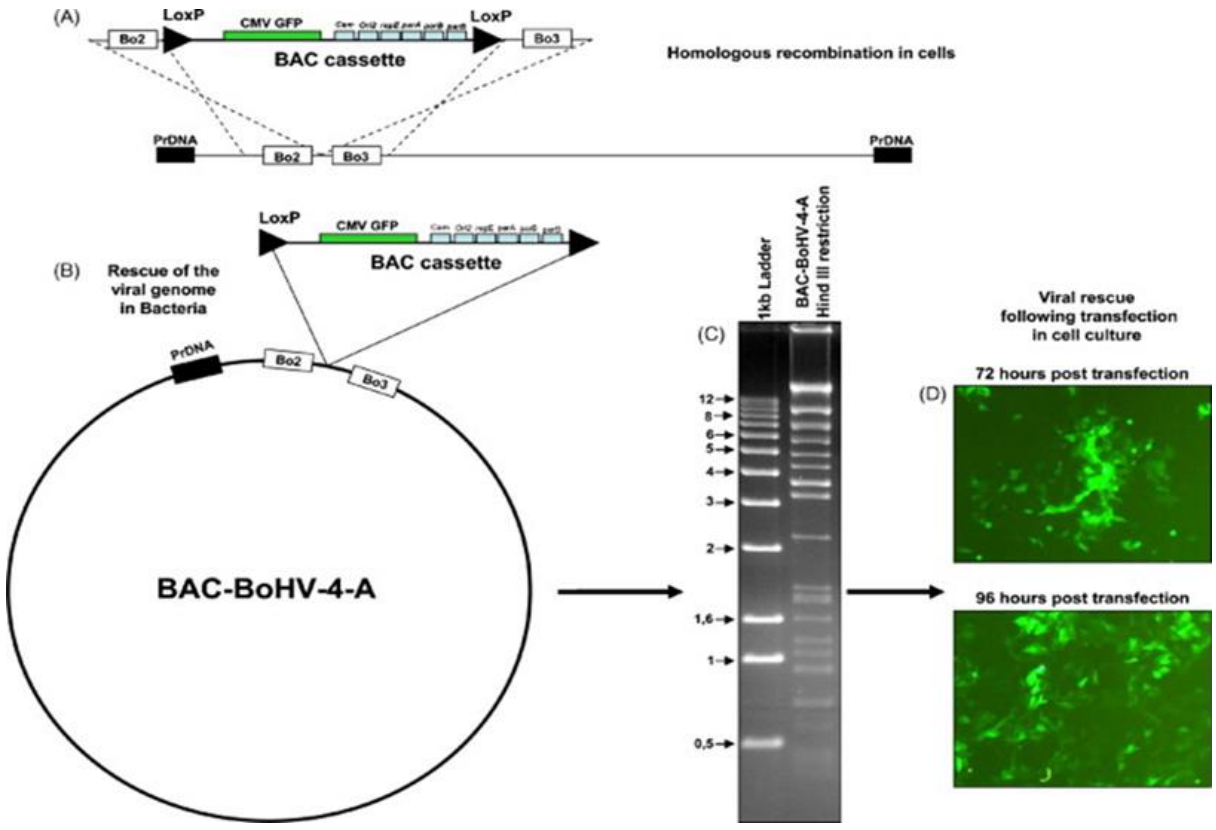
Bacterial Artificial Chromosome (BAC) system and possibility of Manipulation of BoHV-4 genome.

Bacterial Artificial Chromosomes (BAC), are fertility - (F-) factor-based plasmids and are able to stably replicate in low copy numbers. and to transport large genomic fragments, up to 300 Kbp.
The genomic insertions may contain several regulator elements, such as: promoter, terminator and enhancers. BACs can also leave the cloned gene expression under its own regulatory, thus mimicking its endogenous expression pattern.
Several properties, such as: simple high-quality DNA purification, stably clone methylated eukaryotic DNAs and high transformation efficiency protocols make BAC plasmids an alternative resource to conventional vector systems used for studies of transgenes expression under transcriptional control of heterologous promoters (Sharan et al. 2009).
The discovery of Recombineering, the recombination-mediated genetic engineering technique, in the late 1990s; allowed to overcome several technical limitations comparing to conventional cloning and mutagenesis. For these reasons it nowadays represents the most routinely exploited subcloning technique (Narayanan e Chen 2011; Sharan et al. 2009).
The main characteristics of the Recombineering technique is that DNA manipulation is independent from E. coli endogenous homologous recombination functions and are adapted from bacteriophages, in fact, the genes responsible for homologous recombination have been moved on mobile plasmids (BACs) and subsequently transferred to Escherichia coli host strains which in turn contain the coliphage λ Red system or the *RecET* system from the *Rac* prophage.
This technique exposes target DNAs for just a short time to the recombination enzymes so that DNA sustains only stable modifications with no or low risks of rearrangements (Narayanan e Chen 2011).
Furthermore the "targeting constructs", that are target DNAs providing the homologous regions are introduced through electroporation into recombinant E. coli hosts.
The recombination-mediated genetic engineering technique, allows the targeted genetic change at any position on the BAC plasmid, just needing short 50 bp homology arms (Sharan et al. 2009).
BoHV-4-A, a specific "A-pathogenic" BoHV-4 strain, was isolated from the milk cellular fraction of a healthy cow and was cloned to generate a BAC-BoHV-4-Awith no pathogenic risks, in the DH10B-derivative engineered SW102 E.coli strain carrying the λ Red bacteriophage recombination system and the galactokinase (GalK) gene deletion (Capocefalo et al. 2013; Warming et al. 2005).
In particular λ Red bacteriophage system consists in three phage recombination genes:

1. *Gam*; (prevents the action of RecBCD E. coli nuclease, guaranteeing the transformed linear DNA *in vivo* preservation).
2. *Bet*; (encodes for the ssDNA binding Beta protein that promotes the annealing between the two complementary DNA molecules).
3. *Exo*; (possesses a 5′ to 3′ dsDNA exonuclease activity).

These genes were posed under the *lac* promoter transcriptional control and allow the precise linear DNA insertion, inducing the creation of the expected genetic recombinants.
Moreover, λ prophage system expression has a natural strategy through which it regulates its own recombination functions. In E.coli SW102 are present fact two operator sets and a repressor. The *cI857* λ repressor binds the two sets of operator sites located at both *pL* and *pR* promoters, these interact to each other creating a tight protein handcuff impeding the genes expression.
In particular, the repressor is temperature sensitive, it is active at 30-34°C and inactivated when shifting temperature up to 42°C. For this reason, the temperature of the bacterial culture has to be raised to 42°C for 15 minutes (is the time necessary for minimizes cellular stress and accidental

recombinations), the repressor get rapidly inactivated and λ pL promoter induces recombination genes high levels expression (Sharan et al. 2009).
For the identification and metabolic selection of recombinant positive colonies, in SW102 strain, has been inserted the Biotin and Galactose Operon systems (Warming et al. 2005).
In other studies, the Bo2-Bo3 ORFs intergenic region of BoHV-4 genome has been used as the target specific site for the BAC cassette insertion. The BAC cassette was LoxP Floxed (used the Cre-loxP system) and was flanked by BoHV-4-A Bo2-Bo3 homologous regions of approximately 500-1000 bp.
This system contains an Enhanced Green Fluorescent Protein (EGFP) reporter gene under the CMV immediate early gene promoter transcriptional control and the F1 plasmid elements as: origin of replication (Ori2), chloramphenicol resistance gene (Cam), genes repE, parA, parB, parC for partitioning proteins.
Furthermore, when the BAC cassette was obtained, pBo2-EGFP-BAC-Bo3 plasmid and BoHV-4-A purified genome were co-transfected into permissive Bovine Embryo Kidney (BEK) cells and by homologous recombination is obtained the insertion of the BAC cassette into the viral genome of BoHV-4-A. Using a visible green reporter gene allowed the infectious recombinant viral particles detection and its subsequent electroporation of its circular recombinant intermediates into the DH10B E.coli strain to generate of the stable pBAC-BoHV-4-A clone (Donofrio et al. 2009).

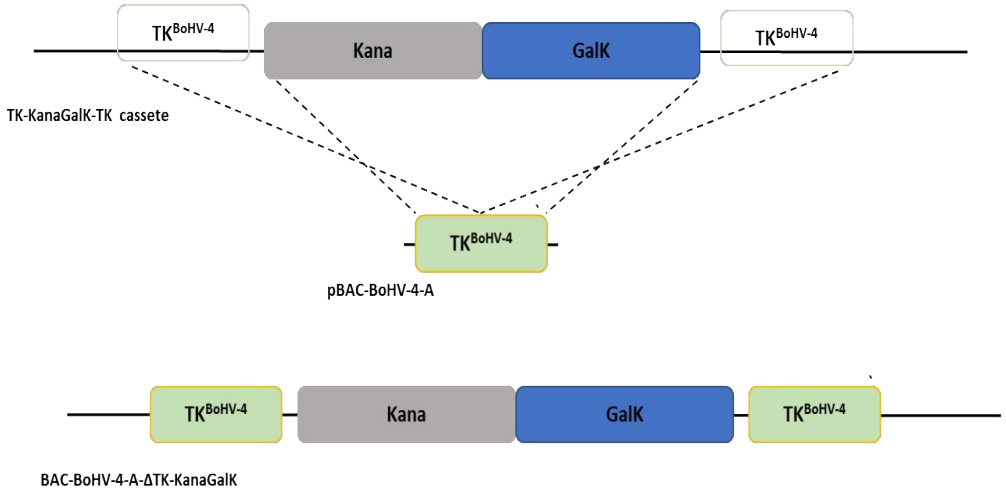


Graphical representation of BoHV-4 genome cloned as a Bacterial artificial chromosome (BAC)
(Donofrio et al. 2008)

After the cloning of a BoHV-4 genome as a BAC, it was necessary to further process and modify this new bacterial clone to be able to use recombinant BoHV-4 clones as a vector to deliver exogenous DNA.
Here we describe the two steps protocol designed by Donofrio et al (Donofrio et al. 2009), to firstly introduce a DNA stuffer into the viral Thymidine Kinase (TK) gene and then to replace that stuffer with the DNA of interest.

- The first step is called "Targeting":

In this process, the Kana/GalK cassette, flanked by two TK homology arms of almost 1Kbp has been produced by PCR and precisely cloned into pBAC-BoHV-4-A exploiting the λ Red-based recombineering system.
In particular, the Kana/GalK cassette is essential for the positively screening of recombined clones and the TK fragments have served as homologous recombination regions (Donofrio et al. 2009). Positive clones selection is performed in plates containing biotin, leucine and galactose as the only carbon source and chloramphenicol for BAC maintenance.

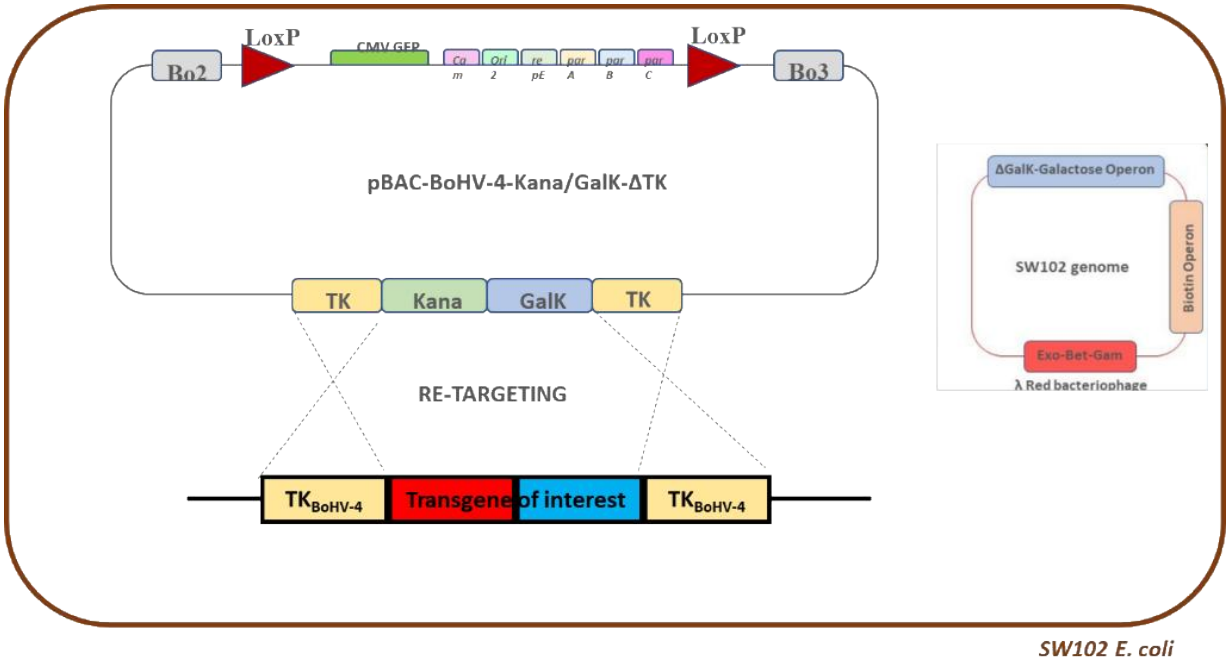


Targeting process

- The last step is the “Re-Targeting”:

During a second-step process, called "Re-Targeting", the Kana/GalK cassette is replaced with another cassette containing the transgene of interest, flanked by the short homology arms of TK gene.
The positively recombined clones are selected through two screening steps:

1. A solid phase selection on plates containing a galactose analogue, the 2-deoxy-galactose (DOG), that get transformed into the 2-deoxy-galactose-1-phosphate toxic compound if phosphorylated by the GalK enzyme thus killing non recombined SW102s.
2. A liquid selective phase, in kanamycine (K) and chloramphenicol (Cl) enriched media, discharging all the K+/Cl+ false positives (Donofrio et al. 2009).



Re-targeting process

The viable viral particles of BoHV-4 recombinant, are obtained by electroporating the purified pBAC-BoHV-4-A- that carries the "transgene of interest" into BEK/fin Cre cells and thanks the Cre-LoxP recombinase system is capable of exciding the floxed BAC cassette from the viral genome (Gillet et al. 2005).

Discovering Open Reading Frame-45 (ORF45).

Open reading frame (ORF45) is a conserved gene, and its product is a tegumental and multifunctional protein found only in Gammaherpesviruses, it is not present in fact in Alpha or Betaherpesviruses, and most importantly, there is no cellular homologue for ORF45.
Co-linearity exists between the various genes among Gammaherpesviruses, including ORF45 which is positioned on the complementary strand of the genome.
Although conserved, the homologs of ORF45 differ considerably in the length of the protein product and most likely carry out different biological activities in the various viruses to which they belong. In addition, other than differing in protein length, the overall sequence homology between homologs is very low. In fact, only a few short and discrete regions can be aligned with each other. Between them, the carboxy-terminal (C-terminal) end has the highest homology, implying the possibility that this region could have a very important biological role (Zhu et al. 2006).
Similarly, another highly conserved region is the one where the nuclear localization (NLS) sequence is present (Zhu et al. 2005, 2006).
Furthermore, the gene product differ in its amino acid composition, in fact in Kaposi sarcoma herpesvirus (KSHV) ORF45 is composed of 407aa and has the longest protein product of the various Gammaherpesviruses, while in BoHV-4 it is only 241aa and in MHV-68 it is 353aa (Bortz et al. 2003; Zhu et al. 2005).
In numerous studies ORF45 is described as an Immediate Early Gene.
Herpesvirus genes have been so far classified in Latent, Immediate-Early (*alpha* or *IE*), Early (*beta* or *E*), and Late (*gamma* or *L*). genes.
ORF45 is considered as immediate-early genes. These genes are expressed soon after viral infection and generally encode for transactivators which induce Early genes expression that encode for the *Beta* gene. *Beta* genes encode proteins that are involved in viral DNA replication but in some cases, can encode also additional transactivators. Furthermore, after the viral DNA replication, it begins the expression of Late gen. In fact, the *Gamma* genes are transcribed only after the initiation of viral DNA synthesis (Lacoste et al. 2004).

<u>The Tegument proteins</u>:

The tegument of the Gammaherpesvirus is very investigated, because little is known about its structure and composition. On the contrary, we know a lot on the function of the tegument proteins in alpha- and betaherpesviruses.
In particular, these proteins are involved in three essential functions in viral replication:

1. structural effects during the entry of virions into naïve cells,
2. the assembly and egress of virions,
3. the translocation of nucleocapsids to the nucleus (Castillo e Kowalik 2002; Subak-Sharpe e Dargan 1998).

Furthermore, also has effects during the immediate-early phase of infection; such as the transactivation of viral immediate-early genes and the possible modulation of host cell gene expression (Jia et al. 2005).

ORF45 in Gammaherpesviruses: Epstein-Barr virus (EBV); Human herpesvirus 8 or Kaposi's sarcoma-associated herpesvirus (HHV-8/KSHV) and Murine gammaherpesvirus 68 (MHV-68).

Two Human Gammaherpesviruses are currently known: Human herpesvirus 8 or Kaposi's sarcoma-associated herpesvirus (HHV-8/KSHV) and Epstein-Barr virus (EBV).
In particular, these viruses can undergo two distinct stages in their life cycle, called: latency and lytic replication (Jia et al. 2005).
These stages are fundamental for the ability to cause benign or malignant tumors in hosts.
HHV-8 is associated with Kaposi's sarcoma, multicentric Castleman's disease and primary effusion lymphoma; while EBV is associated with Burkitt's lymphoma, Hodgkin's disease, lymphoproliferative diseases and nasopharyngeal carcinoma (Humans 2012; Jia et al. 2005).
There are numerous studies that describe the switch from latency to lytic replication in human.
Another important member, which belongs to the Gammaherpesvirus family is Murine gammaherpesvirus 68 (MHV-68), which found natural host in wild rodents (Jia et al. 2005).
In KSHV, ORF45 is described and reported to be an immediately early gene and other studies have indicated that KSHV ORF45 is expressed during the early stage of viral reactivation (Jenner et al. 2001; Jia et al. 2005).
Furthermore, numerous studies described KSHV ORF45 protein like a component of the viral tegument and plays an important role binding the interferon regulatory factor 7 and interferes with its function (Jia et al. 2005; Zhu et al. 2002).
Although the function of ORF45 in EBV is currently unknown, antibodies against the protein have been found in patients with nasopharyngeal carcinoma and oral hairy leukoplakia (Gan et al. 1994; Jia et al. 2005).
In MHV-68, the ORF45 gene product is a low complexity acid protein that contains a NLS and is highly expressed during the early-late phase of lytic infection and is a component of the virion tegument (Bortz et al. 2003; Jia et al. 2005).
It has been described a 33.0% identity with KSHV ORF45 and 13.6% identity with EBV ORF45. Moreover, the C-terminal region is highly conserved among all gammaherpesviruses and the last 23 amino acids of MHV-68 ORF45 have an identity of 58% of KSHV ORF45 and 50% of that of EBV (Jia et al. 2005).

2. Aim of Work

The potential of BoHV-4 as a viral vector capable of delivering various heterologous genes for immune-prophylaxis and gene therapy has already been well documented in various published works. This, thanks to its favorable molecular and biological characteristics; such as little or no pathogenicity, absence of oncogenicity, ability to harbour large amounts of foreign genetic material and the possibility of being manipulated as Bacterial artificial chromosome (BAC) derived from infectious BoHV-4 (Donofrio et al. 2002a).

Several molecular studies are ongoing on the various Open Reading Frames (ORFs) of BoHV-4, and their gene products, in order to better clarify the interaction mechanisms between viral particles and host cells and to better understand and improve its application as a vector viral.

In particular, in this work we have focused on the study and characterization of the "Open reading Frame-45" (BoHV-4 ORF-45) and its protein product.

ORF45-BoHV-4 encodes a protein (orf45) of unknown function in this virus.

To better understand the role of ORF45 gene product during BoHV-4 lytic replication, a recombinant BoHV-4 was generated by homologous recombination from a BoHV-4 genome cloned as a BAC (pBAC-BoHV-4-A), in where the entire gene sequence of the BoHV-4 ORF45 has been replaced by the insertion of a selectable expression cassette containing the gene for kanamycin resistance and galactokinase expression.

The resulting recombinant BoHV-4 genome (pBAC-BoHV-4-AΔORF45-KanaGalK) was completely unable to reconstitute viral, viable and infectious particles (IRVP) and replicate when transfected into permissive cell lines compared to its revertant clone (pBAC-BoHV-4-ΔORF45-Revertant), where an ORF45 expression cassette, driven by a heterologous promoter, was positioned in the opposite direction to the natural ORF45 in the pBAC-BoHV-4-AΔORF45-KanaGalK genome. Because ORF45 was tagged with an HA epitope (YPYDVPDYA), we were able to demonstrate that the ORF45 gene product is associated with virion particles.

This work demonstrates that the replication cycle of BoHV-4 depends on the ORF45 gene product and provides direct evidence that the ORF45 gene product is required for BoHV-4 lytic replication, paving the way to further investigation.

3. Materials and Methods

Bovine ORF45 structure prediction.

The prediction of the complete ORF45 bovine protein tertiary structure by different ab-initio prediction systems only leaded to low-score confidence structures. However, the N-terminal end of the bovine ORF45 was successfully predicted with the Swiss Model 3D structure prediction server (Waterhouse et al. 2018) by using as a template the homologous portion of the ORF45 protein present in human herpesvirus 8 (PDB: 7opo). The predicted ORF45 bovine structure is 47 residues long (from aminoacid 26 to 72) and displayed a RMSD value of 0.105 A. The Modeller comparative modeling program (Webb e Sali 2016) has been used to model the bovine ORF45 FxFP motif to the human corresponding one (PDB: 7opm) bound to ERK2. ChimeraX Software was used to display protein structures (Pettersen et al. 2021).

Cell cultures.

Animal cell lines are grown in plastic flasks: cells form a monolayer that is tightly adherent to the bottom. They replicate until they reach confluence, until they completely cover the internal surface of the flask. If non-neoplastic cell lines are used, contact inhibition remarkably reduces the growth rate at this point, so it is necessary to transfer the culture to a bigger flask. Alternatively, the cells can be split in different flasks: the number depends on the amount of cells and on their growth rate.
Cells are grown in complete Eagle's minimal essential medium (cEMEM: 1 mM sodium pyruvate, 2 mM of L-glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B), supplemented with 10% FBS, and incubated at 37°C/5% CO_2 in a humidified incubator. All the supplements for the culture medium were purchased from Gibco. Splitting of cells from one flask to another one involves proteolytic enzymes, which degrade membrane proteins such as cadherin, responsible of cell-cell interactions, and integrins, involved in cell-flask interactions. A wide variety of enzymes is available, depending on the cell type that is to be treated: one of the most used is the protease trypsin. Prolonged exposure to trypsin may result in cell death instead of the mere detaching from the wall of the flask: the amount of protease and the time of exposure depends on the characteristics of the cell line.
In this study Madin Darby bovine kidney (MDBK; ATCC: CCL-22), bovine embryo kidney (BEK; BS CL-94; from IZS (Istituto Zoopofilattico Sperimentale, Brescia), HEK (Human Embryo Kidney, ATCC: CRL-11268) 293T, and BEK/cre expressing cre recombinase (Donofrio et al. 2008) cell lines were used. These cell lines have a high growth rate, independently from their cellular origin. In addition to this HEK 293T cell line has been immortalized with the SV-40 (Simian Virus-40) Large T-antigen, which is also important for episomal replication and amplification of plasmids containing the SV-40 origin of replication.

Cell cultures passage protocol.

1. Completely remove the medium from the flask;
2. wash the monolayer with trypsin (3 ml for a 25 cm^2 flask, 10 ml for 75 and 175 cm^2 ones). This washing step must be short, because its only purpose is to remove the remaining traces of medium;
3. remove trypsin. At this stage, monolayer should be visible as an opaque coating on the bottom of the flask;
4. add a suitable amount of trypsin to detach the cells (0.5 ml for 25 cm^2 flask, 2 ml for 75 ones and 4 ml for 175 ones);

5. shake the flask until complete detaching of cells: anyway, this procedure should not last more than few minutes in order to avoid flocculation and cell death; if the cell line produces a lot of extracellular matrix, to completely detach cell from plastic flask, incubation at 37°C with trypsin may be required;
6. neutralize trypsin by adding fresh medium with 10% FBS: usually 2 to 5 ml are sufficient;
7. wash the flask wall to recover as many cells as possible;
8. split cell suspension to new flasks;
9. add to every flask an adequate amount of medium (about 10 ml for a 25 cm^2 flask, 20-25 ml for a 75 cm^2 flask, 30-40 ml for a 175 one);
10. incubate the flask at 37°C in an incubator with 5% CO_2.

Constructs Generation.

pCMV-ORF45HA was generated amplifying by PCR BoHV-4 ORF45 from pBAC-BoHV-4-A DNA cut with EcoRI. The PCR amplification reaction was carried out in a final volume of 50 µL, containing 20 mM Tris–hydrochloride pH 8.8, 2 mM MgSO4, 10 mM KCl, 10 mM (NH4)2SO4,0.1 mg/mL BSA, 0.1% (v/v) Triton X-100, 5% dimethyl sulfoxide (DMSO), 0.2 mM deoxynucleotide triphosphates, and 0.25 µM of each primer.
As a couple of primers were used ORF45 NheI sense and ORF45HA SmaI antisense, to provide BoHV-4-ORF45 with a NheI site at its amino-terminal and a SmaI site and an HA tag at its carboxyterminal. 100 ng of DNA was amplified over 35 cycles, as follows: 1 minute denaturation at 94 °C, 1 minute annealing at 60 °C, and 50 seconds elongation at 72°C with 1U of Pfu recombinant DNA polymerase (ThermoScientific). The amplicon ORF45HA was cut with NheI/SmaI and inserted in pEGFP-C1 (Clontech), cut with the same enzyme, to generate pCMV-ORF45HA.
pCMV-ORF45HA was then used as a template to amplify again ORF45HA, with the same PCR parameters described above, with these couple of primers: Fusion-XhoI sense and SmaI-HA-antisense (See Table 1). This new amplicon was fused in frame with the GFP ORF, subcloning ORF45, cut with XhoI/SmaI in pEGFP-C1, cut with the same enzymes, generating pEGFP-ORF45-HA.
pTZ-KanaGalK, was generated by sub-cloning the 2232 bp galactokinase prokaryotic expression cassette (GalK), along with the kanamycin resistance expression cassette (Kana), into the pTZ57R shuttle vector (Thermoscientific), cut with KpnI/PstI (Franceschi et al. 2013).
The targeting vector, pORF45Left-KanaGalK-RightORF45, was generated firstly by the insertion of the ~700 bp left ORF45 homology region amplicon (obtained by PCR using ORF45A sense and antisense primers) cut with EcoRI/KpnI, in pTZ-KanaGalK, cut with the same enzymes; in this intermediate construct, cut with PstI/HindIII, was subsequently sub-cloned the ~700 bp right ORF45 homology region amplicon (obtained by PCR amplification with ORF45B sense and antisense primers), cut with the same enzymes.
The retargeting vector, pORF45Left-CMVORF45HA-RightORF45 was obtained subcloning the CMVORF45HA-pA entire expression cassette, excised from AseI/MluI cut pCMV-ORF45HA, in pORF45Left-KanaGalK-RightORF45, deprived of KanaGalK selector cassette, through NdeI/MluI restriction digestion.

Transient Transfection.

HEK 293 T cells were seeded into 25cm^2 flasks (1×10^6 cells/flask) and incubated at 37°C with 5% CO_2. When cells were sub-confluent, the culture medium was removed and the cells transfected with pCMV-ORF45HA or pEGFP-C1 (mock control) using Polyethylenimine (PEI) transfection reagent (Polysciences, Inc.). Briefly, 7,5 µg of DNA were mixed with 18,75 µg of PEI (1 mg/ml) (ratio 1:2.5 DNA:PEI) in 500 µl of Dulbecco's modified essential medium (DMEM) high glucose (Euroclone) without serum. After 15 minutes incubation at room temperature, 2000 µl of medium without serum were added, and the transfection solution was transferred to the cells (monolayer) and left for 6 hours at 37°C with 5% CO_2, in a humidified incubator. The transfection mixture was then replaced with fresh cEMEM medium, with 10% FBS, and incubated for 24 hours at 37°C with 5% CO_2. To analyze the subcellular localization of BoHV-4 ORF45, HEK 293 T cells were also transfected with pEGFP-ORF45-HA or pEGFP-C1, as a mock control. Twentyfour hours after the transfection, the cells were counterstained with DAPI (Thermo Scientific) and observed with a confocal microscope (Leica Microsystems).

Western Immunoblotting.

Western immunoblotting analysis was performed on protein cell extracts from 25 cm^2 flasks of HEK 293 T cells transfected with pCMV-ORF45HA or mock transfected. For protein extraction, 100 µl of cell extraction buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% NP-40; pH 8) was added on each pellet and total protein quantification was performed using BCA Protein Assay kit (Pierce™, Thermo Fisher Scientific). Before immunobloting analysis, pCMV-ORF45HA transfected cells extract was enriched in phosphorylated protein, passing through a Phosphoprotein Chelating metal resin, enrichment Kit (Pierce, Thermoscientific), following the protocol suggested by the manufacturers. Different amounts of protein samples were electrophoresed on 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, Merck) by electroblotting. The membrane was blocked in 5% skim milk (BD), incubated 1 hour with primary mouse monoclonal antibody anti-HA tag (G036, Abm Inc.) diluted 1:10,000 and then probed with horseradish peroxidase-labeled anti-mouse immunoglobulin (A9044, Sigma), diluted 1:15,000, and finally visualized by enhanced chemiluminescence (Clarity Max Western ECL substrate, Bio-Rad).

Western Immunoblotting protocol.

1. Assemble the glass plates according to the manifacturer's instruction (Hoefer);
2. prepare all the component of a 10% resolving gel as follows: 4.8 ml of deionized water, 2.5 ml of Acrylamide Mix (Acrylamide-bis-acrylamide 40% solution, 37.5:1), 2.5 ml TrisHCl 1.5M, pH 8.8; 100 µl SDS (10% solution), 150µl Ammonium persulfate, APS, (10% solution in water, APS provides the free radicals that drive polymerization) and 6 µl of TEMED (N,N,N',N'-tetramethylethylenediamine accelerates the polymerization by catalyzing the formation of free radicals from APS). Mix the components and pour the solution as soon as possible into the gap generated between the glass plates. Deposit on the surface of the gel a thin layer of isobuthanol, to seal the gel;
3. after the polymerization process is completed, pour off the overlay and wash the top of the gel several times with deionized water; remove any traces of water with the edge of a paper towel;

4. prepare the stacking gel as follows: 2.87 ml deionized water, 500µl of Acrylamide Mix, 500 µl of TrisHCl 1.0M, pH 6.8, 40µl of SDS (10% solution), 60µl of APS and 6µl of TEMED. Pour the gel through the spaces of the comb to completely fill in, being careful to avoid trapping air bubbles;
5. when polymerization is complete, wash the wells several times with running buffer to remove any un-polymerized acrylamide, after mounting the gel in the electrophoresis apparatus;
6. load up to 20 µl of each of the samples in to the bottom of the wells; attach the apparatus to an electric power supply and run until the bromophenol blue reaches the bottom of the resolving gel;
7. remove the glass plates from the apparatus and prepare for electric transfer as follows: a sandwich of gel and PVDF membrane (Millipore), between two pieces of towel paper and two sponges is compressed in a cassette and immersed in Transfer Buffer 1X in a transfer apparatus (Hoefer). Constant Voltage of 100 V is applied for 1 hour;
8. the membrane is then incubated at least for 1 hour with a 5% skim milk solution in TBST 1X;
9. skim milk is discharged and primary antibodies is added, antibody is diluted in 20 ml of a 1% skim milk solution in TBST1X. The membrane has to be incubated in gentle agitation for 1 hour at room temperature or overnight at 4°C;
10. the blot is then washed three times in TBST1X, and then secondary antibody is added to the membrane. After 1 hour incubation, the membrane is washed three time in TBST 1X, then detection is performed;
11. place the membrane between two sheets of acetate. Gently lift the top sheet and with a pipette drop the chemiluminescent substrate (mix 1:1 the two solutions of luminol and peroxide, from Biorad) on top of the membrane, scattering the drops over the surface of the membrane. Cover the membrane with the top sheet of plastic and remove all the bubbles present under the sheet, to create a liquid seal around the membrane;
12. expose the membrane in the ChemiDoc XRS (Biorad) for 2 to 60 seconds.

Buffer required:

- Running Buffer 10X (250mM Tris, 206g glycine, 1%SDS, water to 1L);
- Transfer Buffer 10X (250mM Tris, 144,1g Glycine and water to 1L);
- Transfer Buffer 1X (200 ml Transfer Buffer 10X, 400 ml Methanol, water to 2 L);
- TBST (100 ml TrisHCl 1M, pH 7.8, NaCl 1M, 0.5% Tween20, water to 1L).

BAC Recombineering and Selection.

Recombineering was performed as previously described (Warming et al. 2005) with some modifications. For heat-inducible homologous recombination in SW102 Escherichia coli (E. coli), containing BoHV-4-A genome, cloned as a BAC, pBAC-BoHV-4-A, was used the double selector targeting cassette Left-KanaGalK-Right, which was excised from the plasmid backbone pORF45Left-KanaGalK-RightORF45, cut with EcoRI/HindIII.

- TARGETING

Five hundred µl of a 32°C overnight culture of SW102 containing BAC-BoHV-4-A, were diluted in 25 ml Luria-Bertani (LB) medium with or without chloramphenicol (SIGMA) selection (12.5 µg/ml) in a 50 ml baffled conical flask and grown at 32°C in a shaking water bath to an OD600 of 0.6. Then, 10 ml were transferred to another baffled 50 ml conical flask and heat-shocked at 42°C for exactly 15 min in a shaking water bath. The remaining culture was left at 32°C as the un-induced control.

After 15 minutes the two samples, induced and un-induced, were briefly cooled in ice/water bath slurry and then transferred to two 15 ml Falcon tubes and pelleted using 5000 rpm at 4°C for 5 min. The supernatant was poured off and the pellet was resuspended in 1 ml ice-cold ddH2O by gently swirling the tubes in ice/water bath slurry. Subsequently, 9 ml ice-cold ddH2O was added and the samples pelleted again. This step was repeated once more, the supernatant was removed and the pellet (50 µl each) was kept on ice. Electroporate SW102 bacteria with gel-purified fragment (Left-KanaGalK-Right) obtained by cutting pORF45Left-KanaGalK-RightORF45, with EcoRI/HindIII (Fermentas). An aliquot of 25 µl of SW102 was used for each electroporation in a 0.1 cm cuvette at 25 µF, 2.5 kV and 201 Ω. After electroporation, the bacteria were recovered in 1 ml LB (15 ml Falcon tube) for 1 h in a 32°C shaking water bath. For the counter selection step (see below), the bacteria were recovered in 10 ml LB in a 50 ml baffled conical flask and incubated for 4.5 h in a 32°C shaking water bath.
After the recovery period, the bacteria were washed twice in sterile 1x M9 salts (6 g/l Na2HPO4, 3 g/l KH2PO4, 1 g/l NH4Cl, 0.5 g/l NaCl,) (SIGMA) as follows: 1 ml culture was pelleted in an eppendorf tube at 13,200 r.p.m. for 15 sec and the supernatant was removed with a pipette. The pellet was resuspended in 1 ml of 1x M9 salts, and pelleted again. This washing step was repeated once more. After the second wash, the supernatant was removed and the pellet was resuspended in 1 ml of 1x M9 salts. Plate 100 µl of each serial dilutions (1:10, 1:100 and 1:1000) on M63 minimal medium plates [15 g/l agar (DIFCO, BD Biosciences), 0.2% D-galactose (SIGMA), 1 mg/l D-biotin (SIGMA), 45 mg/l L-leucine (SIGMA) and 12,5 mg/l chloramphenicol (SIGMA)]. Washing in M9 salts is necessary to remove any rich media from the bacteria prior to selection on minimal medium plates. Plates were incubated 3-5 days at 32°C.
After recombineering, only those colonies that were kanamycin and chloramphenicol positive were kept and grown overnight in 5 ml of LB containing 12.5 µg/ml of chloramphenicol or 50 µg/ml kanamycin. BAC-DNA was purified and analyzed through HindIII restriction enzyme digestion for Left-KanaGalK-Right fragment targeted integration into the BoHV-4-A ORF73 locus.

- BAC-BoHV-4 RE-TARGETING

For the retargeting step SW102 bacteria containing BAC-BoHV-4-A-ΔORF45KanaGalK genome were also grown, heat induced and electroporated with HindIII linearized pORF45Left-CMVORF45HA-RightORF45 For the counter selection step, the bacteria were recovered in 10 ml LB in a 50 ml baffled conical flask and incubated for 4.5 h in a 32 °C shaking water bath. Bacterial serial dilutions were plated on M63 minimal medium plates containing 15 g/l agar, 0.2% glycerol (SIGMA), 1 mg/l d-biotin, 45 mg/l l-leucine, 0.2% 2-deoxy-galactose (DOG, SIGMA) and 25 µg/ml chloramphenicol. Plates were incubated 3–5 days at 32 °C. Several selected colonies were picked up, streaked on McConkey agar indicator plates (DIFCO, BD Biosciences) containing 20 µg/ml of chloramphenicol and incubated at 32 °C for 3 days until white colonies appeared. White colonies were grown in duplicate for 5-8 h in 1 ml of LB containing 50 µg/ml of kanamycin or LB containing 12,5 µg/ml of chloramphenicol. Only those colonies growing on chloramphenicol and not on kanamycin were kept and grown overnight in 5 ml of LB containing 12,5 µg/ml of chloramphenicol. BAC-BoHV-4-A-revORF45HA DNA was purified and analyzed through HindIII restriction enzyme digestion for CMVORF45HA locus fragment targeted integration.

Original more detailed protocols for recombineering can also be found at the recombineering website (https://redrecombineering.ncifcrf.gov/).

Bac DNA purification.

Since Bacterial Artificial Chromosome (BAC) size is larger than commonly used plasmids, its purification requires more care to avoid shearing. BAC extraction protocol, is adapted from Warming et al. 2005:

1. In a 50 ml test tube, grow a 5 ml overnight culture in LB with selected antibiotics at 30°C in agitation. The temperature is strictly restricted to 30-32°C;
2. centrifuge at 6000 rpm) for 5 minutes at 4°C;
3. pour off the supernatant and put the test tube upside down on blotting paper in order to remove all traces of medium;
4. resuspend the pellet in 250 µl of resuspension solution (50 mM Tris-HCl pH 8, 10 mM EDTA, 100 µg/ml RNAse A) and transfer to a clean 1.5 ml test tube;
5. add 250 µl of lysis solution (200 mM NaOH, 1% SDS) and mix gently by inverting the test tube. Incubate for no longer than 4 minutes at room temperature;
6. add 250 µl of sodium acetate, mix very gently and incubate on ice or at -80°c for 15 minutes;
7. centrifuge at 14000 rpm for 5 minutes and transfer the supernatant to a clean 1.5 test tube;
8. repeat step 7;
9. add 750 µl of isopropanol to precipitate DNA, mix gently and incubate on ice or at -80°C for 10 minutes;
10. centrifuge at 14000 rpm for 10 minutes at 4°C;
11. remove the supernatant by inverting the tube and wash the pellet with 0.5 ml of a 70% ethanol solution;
12. remove all traces of ethanol and air dry the pellet;
13. dissolve the pellet in 50 µl of sterile filtered water.

Southern blotting.

Southern blotting is a useful and powerful technique to identify and locate DNA sequences in large genomes. First of all, DNA which has to be analyzed must be broken up through restriction enzyme digestion to generate small fragments. These fragments will then be separated in an agarose gel by electrophoresis, denaturated and transferred to a positively charged nylon membrane; UV treatment will result in immobilization of the fragment on the membrane. A labelled probe is then used to verify the presence of the desired sequence: under stringent conditions the synthetic oligonucleotide will pair only to perfectly complementary sequences present on the membrane. Subsequent revealing of the probe tells whether the searched sequence was present or not in the analyzed genome. Different labelling methods are used: the first one involves radioisotopes and revealing of DNA duplex is obtained through autoradiography.

For this study a non radioactive labelling methods was used: the sequence of interest has been amplified through PCR, but in the reaction mixture digoxigenin labelled deoxy-Uridine triphosphate (dUTP) was added. The result is an oligonucleotide with several thymines replaced by this modified nucleotide: revealing of the probe is carried out by a specific antibody directed against digoxigenin conjugated with alkaline phosphatase. Giving the adequate substrate (CSPD from Roche), the phosphatase converts it to a luminescent molecule: subsequent exposure of an X-ray sheet to the membrane results in revealing of DNA duplex.

In this study purified BAC clones from E. coli have been digested for 6 hours with HindIII, then the fragments have been separated overnight through agarose gel electrophoresis with a voltage of 25 V and transferred (according to the protocol) to a nylon membrane (by Boehringer Mannheim).
Here a protocol to produce labelled probes is presented along with a southern blotting protocol (Sambrook e Russell 2006).

Probe labelling PCR reaction:

- 5 µl of dNTPs mix 10X (2mM);
- 5 µl of Taq buffer 10X (from Invitrogen);
- 5 µl of primers 10X (2.5 µM each);
- 1 µl of template (~1 µg of plasmid or pre-amplified fragment);
- o1 µl of Digoxigenin-11-dUTP alkaline labile (Roche);
- 2 µl of Taq polimerase 1U/µl (Invitrogen);
- H2O to 50 µl.

Every cycle comprises 1 minute at 94°C, 1 minute at 60°C, 1 minute at 72°C; the number of cycles is 35.
Because incorporation of digoxigenin-dUTP results in a higher molecular weight than non labelled oligonucleotides, a parallel reaction without this modified nucleotide should be performed to check incorporation of digoxigenin-dUTP: the labelled amplicon should migrate less than the control in an agarose gel electrophoresis.

Southern blotting protocol.

1. After separation of DNA fragments by electrophoresis, put the agarose gel (upside down) in a container with 250 ml of depurination solution (0.25 M HCl) in a shaking waterbath. This solution will remove some purines from DNA, preserving the deoxyribose-phosphate backbone, to facilitate transfer of the DNA to the membrane. Incubate for 15 minutes or until the dye from the loading buffer changes color from blue to yellow;
2. remove depurination solution and add 250 ml of denaturation solution (1.5 M NaCl, 0.5 M NaOH). Incubate for 15 minutes in a shaking waterbath or until the dye changes back its color to blue;
3. remove denaturation solution and add 250 ml of neutralization solution: 1.5 M NaCl, 0.5 M Tris pH 7.5, 1mM EDTA. Incubate for at least 20 minutes;
4. bring agarose gel into contact with the nylon membrane to transfer the DNA by capillarity from the agarose gel to the positively charged nylon membrane;
SSC 20X has the following composition: 3 M NaCl, 0.3 M sodium citrate. Leave overnight: SSC buffer will be drained from the lower reservoir by capillarity and it will transfer DNA from the gel to the membrane.
5. transfer the membrane to an ultraviolet oven and crosslink DNA in order to covalently bind it to the membrane;
6. put the membrane in a glass tube and add 50 ml of pre-hybridization solution (7% SDS, 0.5 M Na2HPO4). Incubate at 65°C in a rotisserie for half an hour;
7. put 5 µl of PCR reaction contained the digoxigenin labelled probe and 500 µl of sterile filtered water in a 2 ml screw cap test tube. Put the test tube in boiling water for 5 minutes to denature DNA, then put it immediately on ice;
8. transfer the probe to 50 ml of hybridization solution (7% SDS, 0.5 M Na2HPO4, 1mM EDTA);
9. replace the pre-hybridization solution in the glass tube with the hybridization solution. Incubate overnight at 65°C in a rotisserie (Tecna instrument oven);

10. pour off hybridization solution;
11. wash the membrane with 100 ml of washing solution 1 (0.5X SSC, 0.1% SDS) for at least 15 minutes at 65°C;
12. pour off washing solution 1 and repeat step 11;
13. remove washing solution 1 and add 100 ml of washing solution 2 (40 mM PO43- pH 7.2, 0.05% SDS). Incubate for at least 15 minutes at 65°C;
14. repeat step 13;
15. pour off washing solution 2 and equilibrate the membrane with washing solution 3 (100 mM maleic acid, 150 mM NaCl, 0.3% Tween20) for 1 minute at room temperature in a shaker;
16. remove washing solution 3 and block the membrane by gentle agitation for 30-60 minutes in blocking solution (100 mM Maleic acid, 150 mM NaCl and 1% of blocking reagent from Roche);
17. dilute the Anti-Digoxigenin-AP (Fab fragment 150U/200 µl, Roche) 1/15000 in 50 ml of blocking solution. Pour off the blocking solution and incubate the membrane for 30 minutes in the antibody solution;
18. discard the antibody solution. Gently wash the membrane twice, 15 minutes per wash, with 100 ml of washing buffer 3;
19. pour off washing buffer 3 and equilibrate the membrane in detection buffer (100 mM Tris/HCl, 1 mM EDTA pH 9.5) for two minutes;
20. place the membrane between two sheets of acetate. Gently lift the top sheet and with a pipette drop the chemiluminescent substrate (CSPD from Roche) on top of the membrane, scattering the drops over the surface of the membrane. Cover the membrane with the top sheet of plastic and remove all the bubbles present under the sheet, to create a liquid seal around the membrane. Leave the membrane at room temperature for 20 minutes;
21. expose the membrane in the ChemiDoc XRS (Biorad) for 5 to 20 minutes.
Primers used for the Southern Blot are the same used for the PCR, the probe was infact designed to amplify and reveal the presence of ORF45 gene promoter in the retargeted BACs.

Cell Culture Electroporation and Recombinant Virus Reconstitution.

BEK or BEK cre cells were maintained as a monolayer with cEMEM growth medium with 10% FBS. When cells were sub-confluent (70–90%) they were split to a fresh culture flask (i.e., every 3–5 days) and were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. pBAC-BoHV-4-A, pBAC-BoHV-4-A-revORF45HA and pBAC-BoHV-4-A-ΔORF45KanaGalK DNAs (5 µg) were electroporated in 600 µl DMEM high without serum (Biorad, Gene Pulser XCell, 270 V, 960 mF, 4-mm gap cuvettes) into BEK and BEK cre cells from a confluent 25-cm^2 flask. Electroporated cells were transferred to new flasks, after 24 hours the medium was replaced with fresh cEMEM, and cells were split 1:2 when they reached confluence at 2 days post-electroporation. Cells were grown until the appearance of cytopathic effect (CPE).

Viruses and Viral Replication.

BoHV-4-A and BAC-BoHV-4-A-revORF45 were propagated by infecting confluent monolayers of BEK or MDBK cells at a multiplicity of infection (M.O.I.) of 0.5 tissue culture infectious doses 50 ($TCID_{50}$) per cell and maintained in cEMEM with only 2% FBS for 2 hours. The medium was then removed and replaced with fresh cEMEM with 10% FBS. When CPE affected the majority of the cell monolayer (~72 hours post infection), the virus was prepared by freezing and thawing cells three

times and pelleting the virions through a 30% sucrose cushion, as previously described (Donofrio et al., 2006). Virus pellets were then resuspended in cold cEMEM without FBS. Viral pellets that were loaded in SDS-PAGE gel were resuspended in 100 µl of cell extraction buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% NP-40; pH 8) and heat denatured. $TCID_{50}$ were determined on BEK cells by limiting dilution.

Viral Growth Curves.

BEK cells were infected with BoHV-4-A and BAC-BoHV-4-A-revORF45 at a M.O.I. of 0.1 $TCID_{50}$/cell and incubated at 37°C for 3 hours. Infected cells were washed with serum-free EMEM and then overlaid with cEMEM with 10% FBS. The supernatants of infected cultures were harvested at scheduled time points (24, 48, 72, and 96 hours post infection), and the amount of infectious virus was determined by limiting dilution on BEK or MDBK cells. Viral titer differences between each time point are the averages of triplicate measurements ± standard errors of the mean ($p > 0.05$ for all time points as measured by Student's t-test).

RNA isolation.

Five-millions of cells were resuspended in 1ml of Trizol (Invitrogen) and stored at -80°C until extraction. Total RNA was isolated by NucleoSpin miRNA kit (Macherey–Nagel), using the protocol combined with TRIzol lysis (Invitrogen) and small and large RNA recovery in one fraction. Concentration and quality of RNA were determined by Agilent 2100.The isolated RNAs were stored at − 80 °C until use.

Library preparation and sequencing.

RNA samples (RIN >7.5) from four replicates (n = 4) for each condition (Control and Treated) were used for library preparation. RNA-Seq libraries were obtained with the Illumina TruSeq RNA Sample Preparation v2 Kit. Concentration and quality check of libraries were determined by Agilent 2100 Bioanalyzer. Sequencing was performed on a single lane of Illumina HiSeq X, 150 cycles paired end.

Data analysis.

RNA-Seq analysis was run with the nf-core/rnaseq v.3.8.1 pipeline (https://nf-co.re/rnaseq). The pipeline integrates TrimGalore v0.6.7 for sequence trimming and STAR v2.7.10a (Dobin et al., 2013) for sequence alignment. Sequences were aligned to the human GRCh38.p13 reference genome. Salmon v1.5.2 (https://combine-lab.github.io/salmon/) was used to quantify alignments to gene regions.
The EdgeR Bioconductor package v3.6 (Bioconductor, https://bioconductor.org/packages/release/bioc/html/edgeR.html) was used to estimate differential expression between control and treated samples. Hierarchical cluster analysis was performed with Genesis, (Sturn et al., 2002). Differentially expressed genes (DEGs) were submitted to GO analysis using the Cytoscape (version.3.2.1) plug-in ClueGO (version 2.3.5) (Bindea et al. 2009).

4. Results

BoHV-4 ORF45 has a low protein homology with other Rhadinovirus ORF45s but surprisingly is structurally related to KHSV ORF45.

BoHV-4 belongs to the genus Rhadinovirus; as the viruses belonging to this genus all have high homology, also the BoHV-4 ORF45 gene, in the BoHV-4 genome, has the same locus position (orf44-orf45-orf46-orf47).
Furthermore, in these viruses the nucleotide sequences of ORF45 and their protein products often have a weak percentage of identity with each other.
In particular, at the protein level, the identity rate of BoHV-4 ORF45 was identified as 24.9, 21.74 and 23.38% with KSHV, RRV and MHV68, respectively.
In all Rhadinoviruses isolated to date, the ORF45 genes have been sequenced and annotated, and their protein products deduced (Alexa et al. 2022).

Fig. 1. *Alignment of representative Rhadinovirus ORF45 protein sequences. Alignment of the bovine gammaherpesvirus 4 ORF45 protein (BoHV-4 ORF45; Acc. n. AEL29789.1) sequence with a subset of protein homologs, hypothetical protein Saimirine gammaherpesvirus 2 (SaHV-2 ORF45; Acc. n. CAC84340.1), Ateline gammaherpesvirus 3 (AtHV-3 orf45; Acc. n. NP_048018.1), Otarine gammaherpesvirus 4 (OtHV-4 ORF45; Acc. n. QRE02526.1), Kaposi Sarcoma Associate Herpesvirus (KSHV ORF45; Acc. n. BAV17895.1), Murid herpesvirus 4 (MHV-68 ORF45; NP_044882.1), Rhesus monkey rhadinovirus (RRV ORF45; Acc. n. AAF60024.1). Conserved amino acids are drawn according to their percentage similarity with the consensus sequence (>80% dark-blue, >60% medium blue, >40% light-blue, <40% white). Conserved functional sites and nuclear localization signals are marked with red and green bars, respectively.*

However, only KSHV ORF45 has been characterized in terms of structure (Alexa et al. 2022).
HHpred analysis found in the KHSV N-terminal fragment ORF45, containing the binding domain for p90 ribosomal S6 kinase (RSK) and signaling regulated kinase (ERK) complex (Alexa et al. 2022), significant homology (~30%) with BoHV-4 ORF45, which has been used as a potential structural model; surprisingly the BLAST analysis of the BoHV-4 ORF45 protein sequence identified low-scoring partial homology sequences and no hits in the pdb database.
The N-terminal structure of BoHV-4 ORF45 was successfully predicted with Swiss Model 3D prediction server (Waterhouse et al. 2018).
The predicted BoHV-4 ORF45 structure is well superimposed (RMSD 0.1 A) to the corresponding KHSV ORF45 orthologue bound to RSK2 kinase.
Further, the key Val and Phe (VP) interacting motif residues are also conserved in the BoHV-4 ORF45 and well fits the hydrophobic pocket located in the N-terminal of AGC-type kinase domain (NTK) of RSK.
Therefore, BoHV-4 ORF45 protein is expected to bind to phosphorylated ERK2, since the substrate-mimicking FxFP motif present in the KHSV ORF45 is also conserved in the BoHV-4 ORF45 and well fits the hydrophobic F-site of ERK2.

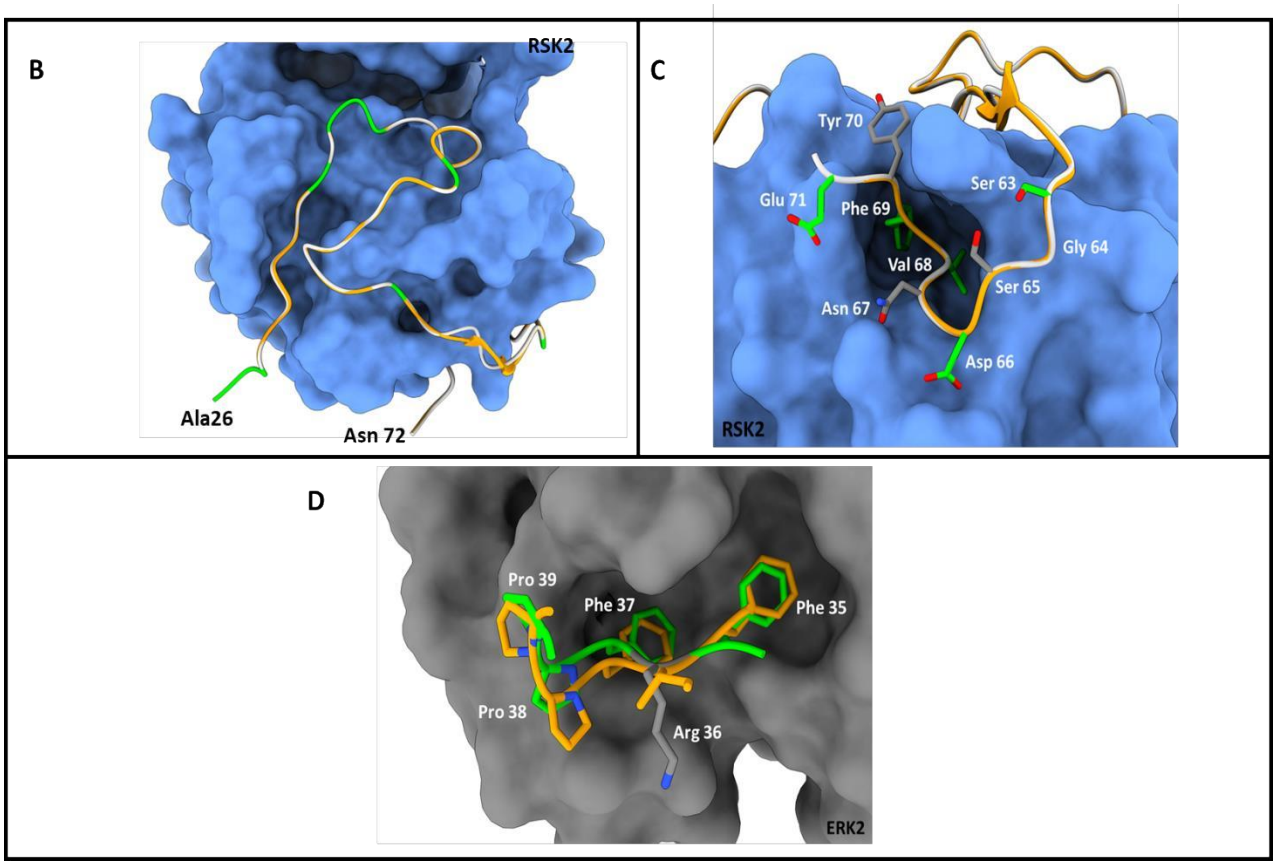


Fig.2. *B. The predicted Ca backbone of BoHV-4 ORF45 (in green the conserved residues; in white the non-conserved residues) is superimposed onto the human ORF45 Ca structure (in orange) bound to RSK2 (protein surface in blue) (PDB: 7opm). The N- and C-termini of the bovine protein are labelled. C. Particular of the predicted BoHV-4 ORF45 3D structure (Ca and side chains) superimposed onto Ca KHSV ORF45 Val-Phe binding motif bound to RSK2. Color code of Ca chains as in "B", while the bovine ORF45 side chains are rendered in in green/CPK or gray/CPK sticks, if conserved or non-conserved, respectively. D. Superposition of the predicted BoHV-4 ORF45 3D structure FxFP motif onto the corresponding human ORF45 (PDB: 7opm) residues bound to the hydrophobic F-site of ERK2 (protein surface in grey) (PDB: 7opm). Human ORF45 Ca and side chains are in orange/CPK sticks, the bovine Ca and side chain residues are in green/CPK or gray/CPK sticks if conserved or non-conserved, respectively.*

Study and characterization of BoHV-4 ORF45: is a phosphoprotein and is localized to the cell nucleus.

In silico the predicted length for BoHV-4 ORF45 starting from its nucleotide sequence (orf45) (Palmeira et al. 2011; Zimmermann et al. 2001), shows that it is composed by 241 aminoacidic residues, with an isoelectric point (IP) of 4.76, a mass of 27.142 kDa, an aliphatic index of 40.46 and is lacking a putative signal peptide.
According to the data, BoHV-4 ORF45 could be described as an intracellular soluble acidic protein.
BoHV-4 ORF45 has not been characterized, for this reason no specific antibodies have been developed and to follow its expression in mammalian cells, a carboxyterminal HA tagged ORF45, called pCMV-ORF45HA, was constructed.
Moreover, after SDS-PAGE migration, we observed that the molecular weight of BoHV-4 ORF45 was almost the double than predicted, ~55 kDa, this is probably due to some post-transcriptional modifications and in agreement with what was found for MHV68 ORF45 (Jia et al. 2005) and KSHV ORF45 (Zhu e Yuan 2003).

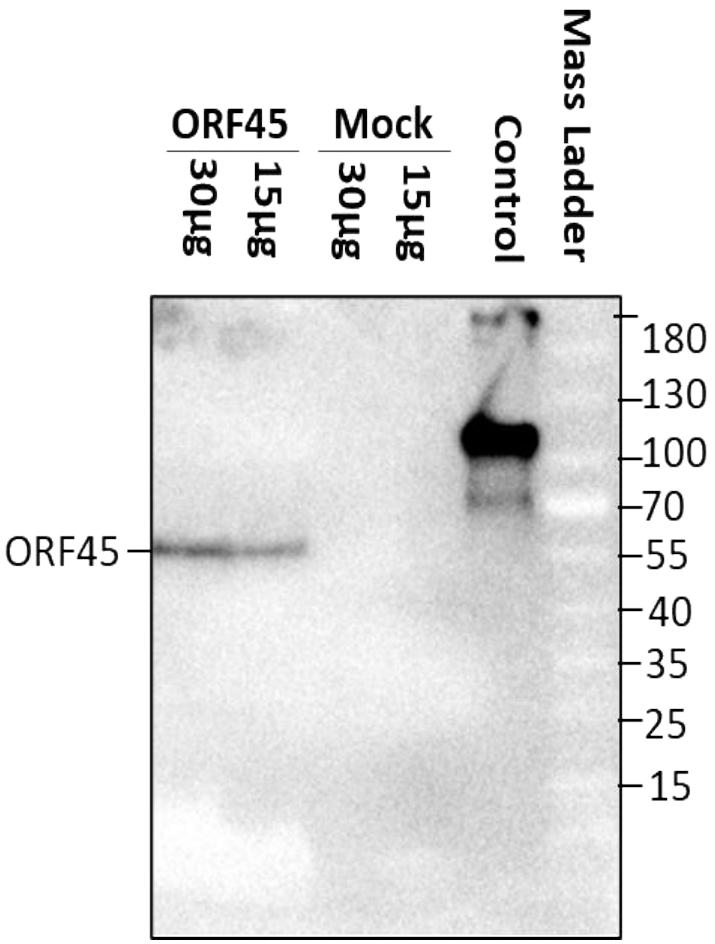


Fig.3.*Western immunoblotting of pCMV-ORF45HA (ORF45) and pEGFP-C1 (Mock) transfected cells protein extracts (30 and 15 µg of total protein extract). A positive antibody control was established with an HA tagged unrelated protein.*

Trought NetPhos-3.1(Blom, Gammeltoft, e Brunak 1999; N et al. 2004) BoHV-4 ORF45 analysis indicated the presence of several phosphorylation sites, where serine and threonine were mainly involved, although with a lower frequency and score and also tyrosine.

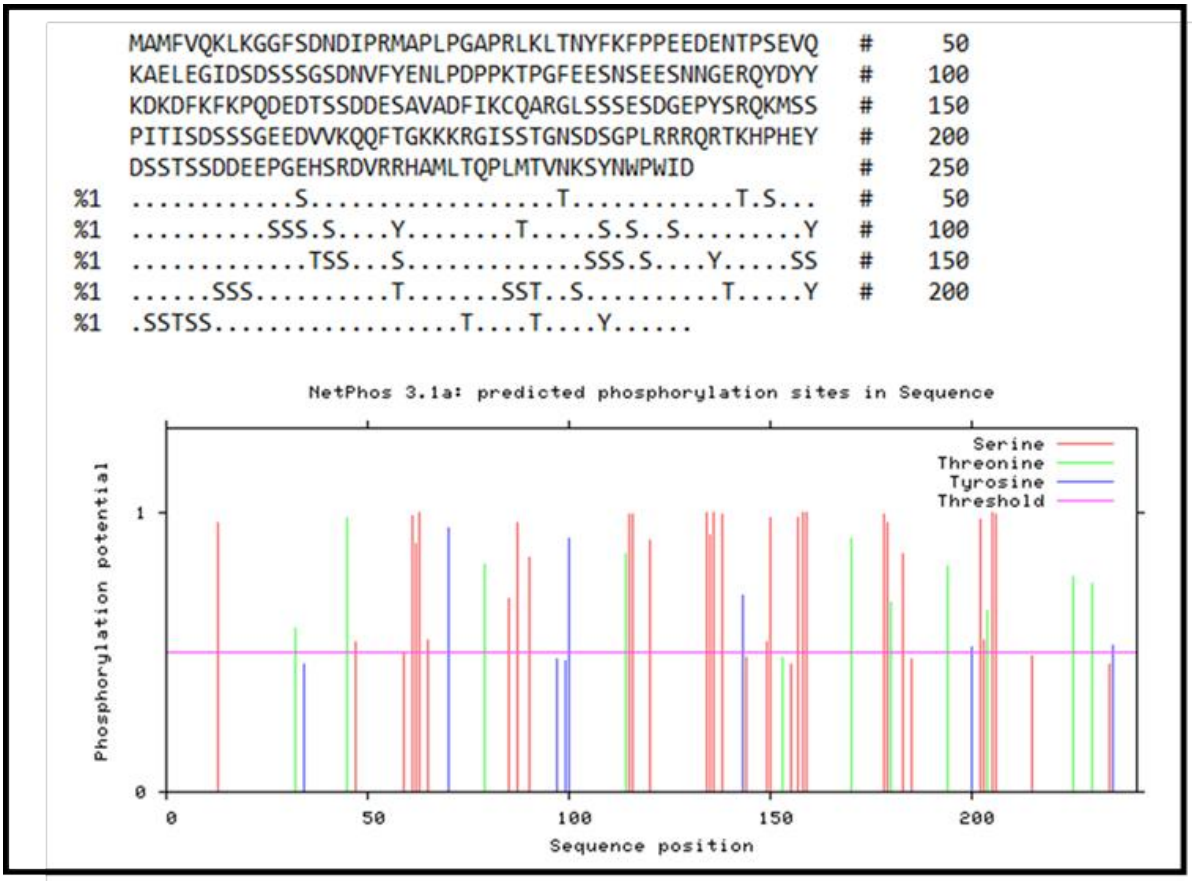


Fig.4.*BoHV-4 ORF45 protein sequence along with Serine (S) Threonine (T) and Tyrosine (Y) residues potentially phosphorylated as predicted by NethPhos 3.1, where the scores above 0.500 indicates positive predictions.*

BoHV-4 ORF45 phosphorylation was confirmed by a phosphoprotein affinity resin which was able to retain BoHV-4 ORF45. The presence of glycosylation sites was not predicted and not identified when BoHV-4 ORF45 was treated with endoglycosidases.
In BoHV-4 ORF45, thanks by PSORT sequence analysis (Nakai 2000) a nuclear localization signal was identified (78%; K=9/23; consensus: KKKR at 172 and PLRRRQR at 187) but not a nuclear export signal as detected in KSHV ORF45 (Li e Zhu 2009).

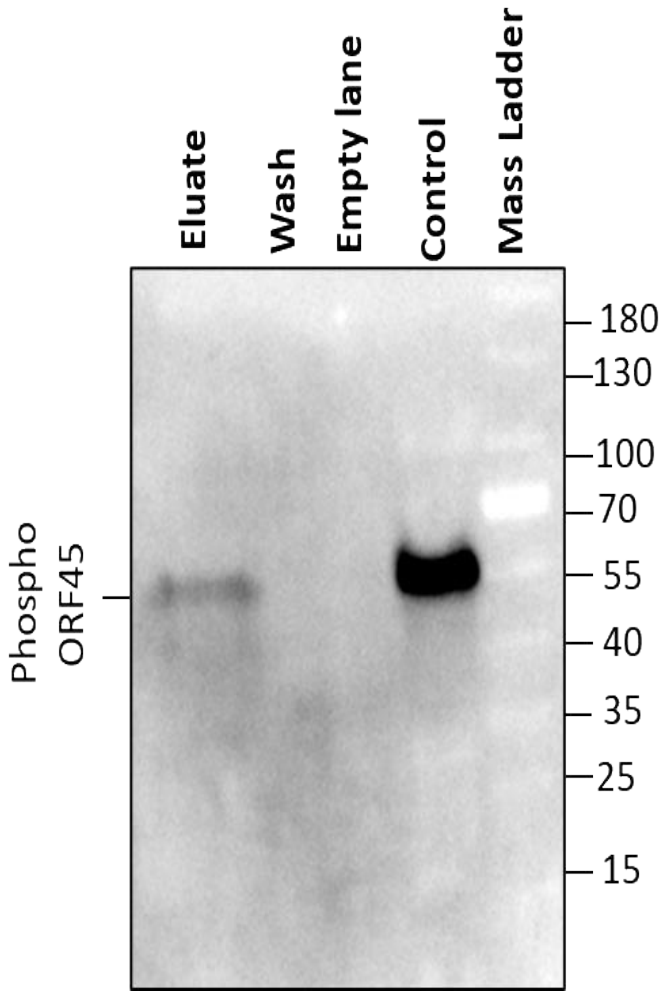


Fig5. Western immunoblotting of concentrated eluate and wash of pCMV-ORF45HA transfected cells extract, passed through a Phosphoprotein Chelating metal resin. A positive antibody control was established with pCMV-ORF45HA transfected cells extract.

For characterize the capability of BoHV-4 ORF45 to localize to the nucleus, a plasmid construct pEGFP-ORF45-HA was generated, where GFP orf was fused *in frame* with ORF45 tagged with HA. Subsequently, after twenty-four hours, the cells transfected with pEGFP-ORF45-HA, were visualized with a confocal microscope where GFP fluorescent signal was well observed within the cell nuclei. This result shows us that BoHV-4 ORF45 was able to deliver GFP, known to be a cytoplasmic protein, into the cell nucleus.

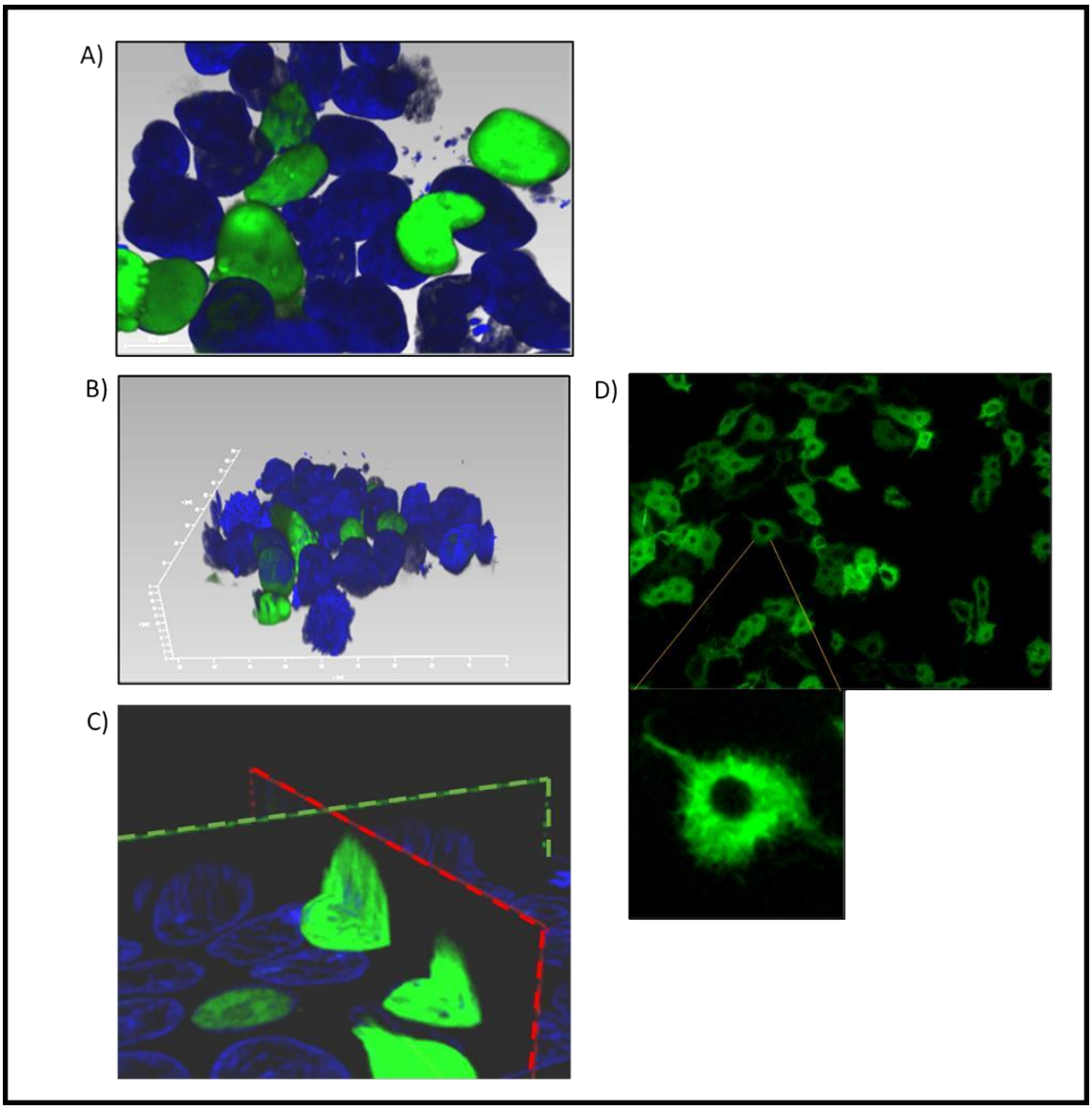


Fig.6.*Subcellular localization of BoHV-4 ORF45. Cells were transfected with a construct, pGFP-ORF45, where GFP orf was fused to the 5' end of BoHV-4ORF45 orf. Transfected cells were counterstained with DAPI and observed with a confocal microscope at 24 hours post transfection. A) 2D image of pEGFP-ORF45-HA transfected cells where GFP-ORF45 is localized within the nuclei (green). B) 3D image of cell nuclei containing GFP-ORF45 (green). C) Sagittal (green dashed line) and transverse (red dashed line) image of cell nuclei containing GFP-ORF45. D) 2D image of pEGFP-C1 transfected cells control, where GFP is only localized within the cytoplasm (green).*

BoHV-4 ORF45 is an essential gene for BoHV-4 lytic replication and its protein product is associated to the virion.

BoHV-4 ORF45 gene is transcribed from the opposite BoHV-4 genome DNA strand (Palmeira et al. 2011; Zimmermann et al. 2001) like the ORF46 but in contrast to ORF44.
To investigate the role of this gene during viral lytic replication in BoHV-4, the gene was deleted by site-specific insertional mutagenesis mediated by heat inducible homologous recombination (Warming et al. 2005) in the genome of BoHV-4 cloned as a BAC (BAC-BoHV-4-A).
BAC-BoHV-4-A was originally derived from a non-pathogenic strain of BoHV-4 isolated from the milk cellular fraction of a healthy cow (Donofrio et al. 2008).
The targeting cassette, Left-KanaGalK-Right, containing the 2232-bp KanaGalK DNA stuffer double selecting cassette (Donofrio et al. 2007) flanked by two BoHV-4 ORF45 gene homologous flanking regions, was generated to mediate insertion and deletion of the BoHV-4 ORF45 coding region.
In particular, although a large deletion and insertion were made, this should not affect ORF44 and ORF46 transcription/translation and the viral phenotype, unable to replicate, obtained should be exclusively due to the loss of ORF45.
The Left-KanaGalK-Right targeting cassette was excised from the plasmid back-bone and electroporated into SW102 E. coli containing pBAC-BoHV-4-A, to generate pBAC-BoHV-4-A-ΔORF45KanaGalK. Selected targeted clones (on where the gene has been replaced) were analyzed by PCR, sequencing and HindIII restriction enzyme digestion.
Moreover, to confirm the authenticity of pBAC-BoHV-4-A-KΔORF45-KanaGalK phenotype is due was exclusively due to the loss of ORF45, and not a mere artifact, we have generated a control BoHV-4 with a carboxyterminal HA tagged ORF 45.
The gene was transcriptionally driven in an opposite direction respect to the natural ORF45, by a heterologous promoter (CMV).
The retargeting cassette, Left-CMV-ORF45-HA-Right, was excised out from the plasmid backbone and electroporated into SW102 E. coli containing pBAC-BoHV-4-A-ΔORF45KanaGalK and thanks at by heat inducible homologous recombination (Warming et al. 2005) was obtained pBAC-BoHV-4-A-revORF45-HA.

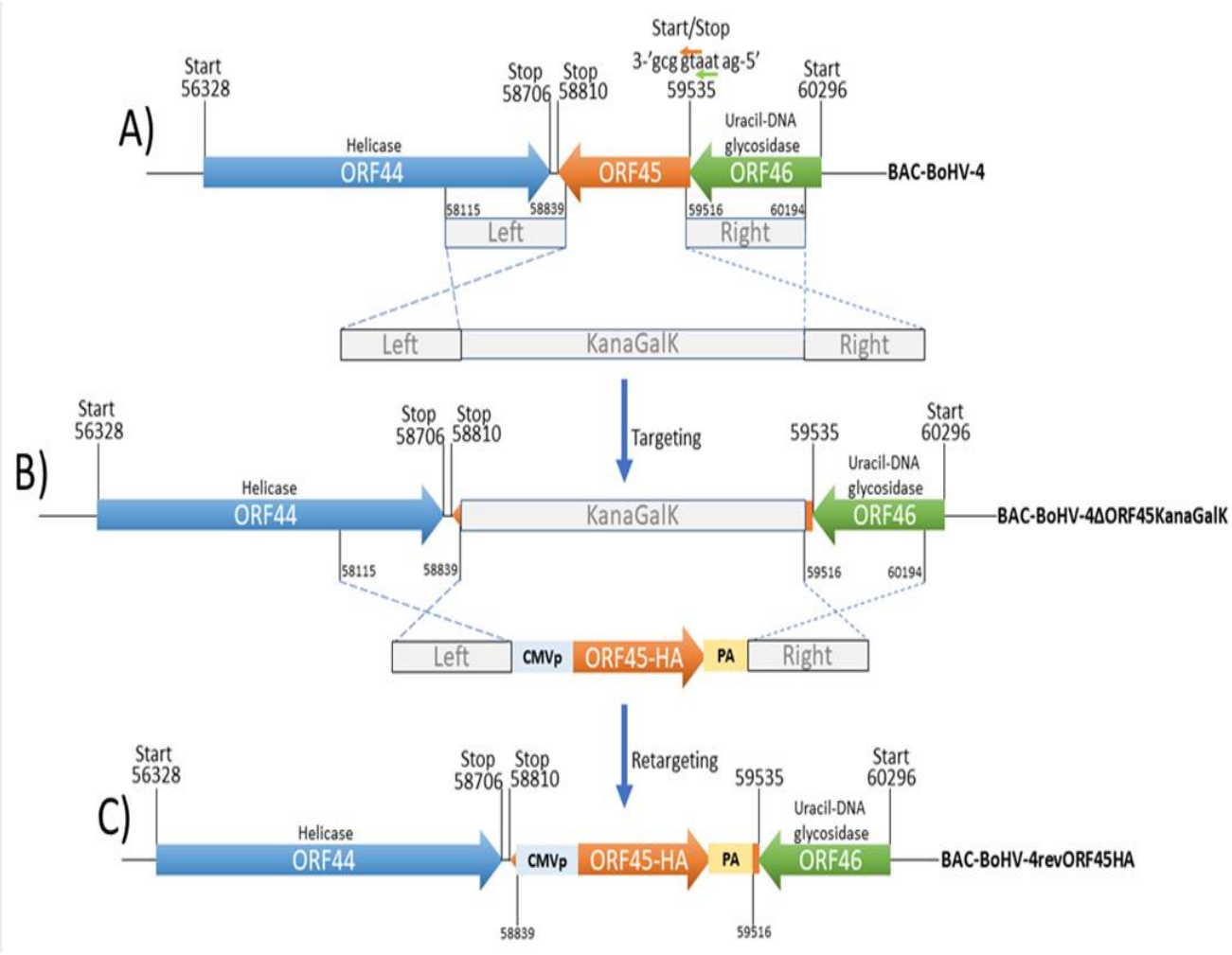


Fig.7.*Overall strategy to disrupt BoHV-4 ORF45 gene via heat inducible homologous recombination. (A and B) Diagram (not to scale) of the BoHV-4 ORF45 gene (orange arrow; nucleotide 59535 and 58810) positioned between ORF 44 (blue arrow; nucleotide 56328 and 58706) and ORF46 (green arrow; nucleotide 60296 and 59535). The last nucleotide of the ORF46 stop codon and the first nucleotide of the ORF45 start codon (a) is overlapped. ORF46 and ORF45 are transcribed on the opposite direction respec t to the ORF44 [based on the complete genome published sequence (GenBank accession number AF318573)]. The 2232-bp Kana/GalK selectable DNA stuffer (gray), flanked by left (nucleotide 58115 and 58839; 724 bp) and right homologous regions (nucleotide 58516 and 60194; 678 bp), was introduced between the positions 58839 and 59516, deleting most of the ORF45 sequence but leaving intact ORF44 and ORF46 and BoHV-4ΔORF45 was generated. B) CMVORF45HA expression cassette flanked by the left and right homologous regions (grey) was used to replace 2232-bp Kana/GalK selectable DNA stuffer (gray) and BoHV-4revORF45HA was so generated C).*

Even in this case, the selected targeted clones were analyzed by PCR, sequencing and HindIII restriction enzyme digestion.

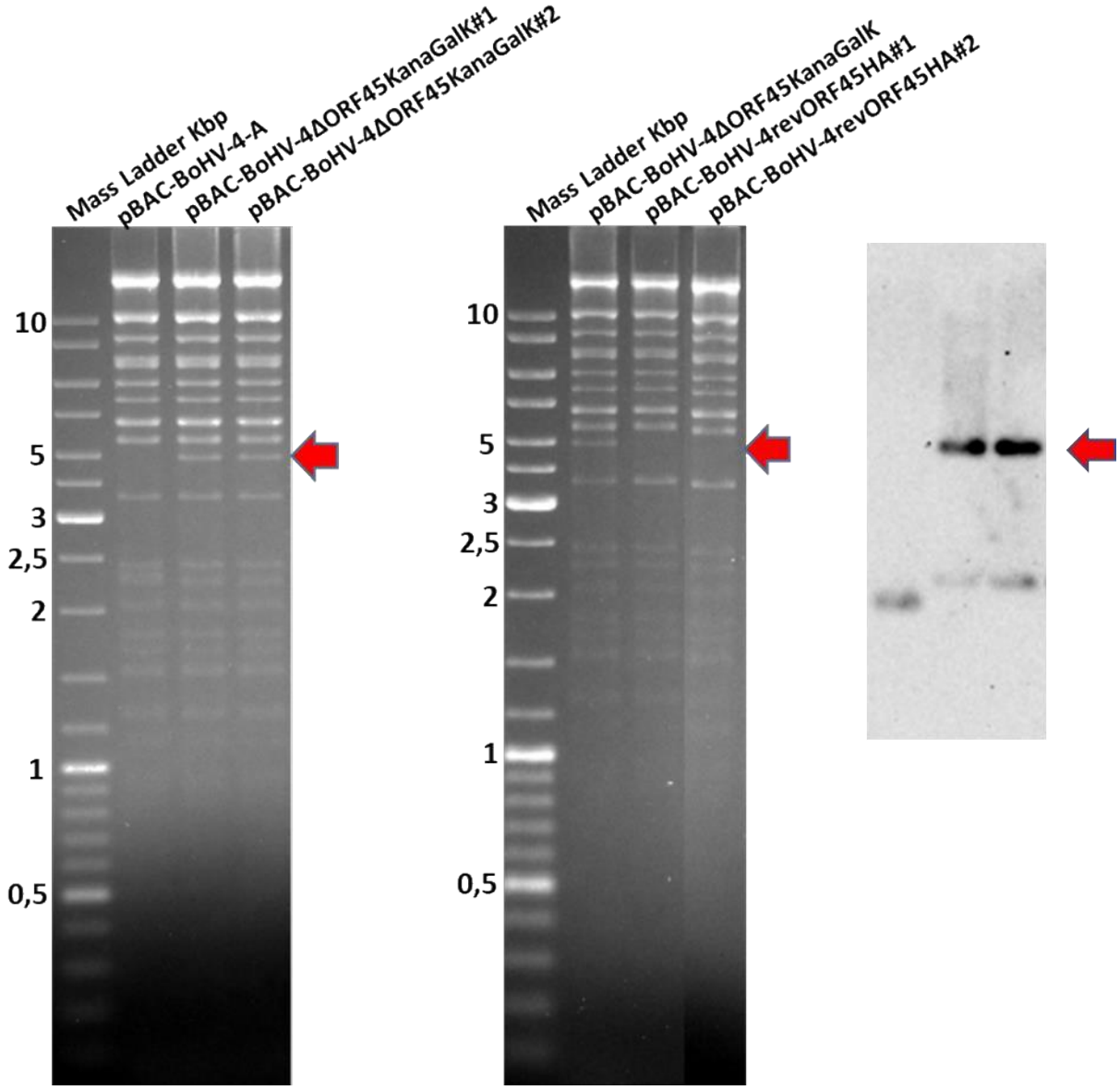


Fig.8.*HindIII restriction enzyme profile of two representative pBAC-BoHV-4ΔORF45 clones (#1 and #2) compared with the parental pBAC-BoHV-4-A. The diagnostic band, indicated by a red arrow, was well observable in pBAC-BoHV-4ΔORF45 respect to pBAC-BoHV-4-A. E) HindIII restriction enzyme profile of two representative pBAC-BoHV-4revORF45HA clones (#1 and #2) compared with the derivative pBAC-BoHV-4ΔORF45. The missing band, indicated by a red arrow, was well observable in pBAC-BoHV-4revORF45HA respect to pBAC-BoHV-4ΔORF45 and Southern blotting analysis performed with specific probes for the Orf45.*

When pBAC-BoHV-4-A-ΔORF45KanaGalK and pBAC-BoHV-4-A-revORF45HA were electroporated into BEK or BEK/cre cells (these cells are used to excise out the BAC cassette), plaques from the viable virus were obtained only on pBAC-BoHV-4-A-revORF45HA transfected cells monolayers but not on those transfected with pBAC-BoHV-4-A-ΔORF45KanaGalK.
This data showed that the ORF45 deletion rendered BoHV-4-A unable to be reconstituted and replicated, such phenotype was rescued by CMV-ORF45HA expression cassette, thus showing ORF45 indispensability in the contest of BoHV-4 lytic replication and in line with data present in literature for KHSV ORF45 (Fu et al. 2014) and MHV68 OFR45 (Jia et al. 2005).

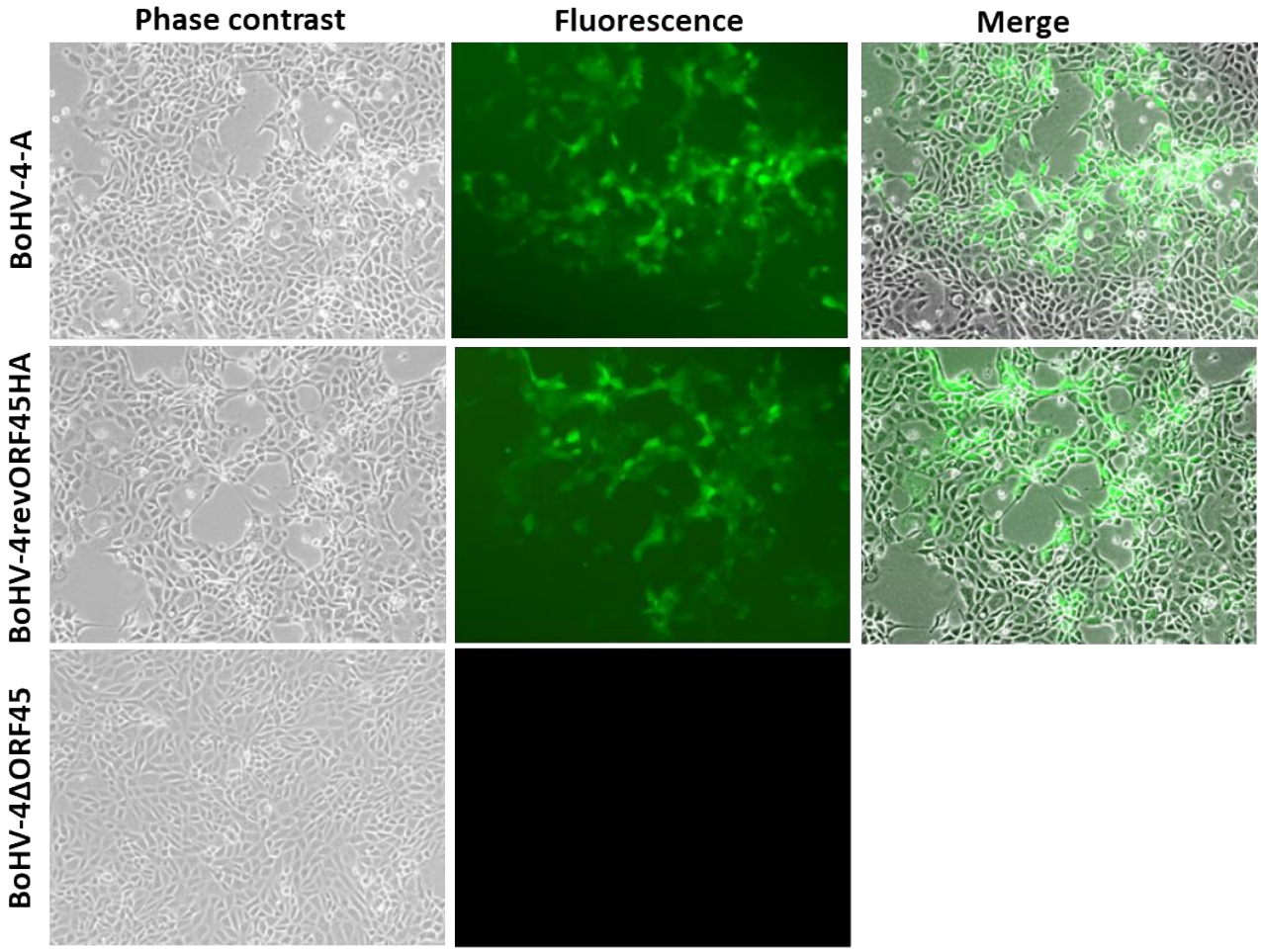


Fig.9.*BoHV-4 recombinants reconstitution and replication. A) Representative images (phase contrast, fluorescence and merged; 10×) of BEK cells electroporated with pBAC-BoHV-4-A, pBAC-BoHV-4revORF45HA and pBAC-BoHV-4-ΔORF45. CPE induced by reconstitution of IRVPs is recognizable only for pBAC-BoHV-4-A, pBAC-BoHV-4revORF45HA as revealed by green plaques.*

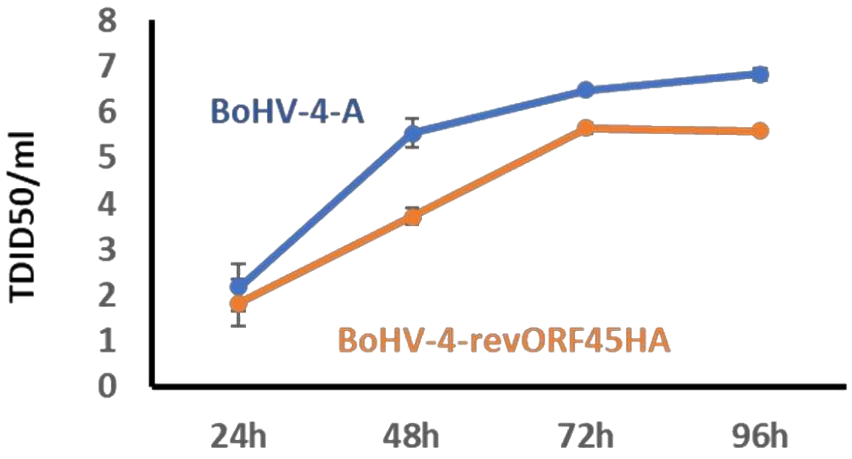


Fig.10.*Replication kinetics of BoHV-4-A and pBAC-BoHV-4revORF45.*

Furthermore, we investigated if BoHV-4 ORF45 is associated with the virus as previously observed by Palmeira et al. (Lété, Palmeira, et al. 2012) in a proteomic analysis setting.
Exploiting the BoHV-4-A-revORF45HA infectious virus that expresses an HA-tagged form of ORF45, when sucrose gradient purified virus was analyzed by SDS-PAGE and western blotting, detectable by an anti HA mAb, it was possible observed a specific band corresponding to ORF45, well evidenced.

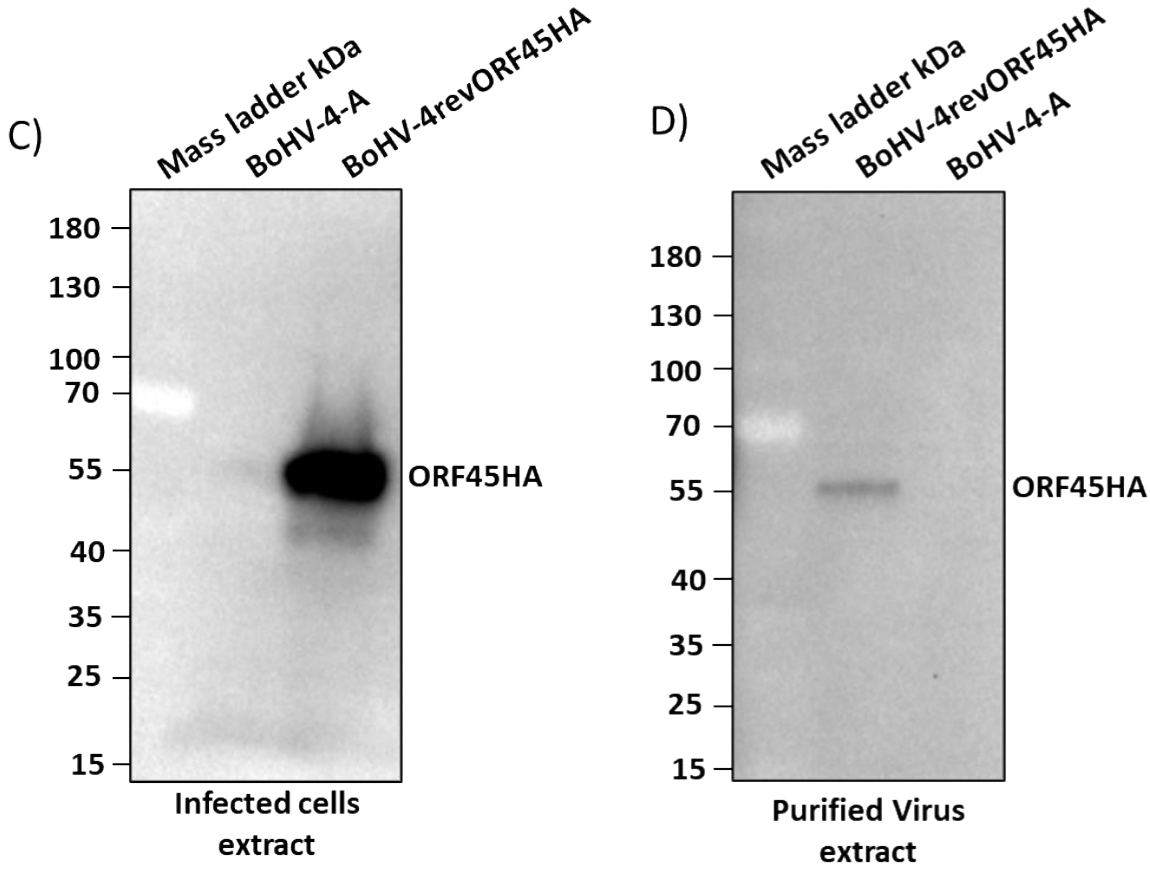


Fig.11.*C) Western immunoblotting of BoHV-4-A and BoHV-4revORF45HA infected cells extract. D) Western immunoblotting of BoHV-4-A and BoHV-4revORF45HA purified virus extract. All experiments were repeated three times with identical results.*

The cellular transcriptome: the possibility that BoHV-4-ORF45 expression alters the trascriptome.

The BoHV-4 ORF45 protein is phosphorylated and localizes into the cell nucleus.
These characteristics would suggest that BoHV-4 ORF45 could take part in a transcriptional regulatory network, indirectly through protein/protein interactions with other transcription factors.
In this regard, we made a comparative transcriptome analysis of cells expressing BoHV-4 ORF45 versus BoHV-4 ORF45 un-expressing cells was performed. RNA-seq analysis generated an average number of 55.8 M (111.56 M paired reads) reads per sample, with about 81.4% of the total reads that were correctly mapped on the human reference genome.
A total of 30738 unique genes were present in both groups, whether 94 and 190 genes were only detected in treated and control samples, respectively.
The principal component analysis (PCA) clearly separates control vs treated samples.
From the results it is possible to observe a total of 984 differentially expressed genes (DEGs), (FDR < 0.05).

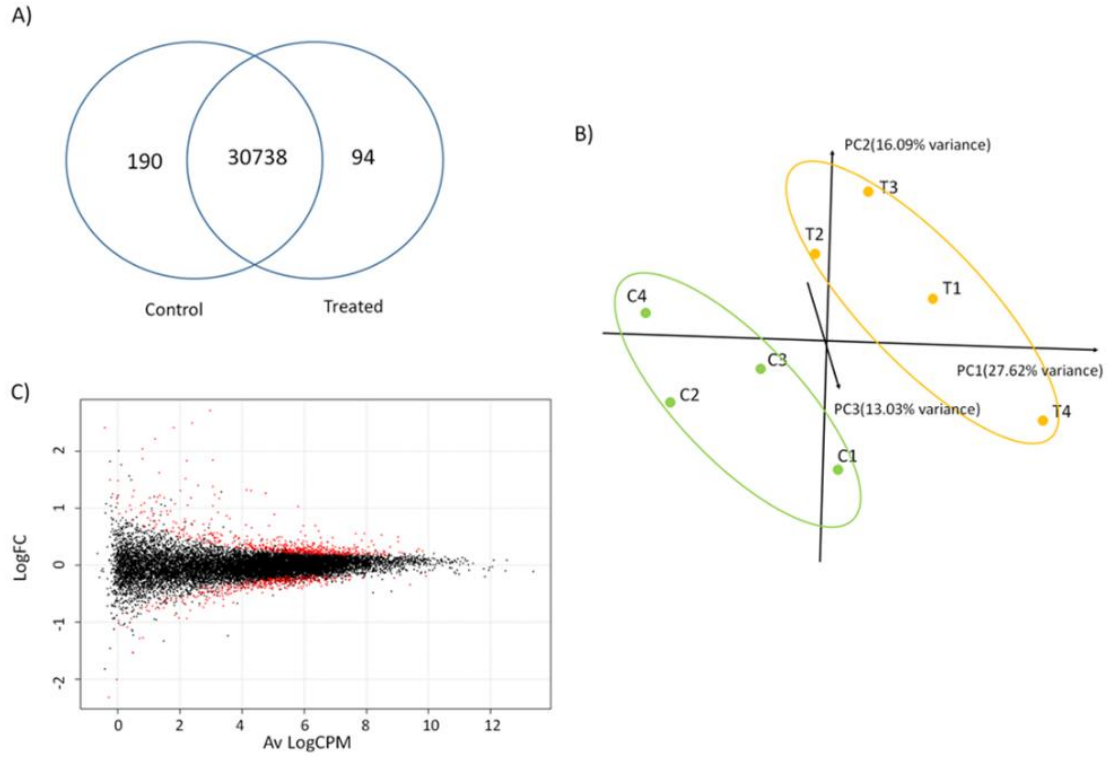


Fig.12. *Transcriptome analysis. A) Shared and unique genes identified in control and treated samples; B) Principal Component Analysis of normalized reads for treated (T1-T4) and control (C1-C4) samples; C) Smear-Plot representing the average logarithmic count per millions (Av LogCPM) gene expression and logaritmic fold change variation (LogFC) between control and treated samples calculated with EdgeR. In red Differentially expressed genes (DEGs).*

As reported in literature, where the functions to KSHV ORF45 are described as the interaction with p90 ribosomal S6 kinase (RSK) and signal-regulated kinase (ERK) complex (Atyeo e Papp 2022), also BoHV-4 ORF45 is present in the same pathway.
In particular, Gene Ontology analysis (GO) showed variation in genes involved in mitotic DNA damage checkpoint and response to DNA damage by p53 and pathway restricted to SMAD phosphorylation.

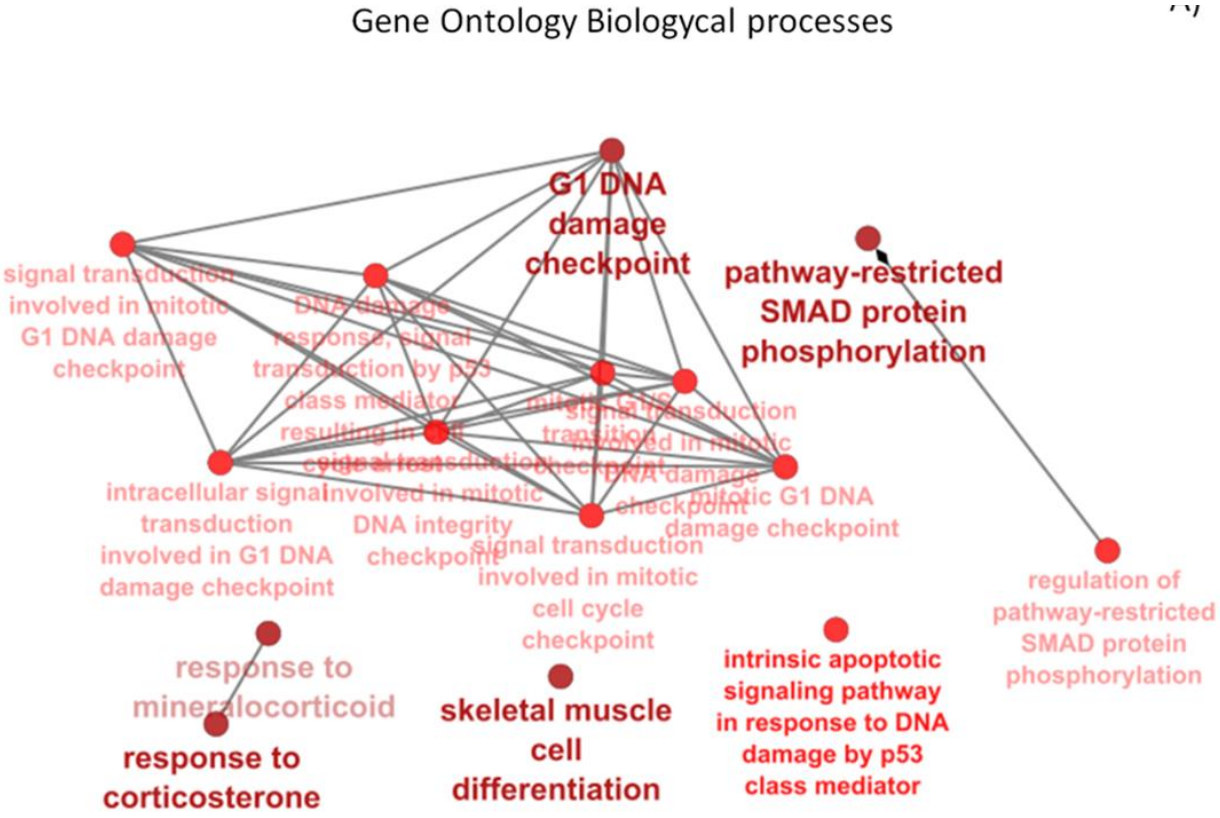


Fig.13.*Biological processes in Gene Ontology (GO) analysis.*

Indeed, reactome pathway database interrogation identified genes related to TP53 regulation of cell cycle, kinase-mediated activation of nuclear transcription and DNA damage senescence response. Finally, biological pathway analysis was performed for high significant DEGs (n=113, FDR < 0.01 and LogFC<|0.5|).

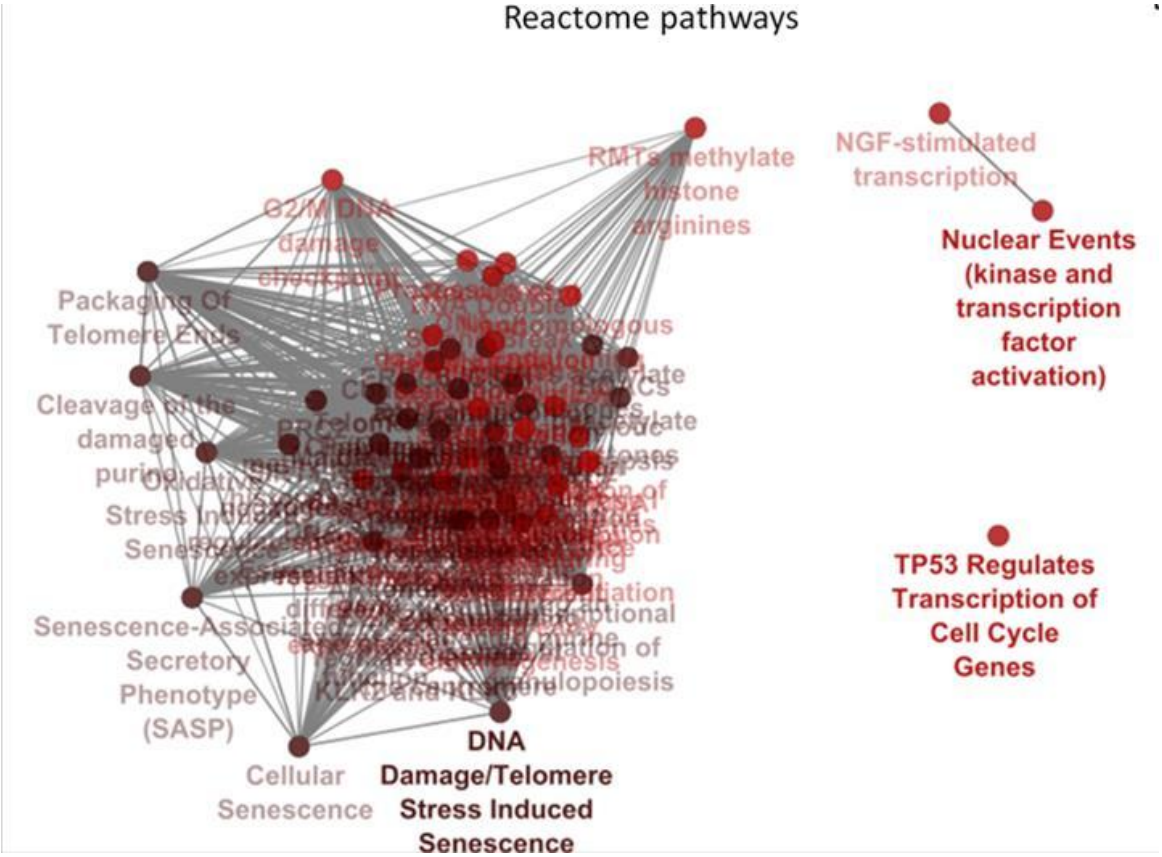


Fig.14.*Reactome pathways for DEGs (FDR<0.01, LogFC|0.5|).*

5.Discussion and Conclusion

Discussion and Conclusion.

The Herpesviridae family is characterized by a well-defined biological structure.
A tidy tegument is described as a fibrous poorly defined layer and it is situated between the capsid and envelope, its thickness could be variable, for example in some classes of virus is smaller in virion accumulating in perinuclear space, compared to those that accumulate in cytoplasmic vacuoles (Falke 1997; Morgan et al. 1959). Proteomic analysis of BoHV-4 mature virions, identified 5 envelope proteins; 9 capsid proteins and 13 tegument proteins (Lété, Palmeira, et al. 2012).
In particular, the tegument proteins ORF45 is one of the most abundant.
Moreover, the criteria utilized to characterize BoHV-4 tegument proteins were based on resistance to protease digestion in absence of detergent and susceptibility to protease digestion following treatment with envelope dissolving detergent.
For absence of experimental data BoHV-4 ORF45 sequence similarity with other previously characterized Rhadinovirus ORF45s were not strong enough to attribute with certainty structural and functional characteristics of an authentic ORF45.
Furthermore, in this work, thanks to the comparison between BoHV-4 ORF45 amino terminal region with that of KHSV ORF45, a structure has been predicted with potential similar functions and interactive pathways to those of KHSV ORF45.
As a results of the overexpression of HA tagged or GFP fused BoHV-4 ORF45 was identified as a phosphoprotein who localizes to the host cell nucleus as it was the case for KSHV, RRV and MHV68.
Thanks to the studies present in this work and in particular, to the generation and analysis of a BoHV-4 ORF45-null mutant ad its para-revertant by BAC recombineering system (Donofrio et al. 2002b), it was demonstrated that the ORF45 null mutant was uncapable of virions production, indicating that the synthetized ORF45 is essential for BoHV-4 lytic replication and such defect could be rescued *in cis* by reintroducing ORF45.
Moreover, the HA tagged ORF45 para-revertant virus allowed to experimentally demonstrate that BoHV-4 ORF45 is associated with the virus particle, corroborating the notion, previously partially demonstrated, that evidenced BoHV-4 ORF45 as a part of the tegument (Lété, Machiels, et al. 2012).
Tegument proteins have several functions, as they are structural viral proteins essentially implicated in viral entry, replication, morphogenesis and egress.
In fact, these proteins are the first viral proteins to be delivered inside the host cells immediately after virus attachment, membrane fusion and uncoating.
Furthermore, as reported in the literature, in many viruses, these proteins can specifically modify, for increasing infection fitness, the host intracellular environment during the establishment of *de novo* infection and modify through a specific interactome the host signaling, epigenetic and transcriptome (Atyeo e Papp 2022; Sathish, Wang, e Yuan 2012).
One of the most investigated proteins is KSHV ORF45 and is one of the virus tegument protein.
Moreover, the most studied function is its interaction with p90 ribosomal S6 kinase (RSK) and signal-regulated kinase (ERK) complex (RSK/ERK).
In particular, since BoHV-4 ORF45 structure was well superimposed (RMSD 0.1 A) to the corresponding KHSV ORF45 orthologue and the key Val and Phe (VP) interacting motif residues are also conserved in the BoHV-4 ORF45.
These residues well fit the hydrophobic pocket located in the N-terminal of AGC-type kinase domain (NTK) of RSK.
Thanks to all these similar functions for BoHV-4 ORF45, one might assume that it has similar functions.

ORF45 performs several functions, such as: sustains activation of the p90 ribosomal S6 kinase (RSK) and signal-regulated kinase (ERK) MAP kinase pathway by binding to the RSK/ERK complex and preventing their dephosphorylation (Alexa et al. 2022; Kuang, Wu, e Zhu 2009) or acting as a SUMO ligase and SUMOylates RSK to promote its kinase activity (Liu et al. 2022).
In particular, one of the downstream targets of phosphorylated RSK/ERK complex is the cellular transcription by direct phosphorylation of transcription factors.
Several studies describe how RSKs regulate several transcription factors, such as: CREB, serum response factor (SRF), NFATc4, NFAT3, the transcription initiation factor TIF1A, ER8, the estrogen receptor-α (ERα) and nuclear factor-κB (NF-κB) (Romeo, Zhang, e Roux 2012).
Thanks to this information, we thought to investigate the impact of BoHV-4 ORF45 on cellular transcriptome because is something that was little studied or not at all for ORF45 belonging to other Gammaherpesvirus.
Moreover, for this study a stringent selection was applied, (DEGs; FDR < 0.01 and LogFC<|0.5|), for biological pathways and identification gene, ontology analysis and reactome pathways database interrogation.
In particular, it was possible to observe a strong transcriptome alteration that was identified in cells expressing BoHV-4 ORF45.
Furthermore, it was surprising demonstrated that the most modified pathways included the cell differentiation, pathways-restricted to SMAD protein phosphorylation and stress response.
Nonetheless, as reported in literature, TP53 regulates transcription of cell cycle genes and DNA damage checkpoint pathways (Miciak e Bunz 2016).
In this work, it was possible to describe that alterations in cells expressing BoHV-4 ORF45 that are consistent with cellular functional modulation in response to RSK/ERK activation.
In the pathway RSK/ERK, RSK binds and enhances the function of the transcriptional co-activators CREB-binding protein (CBP) and p300 (Nakajima et al. 1996; Wang et al. 2003).
Specifically, these proteins are large homologous molecules that facilitate complex formation between different components of the transcriptional machinery.
Several studies, describe various components in this pathway, such as: Fos Proto-Oncogene, AP-1 Transcription Factor Subunit FOS, AP-1 Transcription Factor Subunit JUN, Signal Transducer and Activator of Transcription (STAT), Myogenic Differentiation protein (MyoD), E2F Transcription Factor (E2F), the transcription factors that associate with CBP, p300 and cAMP are response element-binding protein (CREB), NF-κB and steroid receptors including Estrogen receptor alpha (Erα) and un Proto-Oncogene (Romeo et al. 2012).
In our study we have identified the top 200 most significant DEGs, such as: CREB5, JUN, NFKBIL1, FOS, FOSL1.
All these genes showed variation in two group of C and T samples.
These data support that some transcription factors, such as: NF-κB and CREB, SMAD (Freudlsperger et al. 2013), MyoD (cell differentiation) (Noda et al. 2009), E2F (cell cycle progression), CREB/FOS/JUN and TP53 (regulate transcription of cell cycle genes and DNA repair and replication) (Giebler, Lemasson, e Nyborg 2000; Schreiber et al. 1999), CREB/Erα (corticosteroids stress response) (Jing et al. 2016) and G/M checkpoints (Ren et al. 2002); could be directly or indirectly associated to one of the pathways transcriptionally impacted by BoHV-4 ORF45.
In this work, we observed up-regulation of the ETS Variant Transcription Factors, ETV4 and ETV5, in cells expressing BoHV-4 ORF45, likely associated to RSK/ERK activation.
Several studies on cancer, in particular, in cancer cell lines, describe how the expression of endogenous retroviruses envelope proteins in different human cancer cell lines was observed to

induce the transcription of ETV4 and ETV5 which are downstream effectors of the MAPK-ERK1/2 (Lemaître et al. 2017:2).
Furthermore, in cancer cell lines it was reported that αvβ3 integrin signaling may activate ERK pathway, resulting in ETV4 transcription, PD-L1/L2 expression and evasion attacks from the immune system (Ma et al. 2021).

In conclusion, in this PhD work it was described and characterized, for the first time, from the structural and functional point of view, BoHV-4 ORF45.
We have demonstrated numerous biological and functional characteristics, posing the base for further investigation.
In particular, the data obtained indicate that the BoHV-4 ORF45 characteristics can be ascribed to those of KHSV ORF45, already described in various studies.

Appendix.

1) List of primers used in this work:

Primer name	Sequence 5'-3'
ORF45 A sense	CCCGAATTCTGGGACATCTTTTCTTCAAAAAAACTTTGT
ORF45 A antisense	CCCGGTACCCATATGAAAAGTTACAATTGGCCATGGATTGACTGA
ORF45 B sense	CCCCTGCAGACGCGTCCCCTTGAGCTTCTGCACAAACATCGCCAT
ORF45 B antisense	CCCAAGCTTCTACAGTTATCCCCATTTATGAAACAAAAA
ORF45NheI sense	GGGGCTAGCCCACCATGGCGATGTTTGTGCAGAAG
ORF45HA SmaI anti	CCCCCCGGGTTAGGCGTAGTCGGGCACGTCGTAGGGGTAGTCAATC CATGGCCAATTGTAACTTTT
Fusion XhoI sense	CTCAGATCTCGAGCTGCGATGTTTGTGCAGAAGCTCAAGGGGGG

2) List of Differentially Expressed Genes (DEGs) between control and treated samples (FDR<0.05). Gene name (gene_name), gene identification code (gene_id), logarithmic value of fold change expression variation between two groups (logFC) and False Discovery Rate value for significance (FDR) were reported:

gene_name	gene_id	logFC	FDR
H1-2	ENSG00000187837	1,250184	3,69E-48
DHRS2	ENSG00000100867	1,306915	2,12E-46
ENSG00000289609	ENSG00000289609	1,833162	4,63E-46
ETV4	ENSG00000175832	2,39812	6,62E-36
ETV5	ENSG00000244405	2,483144	3,49E-32
LIF	ENSG00000128342	0,878307	1,21E-27
CYP4F2	ENSG00000186115	1,815211	3,48E-25
BTG2	ENSG00000159388	0,592108	4,28E-23
MAFF	ENSG00000185022	1,226604	7,11E-22
ENSG00000260103	ENSG00000260103	1,510454	9,85E-20
ENSG00000288758	ENSG00000288758	2,206582	1,21E-19
CYP4F3	ENSG00000186529	1,396256	1,32E-19
AEN	ENSG00000181026	0,52144	1,75E-17
DDB2	ENSG00000134574	0,683171	6,60E-17
PHLDA3	ENSG00000174307	0,679982	6,60E-17
GDF15	ENSG00000130513	1,033762	2,42E-16
CDKN1A	ENSG00000124762	0,579161	7,89E-16
SPRY4	ENSG00000187678	2,030078	7,89E-16
ACTA2	ENSG00000107796	0,679625	5,38E-15
SNHG17	ENSG00000196756	0,548122	6,55E-15
ENSG00000289688	ENSG00000289688	1,854089	2,30E-13
GPR3	ENSG00000181773	1,169428	7,56E-13
H1-0	ENSG00000189060	0,488185	9,95E-13
ENSG00000287064	ENSG00000287064	1,605897	1,03E-12
INPP5D	ENSG00000168918	0,808769	1,04E-12

SESN2	ENSG00000130766	0,430181	8,52E-12
SNHG15	ENSG00000232956	0,539891	1,61E-11
ATF3	ENSG00000162772	0,421157	2,14E-11
PLK3	ENSG00000173846	0,69972	1,37E-10
FOSL1	ENSG00000175592	0,920662	1,81E-10
SNHG12	ENSG00000197989	0,478966	3,61E-10
ZNF79	ENSG00000196152	0,484098	1,13E-09
H2BC11	ENSG00000124635	1,292668	1,85E-09
PVT1	ENSG00000249859	0,48707	1,94E-09
CREB5	ENSG00000146592	0,471642	2,08E-09
BYSL	ENSG00000112578	0,390713	3,44E-09
H4C14	ENSG00000270882	1,024172	7,35E-09
NECTIN4	ENSG00000143217	1,041537	1,76E-08
NFKBIL1	ENSG00000204498	0,512233	3,66E-08
PRKAB1	ENSG00000111725	0,389225	7,55E-08
EPB41L4A-AS1	ENSG00000224032	0,442367	7,91E-08
ENSG00000273199	ENSG00000273199	1,169854	8,09E-08
SLC3A2	ENSG00000168003	0,403121	1,39E-07
H2AW	ENSG00000181218	0,646834	1,57E-07
GADD45A	ENSG00000116717	0,399395	1,94E-07
PPFIA4	ENSG00000143847	-0,72668	4,26E-07
ETV1	ENSG00000006468	1,070357	5,40E-07
RAD54L	ENSG00000085999	0,376355	7,13E-07
BBC3	ENSG00000105327	0,479891	1,20E-06
TRMT61A	ENSG00000166166	0,376449	1,71E-06
TRIAP1	ENSG00000170855	0,373423	1,74E-06
FDXR	ENSG00000161513	0,439896	2,02E-06
GOLGA8B	ENSG00000215252	-0,36941	2,02E-06
ZNF764	ENSG00000169951	0,365352	2,98E-06
PPP1R15A	ENSG00000087074	0,354025	3,40E-06
DUSP8	ENSG00000184545	0,360598	4,73E-06
PRR14	ENSG00000156858	0,343673	4,93E-06
CHTOP	ENSG00000160679	0,308596	7,20E-06
ORMDL2	ENSG00000123353	0,516127	9,96E-06
USP30	ENSG00000135093	0,419519	1,11E-05
R3HDM2	ENSG00000179912	-0,40962	1,55E-05
ENSG00000288999	ENSG00000288999	0,700538	1,74E-05
ZBTB17	ENSG00000116809	0,417907	1,78E-05
TOE1	ENSG00000132773	0,346591	2,00E-05
IP6K2	ENSG00000068745	0,308371	2,02E-05
H2BC12	ENSG00000197903	0,493824	2,05E-05
ZNF750	ENSG00000141579	1,179761	2,05E-05
ENSG00000285043	ENSG00000285043	0,382207	2,11E-05
LIF-AS2	ENSG00000268812	1,290888	2,45E-05
ENSG00000284602	ENSG00000284602	0,769043	2,45E-05
SORCS2	ENSG00000184985	0,938784	2,48E-05
PPP2R2A	ENSG00000221914	0,289468	2,85E-05

DNAH3	ENSG00000158486	1,485673	3,19E-05
BRF2	ENSG00000104221	0,429926	3,27E-05
ZNF226	ENSG00000167380	0,43574	3,27E-05
FOS	ENSG00000170345	-0,60171	3,65E-05
RRP7BP	ENSG00000182841	0,350479	3,72E-05
DINOL	ENSG00000285244	1,094767	3,94E-05
ENSG00000285901	ENSG00000285901	1,154259	3,94E-05
PAN3-AS1	ENSG00000261485	0,876738	4,03E-05
NBEAL2	ENSG00000160796	-0,37157	4,59E-05
CARS1	ENSG00000110619	0,288033	4,76E-05
ZNF593	ENSG00000142684	0,416715	5,86E-05
DUSP14	ENSG00000276023	0,328549	5,89E-05
NHLH2	ENSG00000177551	0,533624	6,60E-05
CDT1	ENSG00000167513	0,348884	7,29E-05
ZNF576	ENSG00000124444	0,453363	7,52E-05
CLK3	ENSG00000179335	0,312259	8,72E-05
JMJD7-PLA2G4B	ENSG00000168970	-0,54861	8,74E-05
FAM174A	ENSG00000174132	0,470722	0,000106
ENSG00000276131	ENSG00000276131	0,690424	0,000108
H4C11	ENSG00000197238	1,212138	0,00011
PSMC3IP	ENSG00000131470	0,285472	0,00011
SGPP2	ENSG00000163082	-0,57992	0,000113
EPHA2	ENSG00000142627	0,326389	0,000117
NRAV	ENSG00000248008	0,38373	0,000123
ENSG00000273391	ENSG00000273391	0,796033	0,00014
ZNF408	ENSG00000175213	0,346128	0,00015
LUC7L	ENSG00000007392	0,31215	0,000152
ENSG00000260805	ENSG00000260805	0,74051	0,000152
RIBC2	ENSG00000128408	0,55482	0,00017
CCNB1IP1	ENSG00000100814	0,313485	0,000178
SLC38A2	ENSG00000134294	-0,35298	0,000178
ENSG00000268403	ENSG00000268403	0,53784	0,000178
ZNF335	ENSG00000198026	0,296094	0,000182
ENSG00000230021	ENSG00000230021	-1,5406	0,000182
DDX19B	ENSG00000157349	0,320524	0,000184
AKIRIN2	ENSG00000135334	0,284271	0,000185
POMZP3	ENSG00000146707	0,413644	0,000185
RASSF1	ENSG00000068028	0,302186	0,000186
CLK1	ENSG00000013441	0,282911	0,000204
SNHG32	ENSG00000204387	0,31402	0,000208
ENSG00000271918	ENSG00000271918	0,930686	0,000211
MOK	ENSG00000080823	0,356168	0,000226
C17orf97	ENSG00000187624	0,424903	0,000235
JPT1	ENSG00000189159	0,293565	0,000239
TIPIN	ENSG00000075131	0,302327	0,00024
MUL1	ENSG00000090432	0,306582	0,00024
MED26	ENSG00000105085	0,333149	0,000243

DCAF16	ENSG00000163257	-0,31875	0,00027
COL4A4	ENSG00000081052	-1,53039	0,00027
PRCC	ENSG00000143294	0,291034	0,000271
UBALD2	ENSG00000185262	0,410239	0,000271
TBC1D22A-DT	ENSG00000260708	0,538038	0,000312
EMC9	ENSG00000100908	0,362728	0,000321
PDRG1	ENSG00000088356	0,32951	0,000332
NEB	ENSG00000183091	-0,62418	0,000341
BTNL9	ENSG00000165810	-0,45113	0,000342
SLC16A9	ENSG00000165449	-0,38305	0,000366
SLC12A4	ENSG00000124067	0,440601	0,000389
PRTG	ENSG00000166450	-0,66188	0,000389
ILF3-DT	ENSG00000267100	0,358948	0,000389
AKAP8L	ENSG00000011243	0,302225	0,000403
NTMT1	ENSG00000148335	0,338057	0,000403
AURKAP1	ENSG00000213033	1,472529	0,000413
ENSG00000286001	ENSG00000286001	0,700855	0,000413
CNOT3	ENSG00000088038	0,299398	0,000425
LENG1	ENSG00000105617	0,502342	0,000442
BUD31	ENSG00000106245	0,326277	0,000449
PDE4D	ENSG00000113448	-0,5459	0,000449
ENSG00000264112	ENSG00000264112	-0,36732	0,000449
RBM4	ENSG00000173933	0,273635	0,000457
ENSG00000286116	ENSG00000286116	1,078929	0,000457
BANP	ENSG00000172530	0,33255	0,000463
VPS13C	ENSG00000129003	-0,41014	0,000463
WDR46	ENSG00000227057	0,275405	0,000531
DUS3L	ENSG00000141994	0,307239	0,000551
XRCC6P1	ENSG00000237417	1,045026	0,000551
CCDC174	ENSG00000154781	0,338508	0,000557
SOX4	ENSG00000124766	-0,26468	0,000563
CHIC2	ENSG00000109220	0,374727	0,000568
LTB4R2	ENSG00000213906	-0,52568	0,000568
LINC02846	ENSG00000260193	0,72831	0,000591
HROB	ENSG00000125319	0,345609	0,000594
C1orf50	ENSG00000164008	0,445824	0,000613
ENSG00000283515	ENSG00000283515	0,533379	0,000638
CDIP1	ENSG00000089486	0,330282	0,000646
ENSG00000290058	ENSG00000290058	1,195766	0,000724
SNHG20	ENSG00000234912	0,318458	0,000731
GABARAPL1	ENSG00000139112	0,304206	0,000754
NLE1	ENSG00000073536	0,26388	0,000755
H6PD	ENSG00000049239	-0,36578	0,000776
ACADSB	ENSG00000196177	-0,41993	0,000808
GAS5	ENSG00000234741	0,308511	0,000823
INHBE	ENSG00000139269	0,751107	0,000864
ZNHIT2	ENSG00000174276	0,391963	0,000864

RNF25	ENSG00000163481	0,308139	0,000937
THBS4	ENSG00000113296	-0,69314	0,000938
GRIN2C	ENSG00000161509	-0,47088	0,000994
IP6K1	ENSG00000176095	0,241743	0,000994
ZSCAN21	ENSG00000166529	0,335864	0,00101
BPNT2	ENSG00000104331	-0,28762	0,001078
EIF2B2	ENSG00000119718	0,257215	0,001088
CEP89	ENSG00000121289	0,263358	0,001105
WNK4	ENSG00000126562	-0,49572	0,001123
GRWD1	ENSG00000105447	0,270023	0,001155
SERTAD1	ENSG00000197019	0,505826	0,001155
ZKSCAN2-DT	ENSG00000274925	0,476858	0,001165
TRMT1	ENSG00000104907	0,266229	0,001171
CCDC86	ENSG00000110104	0,270535	0,001171
JUN	ENSG00000177606	0,281259	0,001171
ENSG00000283235	ENSG00000283235	1,157329	0,001177
DTL	ENSG00000143476	0,313492	0,001178
PGF	ENSG00000119630	0,475252	0,001213
ATG101	ENSG00000123395	0,33371	0,001213
ENSG00000228793	ENSG00000228793	1,239987	0,001213
RAE1	ENSG00000101146	0,238811	0,001215
ZNF16	ENSG00000170631	0,334769	0,001259
ATM	ENSG00000149311	-0,41835	0,001398
DHX37	ENSG00000150990	0,23646	0,001462
SHANK2	ENSG00000162105	-0,9295	0,001577
RPP38	ENSG00000152464	0,288122	0,001619
HSPA12A	ENSG00000165868	-0,31739	0,001622
MIR34AHG	ENSG00000228526	0,518119	0,001657
LPP	ENSG00000145012	-0,45874	0,001669
IRS4	ENSG00000133124	-0,34527	0,001695
ZNF689	ENSG00000156853	0,259689	0,001873
CAPN10-DT	ENSG00000260942	0,477122	0,001881
SNHG4	ENSG00000281398	-0,29847	0,001959
UBOX5	ENSG00000185019	0,348924	0,002119
ENSG00000269399	ENSG00000269399	0,552349	0,002163
RPS27L	ENSG00000185088	0,397545	0,002257
PIBF1	ENSG00000083535	0,25392	0,002291
AHI1	ENSG00000135541	-0,35505	0,002319
NR1D2	ENSG00000174738	-0,38141	0,002319
ENSG00000228528	ENSG00000228528	1,03971	0,00241
ZNF48	ENSG00000180035	0,260135	0,002541
FIGNL1	ENSG00000132436	-0,31084	0,002558
TP53I3	ENSG00000115129	0,38593	0,002633
NOP16	ENSG00000048162	0,277671	0,002688
PPP1R37	ENSG00000104866	0,316027	0,002688
REXO4	ENSG00000148300	0,248608	0,002762
TSSC4	ENSG00000184281	0,299457	0,002762

SRSF6	ENSG00000124193	0,235398	0,002791
UTP14A	ENSG00000156697	0,233565	0,002838
ANKRD36BP1	ENSG00000214262	-0,87987	0,002843
STK26	ENSG00000134602	-0,31689	0,002869
ERO1A	ENSG00000197930	-0,27111	0,002912
PHF23	ENSG00000040633	0,282053	0,002925
LAMA3	ENSG00000053747	-0,28489	0,002952
CCDC9	ENSG00000105321	0,292043	0,002952
FKBPL	ENSG00000204315	0,391876	0,002952
LINC01311	ENSG00000260924	0,474468	0,002952
PLK2	ENSG00000145632	0,460385	0,003083
UNC5B	ENSG00000107731	-0,24304	0,003117
AARSD1	ENSG00000266967	0,337894	0,003125
XRCC1	ENSG00000073050	0,24732	0,003181
GLS	ENSG00000115419	-0,35587	0,003186
BRIP1	ENSG00000136492	-0,33905	0,003186
BAK1	ENSG00000030110	0,26195	0,003295
TTLL3	ENSG00000214021	-0,26417	0,003371
KLF13	ENSG00000169926	-0,37235	0,003409
LINC02086	ENSG00000244649	0,653262	0,003409
TRIT1	ENSG00000043514	0,238656	0,003436
SLC16A8	ENSG00000100156	-1,28676	0,003452
POFUT2	ENSG00000186866	0,239733	0,003456
PARTICL	ENSG00000286532	0,508414	0,003458
MFSD2A	ENSG00000168389	0,428111	0,003472
SNX10	ENSG00000086300	-0,44387	0,003539
PKD1	ENSG00000008710	-0,30266	0,003655
ZFAS1	ENSG00000177410	0,338366	0,003655
AAR2	ENSG00000131043	0,249691	0,003756
KMT2E-AS1	ENSG00000239569	0,764497	0,003756
NOL12	ENSG00000273899	0,294788	0,003828
PCBP1	ENSG00000169564	0,238713	0,004036
MARS1	ENSG00000166986	0,222869	0,004074
ENSG00000276791	ENSG00000276791	0,509536	0,00415
WRAP53	ENSG00000141499	0,291763	0,004177
LINC02983	ENSG00000234432	0,647912	0,004177
EGR1	ENSG00000120738	-0,40748	0,004267
LTBP2	ENSG00000119681	-0,48372	0,004311
GALNT8	ENSG00000130035	0,959409	0,004372
SMIM14	ENSG00000163683	-0,7476	0,004394
ZNF473	ENSG00000142528	0,26376	0,00448
LDLRAD4	ENSG00000168675	-0,79879	0,00448
RRN3P1	ENSG00000248124	0,301258	0,00448
ENSG00000257176	ENSG00000257176	-0,76582	0,00448
TARBP1	ENSG00000059588	-0,26993	0,004639
DOLPP1	ENSG00000167130	0,271565	0,004639
PVR	ENSG00000073008	0,236047	0,004711

MRM2	ENSG00000122687	0,24005	0,004866
ENSG00000283341	ENSG00000283341	0,600506	0,004866
PDZK1	ENSG00000174827	0,920861	0,004887
ENSG00000280211	ENSG00000280211	0,264836	0,004889
CRNKL1	ENSG00000101343	0,283614	0,00537
PSMC3	ENSG00000165916	0,246153	0,00537
KTI12	ENSG00000198841	0,288973	0,005434
VPS37B	ENSG00000139722	0,229805	0,005575
GTSE1-DT	ENSG00000277232	1,175864	0,005654
ACAP2	ENSG00000114331	-0,43125	0,005698
CCDC59	ENSG00000133773	0,249849	0,00571
C14orf119	ENSG00000179933	0,266061	0,005763
LINC00641	ENSG00000258441	-0,2501	0,005767
ADCY7	ENSG00000121281	-0,67495	0,005804
POP7	ENSG00000172336	0,281556	0,005897
SCAMP1-AS1	ENSG00000245556	0,411795	0,005897
ENSG00000269937	ENSG00000269937	-0,47762	0,005897
SLC25A25	ENSG00000148339	0,240452	0,005952
PAF1	ENSG00000006712	0,216743	0,006041
PMS2P3	ENSG00000127957	0,338344	0,006041
RBMS1	ENSG00000153250	-0,31467	0,006041
ZNF513	ENSG00000163795	0,35204	0,006041
ZNF581	ENSG00000171425	0,289073	0,006041
KCTD12	ENSG00000178695	-0,30136	0,006041
SCFD2	ENSG00000184178	0,294263	0,006041
ZDHHC23	ENSG00000184307	-0,3897	0,006041
SH3BP5-AS1	ENSG00000224660	-0,42356	0,006041
PAPOLA-DT	ENSG00000260806	0,615697	0,006041
ENSG00000289523	ENSG00000289523	0,928028	0,006045
HSD17B7P2	ENSG00000099251	0,507013	0,006064
MRPL22	ENSG00000082515	0,277192	0,006087
BIVM	ENSG00000134897	-0,34568	0,006104
CLASRP	ENSG00000104859	0,236153	0,006181
TIMM9	ENSG00000100575	0,25922	0,006184
ZNF503-AS2	ENSG00000237149	0,322775	0,006229
RCN2	ENSG00000117906	-0,24656	0,006247
DNHD1	ENSG00000179532	-0,46634	0,006346
ENSG00000288983	ENSG00000288983	0,368361	0,006381
DDX39A	ENSG00000123136	0,217014	0,006391
SCAND1	ENSG00000171222	0,373003	0,006411
KANK3	ENSG00000186994	0,555349	0,006411
RETREG1	ENSG00000154153	-0,47007	0,006465
TK1	ENSG00000167900	0,267839	0,006503
KNTC1	ENSG00000184445	-0,23286	0,006647
MRTO4	ENSG00000053372	0,269145	0,006699
BRIX1	ENSG00000113460	0,220797	0,006699
WDR31	ENSG00000148225	-0,4231	0,006713

ZNF205	ENSG00000122386	0,281593	0,006852
TBRG4	ENSG00000136270	0,234303	0,006852
C18orf21	ENSG00000141428	0,252107	0,006852
C19orf54	ENSG00000188493	0,25139	0,006852
CTH	ENSG00000116761	0,249267	0,006859
HMCN1	ENSG00000143341	-0,93631	0,006972
COL5A2	ENSG00000204262	-0,43452	0,006972
SARNP	ENSG00000205323	0,275617	0,006972
CDADC1	ENSG00000102543	0,411554	0,007037
ZNF584	ENSG00000171574	0,299525	0,007105
KATNAL1	ENSG00000102781	-0,4174	0,007147
ACSBG1	ENSG00000103740	0,323067	0,007147
NXT1	ENSG00000132661	0,327959	0,007147
OSER1	ENSG00000132823	0,290424	0,007147
DNAH17-AS1	ENSG00000267432	0,925733	0,007147
CARNMT1	ENSG00000156017	-0,31493	0,007199
PPP2R3C	ENSG00000092020	0,260481	0,007204
THAP7	ENSG00000184436	0,240414	0,00722
EIF1	ENSG00000173812	0,244677	0,007261
SLC44A5	ENSG00000137968	-0,37014	0,007298
LINC01004	ENSG00000228393	0,467756	0,007309
SNRPA	ENSG00000077312	0,264443	0,007396
KLHL26	ENSG00000167487	0,302934	0,007535
SFPQ	ENSG00000116560	0,242328	0,007539
ZBTB7B	ENSG00000160685	0,276851	0,007579
ZNF672	ENSG00000171161	0,256922	0,007579
CCDC137	ENSG00000185298	0,232705	0,007579
TIGD1	ENSG00000221944	0,287035	0,007662
BRPF1	ENSG00000156983	0,222282	0,007685
ENSG00000289043	ENSG00000289043	0,542721	0,007685
TMSB15B	ENSG00000158427	1,362735	0,007715
SARS1	ENSG00000031698	0,211112	0,00787
H2AC19	ENSG00000288859	2,703408	0,00787
GALNT7	ENSG00000109586	-0,36162	0,007969
EIF1B	ENSG00000114784	0,236189	0,007969
IQCC	ENSG00000160051	0,240718	0,007984
SLC25A19	ENSG00000125454	0,265623	0,007996
NIFK	ENSG00000155438	0,236206	0,007996
COA6	ENSG00000168275	0,260567	0,007996
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H2BU1	ENSG00000196890	0,858979	0,008229
SUCO	ENSG00000094975	-0,32931	0,008289
RABGEF1P1	ENSG00000229180	0,278414	0,008358
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GOLGA6B	ENSG00000215186	-1,28013	0,008709
TOM1	ENSG00000100284	0,280659	0,008723
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CNPY2-AS1	ENSG00000257303	0,503043	0,008723
CDC42EP1	ENSG00000128283	0,265209	0,008772
ZNF337	ENSG00000130684	0,246998	0,008772
RUFY1	ENSG00000176783	0,209687	0,008772
MZT1	ENSG00000204899	-0,2477	0,008772
TCF4	ENSG00000196628	-0,40368	0,008861
NME1-NME2	ENSG00000011052	0,377792	0,008912
H2BC21	ENSG00000184678	0,461292	0,008912
RBPJ	ENSG00000168214	-0,24009	0,009089
TMEM11	ENSG00000178307	0,283077	0,009089
ELMOD2	ENSG00000179387	-0,27242	0,009089
ARPIN-AP3S2	ENSG00000250021	0,256742	0,009089
CFAP43	ENSG00000197748	-0,5189	0,009288
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VWCE	ENSG00000167992	-0,48951	0,009342
DNAH17	ENSG00000187775	0,450284	0,009567
KIAA1109	ENSG00000138688	-0,37014	0,009572
CD109	ENSG00000156535	-0,51379	0,009572
RAP1GAP2	ENSG00000132359	-0,31969	0,009577
PSTK	ENSG00000179988	0,352835	0,009667
BRCA2	ENSG00000139618	-0,28735	0,00986
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GEMIN8	ENSG00000046647	0,313782	0,01028
DNM1	ENSG00000106976	-0,28402	0,01028
RSKR	ENSG00000167524	-0,45679	0,01028
FAM156B	ENSG00000179304	-0,37894	0,010312
ANK2	ENSG00000145362	-0,30404	0,010336
CDK6	ENSG00000105810	-0,31361	0,010378
NXPE3	ENSG00000144815	-0,35382	0,010378
PNO1	ENSG00000115946	0,207225	0,01038
NFYC	ENSG00000066136	0,22609	0,010427
LINC01341	ENSG00000227953	-0,75835	0,010427
LRRC8B	ENSG00000197147	-0,33583	0,010456
EMB	ENSG00000170571	-0,31373	0,010593
SGTA	ENSG00000104969	0,226566	0,010625
PLOD2	ENSG00000152952	-0,26531	0,010625
INTS5	ENSG00000185085	0,237023	0,010625
TMEM123	ENSG00000152558	-0,27781	0,010655
MRPL49	ENSG00000149792	0,223349	0,010917
CAP2	ENSG00000112186	-0,38939	0,011011
SNHG30	ENSG00000267321	0,360253	0,011168
KIF14	ENSG00000118193	-0,31106	0,011234
TRPM7	ENSG00000092439	-0,30117	0,011234
MBNL2	ENSG00000139793	-0,51181	0,011314

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C19orf48	ENSG00000167747	0,238463	0,011608
ATP8A1	ENSG00000124406	-0,49521	0,011611
TMED7	ENSG00000134970	-0,32721	0,011641
DYNLT2	ENSG00000184786	0,388377	0,011641
ZNF117	ENSG00000152926	-0,53772	0,011769
FAM157C	ENSG00000260528	-0,59433	0,011825
EIF3D	ENSG00000100353	0,221323	0,012068
TAS2R5	ENSG00000127366	-0,98121	0,012068
EDA2R	ENSG00000131080	0,580922	0,012068
ALAS1	ENSG00000023330	0,201064	0,012281
SUPT7L	ENSG00000119760	0,21624	0,012281
SNHG10	ENSG00000247092	0,315995	0,012281
AVPR1A	ENSG00000166148	-0,81343	0,012314
GEMIN7	ENSG00000142252	0,270977	0,012388
BLOC1S2	ENSG00000196072	0,240015	0,012399
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TFPT	ENSG00000105619	0,343645	0,012514
BCL7B	ENSG00000106635	0,236496	0,012514
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UIMC1	ENSG00000087206	0,212248	0,012543
FMN2	ENSG00000155816	-0,25667	0,012653
GOT1	ENSG00000120053	0,245159	0,012745
SLAIN2	ENSG00000109171	-0,40803	0,012783
NIPBL-DT	ENSG00000285967	0,274428	0,012939
RIOK1	ENSG00000124784	0,197825	0,013009
CGB7	ENSG00000196337	0,774539	0,013216
DPF2	ENSG00000133884	0,200583	0,013292
C2CD3	ENSG00000168014	0,22869	0,013627
SOX6	ENSG00000110693	-0,6633	0,013653
SAR1B	ENSG00000152700	0,243601	0,013718
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ZNF296	ENSG00000170684	0,320651	0,013775
PEA15	ENSG00000162734	0,204473	0,013793
IPO5P1	ENSG00000269837	0,802385	0,013793
TMEM115	ENSG00000126062	0,232926	0,013842
MAVS	ENSG00000088888	-0,22334	0,013938
NUP58	ENSG00000139496	-0,23306	0,01397
KLF10	ENSG00000155090	-0,26041	0,01397
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OVGP1	ENSG00000085465	0,251434	0,014143
RPP38-DT	ENSG00000176236	0,615862	0,01423
CDCA5	ENSG00000146670	0,199035	0,014331
VLDLR	ENSG00000147852	-0,4826	0,014331
ZNF574	ENSG00000105732	0,245679	0,01444
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SRSF3	ENSG00000112081	0,206245	0,014511
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BEND4	ENSG00000188848	-0,37792	0,014511
DENND1B	ENSG00000213047	-0,41083	0,014511
GTF2F1	ENSG00000125651	0,207439	0,014536
ZNF622	ENSG00000173545	0,263	0,014536
ALKBH1	ENSG00000100601	0,238132	0,014631
PLEKHG4B	ENSG00000153404	-0,6649	0,014845
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PHF5A	ENSG00000100410	0,231629	0,015017
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N6AMT1	ENSG00000156239	0,246672	0,015043
HLF	ENSG00000108924	-0,47537	0,015046
YY1AP1	ENSG00000163374	0,194502	0,015199
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RIMS3	ENSG00000117016	-0,37648	0,015338
NCKAP1	ENSG00000061676	-0,21083	0,015419
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ITGA2	ENSG00000164171	-0,48906	0,015419
FGD6	ENSG00000180263	-0,48966	0,015419
ZNF787	ENSG00000142409	0,267247	0,015441
ABCB10	ENSG00000135776	-0,24869	0,015517
PSMG3	ENSG00000157778	0,270185	0,015517
PBDC1	ENSG00000102390	0,32257	0,015599
ZNF628	ENSG00000197483	0,444885	0,015601
CDK9	ENSG00000136807	0,261949	0,015633
SAC3D1	ENSG00000168061	0,305843	0,015633
AKAP5	ENSG00000179841	-0,46137	0,015633
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MDM1	ENSG00000111554	-0,27853	0,015723
THAP3	ENSG00000041988	0,281487	0,015727
CFAP97	ENSG00000164323	-0,35184	0,015806
OIP5-AS1	ENSG00000247556	-0,23337	0,015898
ECHDC2	ENSG00000121310	-0,38328	0,015947
HRAS	ENSG00000174775	0,276235	0,015963
SEMA3A	ENSG00000075213	-0,57237	0,016167
GPATCH4	ENSG00000160818	0,220279	0,016167
AGAP5	ENSG00000172650	-0,32047	0,016167
LMAN2L	ENSG00000114988	0,232699	0,016211
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TRPS1	ENSG00000104447	-0,40587	0,016258
DRG1	ENSG00000185721	0,219661	0,016258
C19orf47	ENSG00000160392	0,294203	0,016425
OAZ2	ENSG00000180304	0,215402	0,016425
OXLD1	ENSG00000204237	0,290898	0,01657

AUP1	ENSG00000115307	0,212277	0,016617
PCYOX1	ENSG00000116005	-0,26411	0,016617
GRHL3	ENSG00000158055	0,554822	0,016702
CCT6P1	ENSG00000228409	0,220377	0,016926
LRP1	ENSG00000123384	-0,26704	0,017014
ZNF385A	ENSG00000161642	0,338959	0,017122
ANGEL2	ENSG00000174606	-0,23881	0,017163
PRR7	ENSG00000131188	0,314896	0,017262
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MAGEF1	ENSG00000177383	0,205342	0,017266
WASHC4	ENSG00000136051	-0,31488	0,017301
ZNF282	ENSG00000170265	0,203478	0,017319
ZRANB2-DT	ENSG00000229956	-1,05546	0,017482
ZNF768	ENSG00000169957	0,224509	0,017526
ZDHHC21	ENSG00000175893	-0,42946	0,017526
METTL2A	ENSG00000087995	0,217191	0,017691
RPS19BP1	ENSG00000187051	0,251508	0,017775
YARS1	ENSG00000134684	0,200849	0,017896
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SNAP47	ENSG00000143740	0,218388	0,018053
GBF1	ENSG00000107862	0,190138	0,018119
MANEA	ENSG00000172469	-0,39899	0,018119
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GVQW3	ENSG00000179240	-0,32967	0,018119
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MASP2	ENSG00000009724	-0,56729	0,018124
ARSB	ENSG00000113273	-0,3361	0,018124
DMD	ENSG00000198947	-0,35775	0,018257
CYHR1	ENSG00000187954	0,218027	0,018444
TMEM47	ENSG00000147027	-0,36747	0,018575
FREM2	ENSG00000150893	-0,49473	0,018575
ADAM9	ENSG00000168615	-0,2944	0,018575
TMEM79	ENSG00000163472	0,289167	0,018642
MED19	ENSG00000156603	0,317699	0,018761
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FUZ	ENSG00000010361	0,240079	0,019126
ACER3	ENSG00000078124	-0,29413	0,019126
SDHAF1	ENSG00000205138	0,328404	0,019126
FRG1	ENSG00000109536	0,26173	0,019247
CTNNBL1	ENSG00000132792	0,279781	0,019249
ADD3	ENSG00000148700	-0,30775	0,01942
MRPL48	ENSG00000175581	0,259823	0,01942
ID4	ENSG00000172201	-0,20802	0,019433
SPPL2B	ENSG00000005206	-0,20667	0,019529
INTS2	ENSG00000108506	-0,27511	0,019529
FAM217B	ENSG00000196227	-0,33403	0,019569

NDUFC2-KCTD14	ENSG00000259112	0,937007	0,019689
SGF29	ENSG00000176476	0,287366	0,019834
BAG1	ENSG00000107262	0,242454	0,019886
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PRR11	ENSG00000068489	-0,23298	0,019892
MLLT10	ENSG00000078403	-0,244	0,019892
MCOLN3	ENSG00000055732	-0,32895	0,019937
TARS1	ENSG00000113407	0,199996	0,019978
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BCDIN3D	ENSG00000186666	0,284368	0,019978
RRP8	ENSG00000132275	0,220465	0,020033
SPATA2	ENSG00000158480	0,256672	0,020132
CHPF2	ENSG00000033100	0,202674	0,020438
ANK3	ENSG00000151150	-0,26617	0,020439
ZNF256	ENSG00000152454	0,319781	0,020439
NSMCE2	ENSG00000156831	0,270236	0,020439
FCHO2	ENSG00000157107	-0,39976	0,020439
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ICE2	ENSG00000128915	-0,22614	0,020899
MAP1S	ENSG00000130479	0,215372	0,020899
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ZDBF2	ENSG00000204186	-0,40076	0,020953
SLC7A2	ENSG00000003989	-0,33046	0,020972
DUSP12	ENSG00000081721	0,249084	0,020972
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RP9	ENSG00000164610	0,267877	0,020972
GPRASP1	ENSG00000198932	-0,6183	0,020972
SCNM1	ENSG00000163156	0,258478	0,021206
DYNLT3	ENSG00000165169	-0,25871	0,021267
TRMO	ENSG00000136932	0,289473	0,021275
MKLN1	ENSG00000128585	0,24717	0,021339
SAMD5	ENSG00000203727	-0,48768	0,021339
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TRAM1	ENSG00000067167	-0,2156	0,021419
FBXL12	ENSG00000127452	0,23865	0,021419
HOXD10	ENSG00000128710	-0,23387	0,021419
RRS1	ENSG00000179041	0,203689	0,021419
CSF1	ENSG00000184371	0,295437	0,021505
GP1BA	ENSG00000185245	-0,61839	0,021505
COA4	ENSG00000181924	0,210927	0,021512
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CBWD5	ENSG00000147996	0,229466	0,021626
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SMAD9	ENSG00000120693	-0,30697	0,022149
ITPA	ENSG00000125877	0,250609	0,022149
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LYPLA1	ENSG00000120992	-0,28238	0,022235
ATXN7L2	ENSG00000162650	0,290775	0,022235
ZDHHC20	ENSG00000180776	-0,27782	0,022235
MAD1L1	ENSG00000002822	0,28566	0,022269
PML	ENSG00000140464	0,245431	0,022269
CAMK1D	ENSG00000183049	-0,34858	0,022272
STXBP3	ENSG00000116266	-0,25967	0,022416
FAM161A	ENSG00000170264	-0,38435	0,022416
RNF213	ENSG00000173821	-0,24984	0,022416
ADPRM	ENSG00000170222	0,351184	0,022588
CHD1-DT	ENSG00000248489	0,59656	0,022588
PM20D2	ENSG00000146281	-0,25441	0,022704
DYM	ENSG00000141627	0,251257	0,022712
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PMS1	ENSG00000064933	-0,23091	0,02313
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ENSG00000289021	ENSG00000289021	0,498434	0,023185
ARIH2OS	ENSG00000221883	0,499543	0,023191
ESS2	ENSG00000100056	0,232394	0,023193
HOOK3	ENSG00000168172	-0,42809	0,023193
LYSMD3	ENSG00000176018	-0,3341	0,023193
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MID1	ENSG00000101871	-0,29345	0,023327
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RRP12	ENSG00000052749	0,192383	0,023374
TACO1	ENSG00000136463	0,224636	0,023374
DOK3	ENSG00000146094	-0,3992	0,023374
RPS2P55	ENSG00000216866	0,212283	0,023374
SHMT2	ENSG00000182199	0,195147	0,023425
SORBS1	ENSG00000095637	0,370878	0,023516
RHBDD2	ENSG00000005486	0,230316	0,023855
DLC1	ENSG00000164741	-0,26925	0,023855
RABGGTA	ENSG00000100949	0,28091	0,024032
TBX15	ENSG00000092607	0,618312	0,024047
BORCS5	ENSG00000165714	0,414675	0,024047
NEDD4	ENSG00000069869	-0,34582	0,024164
FEN1	ENSG00000168496	0,195197	0,024235
CSTF1	ENSG00000101138	0,211171	0,024312
MRPL27	ENSG00000108826	0,235842	0,024312
TMEM234	ENSG00000160055	0,283736	0,024312
SDHAP1	ENSG00000185485	0,270886	0,024312
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SPRED2	ENSG00000198369	0,355277	0,024519
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PFDN1	ENSG00000113068	0,208013	0,02476

C16orf91	ENSG00000174109	0,299699	0,024963
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SEPTIN11	ENSG00000138758	-0,22942	0,024988
SPA17	ENSG00000064199	0,367728	0,025047
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ATP6V1D	ENSG00000100554	0,2249	0,025206
SCOC	ENSG00000153130	-0,23311	0,02524
EMX2OS	ENSG00000229847	0,413571	0,02524
RPP30	ENSG00000148688	0,202834	0,025253
CBX7	ENSG00000100307	-0,40148	0,025369
PLCE1	ENSG00000138193	-0,28403	0,025442
ZNRD2	ENSG00000173465	0,256953	0,025482
PLEKHA1	ENSG00000107679	-0,29405	0,025655
HGSNAT	ENSG00000165102	-0,24103	0,025655
ZNF521	ENSG00000198795	-0,37176	0,025655
STAM-DT	ENSG00000260589	0,381018	0,025729
LMBR1	ENSG00000105983	-0,27349	0,025754
SAAL1	ENSG00000166788	0,204492	0,025754
FAM126A	ENSG00000122591	-0,28342	0,025785
RPF2	ENSG00000197498	0,202916	0,025785
YBX3	ENSG00000060138	0,198243	0,02583
ZNF165	ENSG00000197279	0,359153	0,025928
SBF2	ENSG00000133812	0,34525	0,025956
FHIP2A	ENSG00000151553	-0,28846	0,026176
TMED8	ENSG00000100580	-0,25617	0,026217
ZNF324	ENSG00000083812	0,239413	0,026275
USP39	ENSG00000168883	0,183042	0,026363
CPOX	ENSG00000080819	-0,24022	0,026377
LLPH	ENSG00000139233	0,232376	0,0266
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PTPRS	ENSG00000105426	-0,27668	0,026707
CACNA2D1	ENSG00000153956	-0,43954	0,026707
ZMAT3	ENSG00000172667	0,293641	0,026707
CHDH	ENSG00000016391	-0,25681	0,02714
SRSF4	ENSG00000116350	0,193915	0,027228
PPM1D	ENSG00000170836	0,202784	0,027228
ARL10	ENSG00000175414	-0,22653	0,027228
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UNG	ENSG00000076248	0,179863	0,027334
SYT16	ENSG00000139973	-0,66071	0,027334
MRPL36	ENSG00000171421	0,28263	0,027334
SLC39A10	ENSG00000196950	-0,24652	0,02767
KRCC1	ENSG00000172086	-0,29055	0,027869
PTER	ENSG00000165983	-0,24116	0,028001
PIH1D1	ENSG00000104872	0,251823	0,028187
INTS12	ENSG00000138785	0,202133	0,028236
RNF207	ENSG00000158286	-0,48621	0,028236

FAS	ENSG00000026103	0,279896	0,028395
DPY19L4	ENSG00000156162	-0,40907	0,028412
WDR13	ENSG00000101940	0,328715	0,028507
ATP9A	ENSG00000054793	-0,26689	0,02862
AGFG1	ENSG00000173744	0,218558	0,028622
MRTFA	ENSG00000196588	0,223207	0,028638
ITGA6	ENSG00000091409	-0,34429	0,028718
C5	ENSG00000106804	-0,5499	0,028718
MIB1	ENSG00000101752	-0,34071	0,028872
TEX15	ENSG00000133863	-0,40893	0,028872
DDX56	ENSG00000136271	0,199202	0,028872
MED8	ENSG00000159479	0,211074	0,028872
AP2A2	ENSG00000183020	0,239851	0,028872
POU2F1-DT	ENSG00000272205	0,578472	0,028888
VPS25	ENSG00000131475	0,202577	0,029089
THG1L	ENSG00000113272	0,257226	0,029279
PRRC1	ENSG00000164244	-0,25013	0,029566
AIMP2	ENSG00000106305	0,192203	0,029743
NUP210	ENSG00000132182	-0,17882	0,029743
EEFSEC	ENSG00000132394	0,24346	0,029743
ACTR2	ENSG00000138071	-0,23264	0,029743
BRAF	ENSG00000157764	0,285688	0,029743
KREMEN1	ENSG00000183762	-0,22655	0,029743
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STX1B	ENSG00000099365	-0,67687	0,029873
ZFP30	ENSG00000120784	-0,2998	0,029873
GPN2	ENSG00000142751	0,218264	0,029873
FBXO41	ENSG00000163013	-0,30325	0,029873
ITGA1	ENSG00000213949	-0,49256	0,029873
AKAP6	ENSG00000151320	-0,65287	0,029926
BNIP1	ENSG00000113734	0,239435	0,030177
TMEM65	ENSG00000164983	-0,24859	0,030182
LMAN1	ENSG00000074695	-0,22832	0,030242
CCNB3	ENSG00000147082	-0,61245	0,030669
SENP3-EIF4A1	ENSG00000277957	0,337952	0,030838
IL6ST	ENSG00000134352	-0,31701	0,030937
PAQR3	ENSG00000163291	-0,25824	0,030973
SLFN5	ENSG00000166750	-0,60072	0,030973
PUF60	ENSG00000179950	0,195984	0,031005
WASH5P	ENSG00000282458	-0,32368	0,031192
NEBL	ENSG00000078114	-0,3947	0,031283
PI4KB	ENSG00000143393	0,181461	0,031285
MOAP1	ENSG00000165943	0,207959	0,031285
MIDEAS	ENSG00000156030	-0,29677	0,031577
UTP3	ENSG00000132467	0,191932	0,031598
MKX	ENSG00000150051	-0,41742	0,031598
WDR73	ENSG00000177082	0,18515	0,031598

MUC19	ENSG00000205592	0,85523	0,031598
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PI4K2B	ENSG00000038210	-0,2796	0,031982
TCFL5	ENSG00000101190	-0,26329	0,032163
PASK	ENSG00000115687	-0,2004	0,032163
DCTN5	ENSG00000166847	0,205654	0,032163
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SEL1L	ENSG00000071537	-0,33665	0,032167
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NOSIP	ENSG00000142546	0,258804	0,032167
PCYOX1L	ENSG00000145882	-0,2737	0,032167
GOLGA8R	ENSG00000186399	-0,69027	0,032167
ZNF444	ENSG00000167685	0,271891	0,032168
EFCAB10	ENSG00000185055	0,50222	0,032562
ILKAP	ENSG00000132323	0,199955	0,032677
MRPS18A	ENSG00000096080	0,211103	0,032792
RNF170	ENSG00000120925	-0,34702	0,032861
SDC3	ENSG00000162512	-0,20833	0,032861
VARS1	ENSG00000204394	0,196714	0,032861
RBMS2	ENSG00000076067	-0,27463	0,032936
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IRAK3	ENSG00000090376	-0,75995	0,033082
ZMIZ1	ENSG00000108175	-0,27646	0,033082
CDK2AP2	ENSG00000167797	0,298134	0,033185
RBM34	ENSG00000188739	0,20858	0,033185
SOWAHC	ENSG00000198142	-0,41945	0,033185
EPB41L2	ENSG00000079819	-0,18158	0,033194
BAIAP2	ENSG00000175866	0,253835	0,033294
PTPN13	ENSG00000163629	-0,2824	0,033307
ERLIN2	ENSG00000147475	-0,23561	0,033395
NIPAL2	ENSG00000104361	-0,43118	0,033405
SGPP1	ENSG00000126821	-0,32784	0,033515
SNHG9	ENSG00000255198	0,393904	0,033515
WDR70	ENSG00000082068	0,366005	0,033637
C12orf29	ENSG00000133641	0,236367	0,033637
ADAMTS1	ENSG00000154734	-0,17877	0,033637
APOOL	ENSG00000155008	-0,24828	0,033637
SLC35A4	ENSG00000176087	0,178017	0,033637
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PCMTD2	ENSG00000203880	-0,32197	0,033637
RSRC2	ENSG00000111011	0,175241	0,033972
DCAF15	ENSG00000132017	0,230412	0,033972
EXOSC9	ENSG00000123737	0,212344	0,034128
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HERC1	ENSG00000103657	-0,37428	0,034557
EXOSC7	ENSG00000075914	0,220917	0,03461
CAMTA2	ENSG00000108509	0,210954	0,03461

STAT2	ENSG00000170581	-0,25621	0,03461
DOHH	ENSG00000129932	0,266493	0,034711
HSF2BP	ENSG00000160207	0,47442	0,03477
SNORD3A	ENSG00000263934	0,67301	0,03477
PER1	ENSG00000179094	0,260269	0,03487
IRF2BP1	ENSG00000170604	0,210875	0,034906
ASB6	ENSG00000148331	0,194088	0,035078
ARMC6	ENSG00000105676	0,180934	0,035104
ATP13A1	ENSG00000105726	0,186915	0,035183
MRPS14	ENSG00000120333	0,211165	0,035228
CAMK4	ENSG00000152495	0,464719	0,035256
IMP4	ENSG00000136718	0,179837	0,035359
DAAM1	ENSG00000100592	-0,35863	0,035428
PDE3B	ENSG00000152270	-0,33873	0,035874
SLC30A5	ENSG00000145740	-0,24616	0,03589
DNAJC30	ENSG00000176410	0,270913	0,035965
KAT5	ENSG00000172977	0,232643	0,036108
ASPH	ENSG00000198363	-0,23979	0,036108
DNAJB12	ENSG00000148719	0,183324	0,036225
POLE3	ENSG00000148229	0,173735	0,036343
NR3C1	ENSG00000113580	-0,29333	0,036402
IVD	ENSG00000128928	-0,18949	0,036402
EFR3A	ENSG00000132294	-0,33502	0,036402
SF3B4	ENSG00000143368	0,199831	0,036402
DNTTIP1	ENSG00000101457	0,213437	0,036721
ARHGAP28	ENSG00000088756	-0,58236	0,036827
DMXL2	ENSG00000104093	-0,2687	0,036827
CNTNAP3	ENSG00000106714	-0,35719	0,036827
IMMP1L	ENSG00000148950	0,298326	0,036827
AP1S2	ENSG00000182287	-0,25349	0,036827
DUXAP8	ENSG00000206195	-0,30597	0,036827
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SMIM15-AS1	ENSG00000251279	0,656006	0,037098
IRF1	ENSG00000125347	0,536292	0,037166
NAALAD2	ENSG00000077616	-0,62713	0,037216
CYTH2	ENSG00000105443	0,202159	0,037216
LPGAT1	ENSG00000123684	-0,23655	0,037216
ST6GALNAC6	ENSG00000160408	0,208256	0,037216
H4C15	ENSG00000270276	1,295085	0,037216
CBX5	ENSG00000094916	-0,21221	0,037293
KDM5B	ENSG00000117139	0,189232	0,037315
COL24A1	ENSG00000171502	-0,77047	0,037315
ZNF222	ENSG00000159885	0,384969	0,037438
C11orf24	ENSG00000171067	0,219628	0,037438
ITSN1	ENSG00000205726	-0,21919	0,037506
PES1	ENSG00000100029	0,213265	0,03751
RPA2	ENSG00000117748	0,180532	0,037637

FBXL17	ENSG00000145743	-0,45055	0,037674
GBX2	ENSG00000168505	0,401731	0,037674
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NBPF15	ENSG00000266338	-0,18478	0,037827
MECOM	ENSG00000085276	-0,29087	0,03785
RTKN2	ENSG00000182010	-0,27442	0,03785
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PAXBP1-AS1	ENSG00000238197	-0,3463	0,037872
EGLN1	ENSG00000135766	-0,18342	0,037979
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LONRF2	ENSG00000170500	-0,31116	0,03801
SPTSSA	ENSG00000165389	-0,26829	0,038083
PAQR8	ENSG00000170915	-0,32941	0,038083
DDX10	ENSG00000178105	0,204064	0,038224
MRPS2	ENSG00000122140	0,201885	0,038276
TOX4	ENSG00000092203	0,182268	0,038346
TUB	ENSG00000166402	-0,25031	0,038527
C16orf95-DT	ENSG00000270006	0,775983	0,038527
PHC3	ENSG00000173889	0,288181	0,038606
NUTM2A-AS1	ENSG00000223482	0,216594	0,038606
CEBPZ	ENSG00000115816	0,208527	0,03877
GPD2	ENSG00000115159	-0,34056	0,039051
RNF32-AS1	ENSG00000224903	-0,52486	0,039051
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ISL2	ENSG00000159556	0,232648	0,039423
PARP12	ENSG00000059378	-0,72507	0,039463
BCS1L	ENSG00000074582	0,215256	0,039696
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TENM1	ENSG00000009694	-0,46694	0,039704
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EIF2B5	ENSG00000145191	0,198205	0,040227
NOPCHAP1	ENSG00000151131	0,281521	0,040237
CHML	ENSG00000203668	-0,29156	0,040258
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UTP4	ENSG00000141076	0,166707	0,040435
LSM1	ENSG00000175324	0,216168	0,040435
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DXO	ENSG00000204348	0,216798	0,040544
DPY19L3	ENSG00000178904	-0,28674	0,040764
PAG1	ENSG00000076641	-0,39724	0,040845
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ZBTB41	ENSG00000177888	-0,36403	0,040909
CHASERR	ENSG00000272888	0,208195	0,040978
ADAMTS10	ENSG00000142303	-0,40082	0,041165
FAM102B	ENSG00000162636	-0,29377	0,041165
RPGRIP1	ENSG00000092200	0,735656	0,041214
HM13	ENSG00000101294	0,194722	0,04125

SMG9	ENSG00000105771	0,219347	0,041602
UBAP1L	ENSG00000246922	-0,55235	0,041643
THUMPD2	ENSG00000138050	0,174561	0,041683
COA6-AS1	ENSG00000231663	0,463966	0,041683
BCAT1	ENSG00000060982	-0,22543	0,041688
LMNA	ENSG00000160789	0,204091	0,041688
NUS1	ENSG00000153989	-0,19708	0,041841
ZMYND8	ENSG00000101040	-0,21108	0,042016
USP24	ENSG00000162402	-0,22435	0,042016
CMTR2	ENSG00000180917	-0,34505	0,042016
SMN2	ENSG00000205571	0,188293	0,042016
ENSG00000277991	ENSG00000277991	0,506068	0,042143
SMAD5	ENSG00000113658	-0,23357	0,042388
COX17	ENSG00000138495	0,238722	0,042388
PPAN	ENSG00000130810	0,191786	0,042481
SURF6	ENSG00000148296	0,201918	0,042565
CFAP251	ENSG00000158023	0,66975	0,043143
SOGA1	ENSG00000149639	-0,26805	0,043482
SPDYE2	ENSG00000205238	-0,54768	0,043493
ETAA1	ENSG00000143971	-0,23925	0,044063
KIAA0232	ENSG00000170871	-0,23057	0,044063
POLR1D	ENSG00000186184	0,174663	0,044063
KCTD3	ENSG00000136636	-0,20015	0,044144
POU3F2	ENSG00000184486	-0,24125	0,044144
SAE1	ENSG00000142230	0,175195	0,044201
OTUD6B	ENSG00000155100	-0,33439	0,044388
MLST8	ENSG00000167965	0,188954	0,044388
TRMT11	ENSG00000066651	0,203798	0,044693
GNB4	ENSG00000114450	-0,29268	0,044693
SH3BGRL	ENSG00000131171	-0,22961	0,044693
SP2	ENSG00000167182	0,196199	0,044693
MECP2	ENSG00000169057	0,179735	0,044693
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NAB1	ENSG00000138386	-0,3463	0,044876
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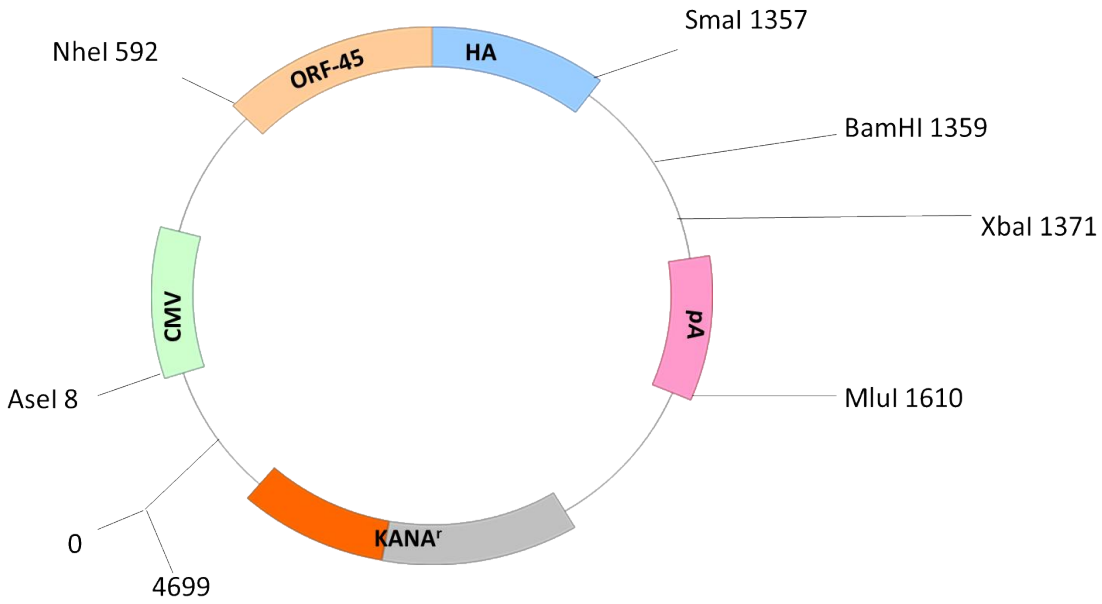
MAP1B	ENSG00000131711	-0,35219	0,045085
LANCL1	ENSG00000115365	-0,26253	0,045089
HSPA1B	ENSG00000204388	-0,19345	0,045089
FAM27E3	ENSG00000274026	0,251182	0,045089
PPIE	ENSG00000084072	0,205093	0,045143
LRFN5	ENSG00000165379	-0,54568	0,045143
IFRD2	ENSG00000214706	0,186333	0,045143
ZSWIM8-AS1	ENSG00000272589	0,477595	0,045209
FBXO22	ENSG00000167196	0,249197	0,045794
DRG2	ENSG00000108591	0,179901	0,045869
ARHGAP29	ENSG00000137962	-0,41306	0,045869
NCOA2	ENSG00000140396	-0,35922	0,045869

RNF150	ENSG00000170153	-0,32358	0,045869
ZNF579	ENSG00000218891	0,244967	0,046077
WDR74	ENSG00000133316	0,202163	0,046094
ZBTB18	ENSG00000179456	-0,2026	0,046409
RBM8A	ENSG00000265241	0,19163	0,046438
DHX30	ENSG00000132153	0,169353	0,046622
ZC3H3	ENSG00000014164	0,19849	0,046622
MCTS1	ENSG00000232119	0,24627	0,046724
GARS1	ENSG00000106105	0,168893	0,046889
AKR1A1	ENSG00000117448	0,20534	0,046889
SOCS7	ENSG00000274211	-0,30021	0,046889
FDX2	ENSG00000267673	0,273026	0,046918
CCDC90B	ENSG00000137500	0,225637	0,04709
ZNF516	ENSG00000101493	-0,32329	0,047171
TRAF3IP1	ENSG00000204104	-0,24838	0,047184
GAS8	ENSG00000141013	0,187193	0,047251
H4C8	ENSG00000158406	0,942485	0,047282
MVP-DT	ENSG00000238045	0,211912	0,047294
OAZ3	ENSG00000143450	0,486563	0,047378
ARC	ENSG00000198576	0,533936	0,047378
RAB4A-AS1	ENSG00000177788	-0,31803	0,047475
USP49	ENSG00000164663	-0,24973	0,047541
SNAPC2	ENSG00000104976	0,264357	0,047578
CHAF1A	ENSG00000167670	0,206304	0,047625
MAD2L1BP	ENSG00000124688	0,207886	0,047864
ENSG00000263585	ENSG00000263585	0,295836	0,047887
PRR3	ENSG00000204576	0,199777	0,047941
MANF	ENSG00000145050	0,196434	0,048085
DSG2	ENSG00000046604	-0,26531	0,048123
ENSG00000283103	ENSG00000283103	0,295853	0,048123
SNRNP40	ENSG00000060688	0,172723	0,048173
MAP7D1	ENSG00000116871	0,185746	0,048173
GPBP1L1	ENSG00000159592	0,200753	0,048173
UBE2J2	ENSG00000160087	0,184856	0,048173
GPR153	ENSG00000158292	-0,3115	0,048208
FOXN3	ENSG00000053254	-0,33134	0,048377
TRIP6	ENSG00000087077	0,203604	0,048377
C1QTNF6	ENSG00000133466	-0,31048	0,048377
ADAMTS3	ENSG00000156140	-0,39498	0,048377
TMEM181	ENSG00000146433	-0,21239	0,04842
MEPCE	ENSG00000146834	0,206625	0,048482
TRAPPC2B	ENSG00000256060	0,378829	0,048482
KIAA0586	ENSG00000100578	-0,2041	0,048507
FBXO30	ENSG00000118496	-0,26711	0,04877
KRAS	ENSG00000133703	-0,22047	0,048783
RHBDD3	ENSG00000100263	0,212976	0,048953
LIPA	ENSG00000107798	-0,20574	0,04905

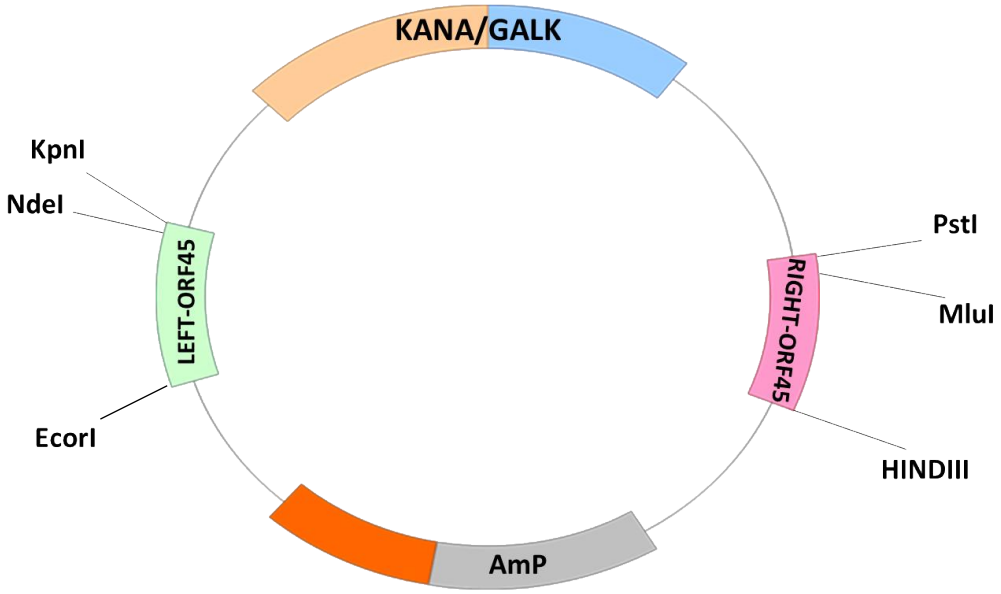
TSIX	ENSG00000270641	-0,23546	0,049158
PRKCI	ENSG00000163558	-0,25403	0,049162
ZNF766	ENSG00000196214	-0,2792	0,049162
ASNSP1	ENSG00000248498	0,428686	0,049162
MAP3K21	ENSG00000143674	-0,22588	0,04928
PRKAA1	ENSG00000132356	-0,33537	0,04938
NECAP2	ENSG00000157191	0,207639	0,049506
H2AC6	ENSG00000180573	0,609061	0,049506
ENSG00000242588	ENSG00000242588	-0,1893	0,049506
SLC33A1	ENSG00000169359	-0,23535	0,049515
ENSG00000288632	ENSG00000288632	0,226051	0,04955
RCBTB1	ENSG00000136144	-0,26907	0,049661
MAPKAPK5-AS1	ENSG00000234608	0,234541	0,049725
POU4F1	ENSG00000152192	-0,18758	0,049809
COPS3	ENSG00000141030	0,168237	0,049818
CEP170	ENSG00000143702	-0,23801	0,049834
ARL4D	ENSG00000175906	0,269721	0,049834
KANSL2	ENSG00000139620	0,19399	0,04984
LMBRD2	ENSG00000164187	-0,31845	0,04984

3) List of constructs used in this work:

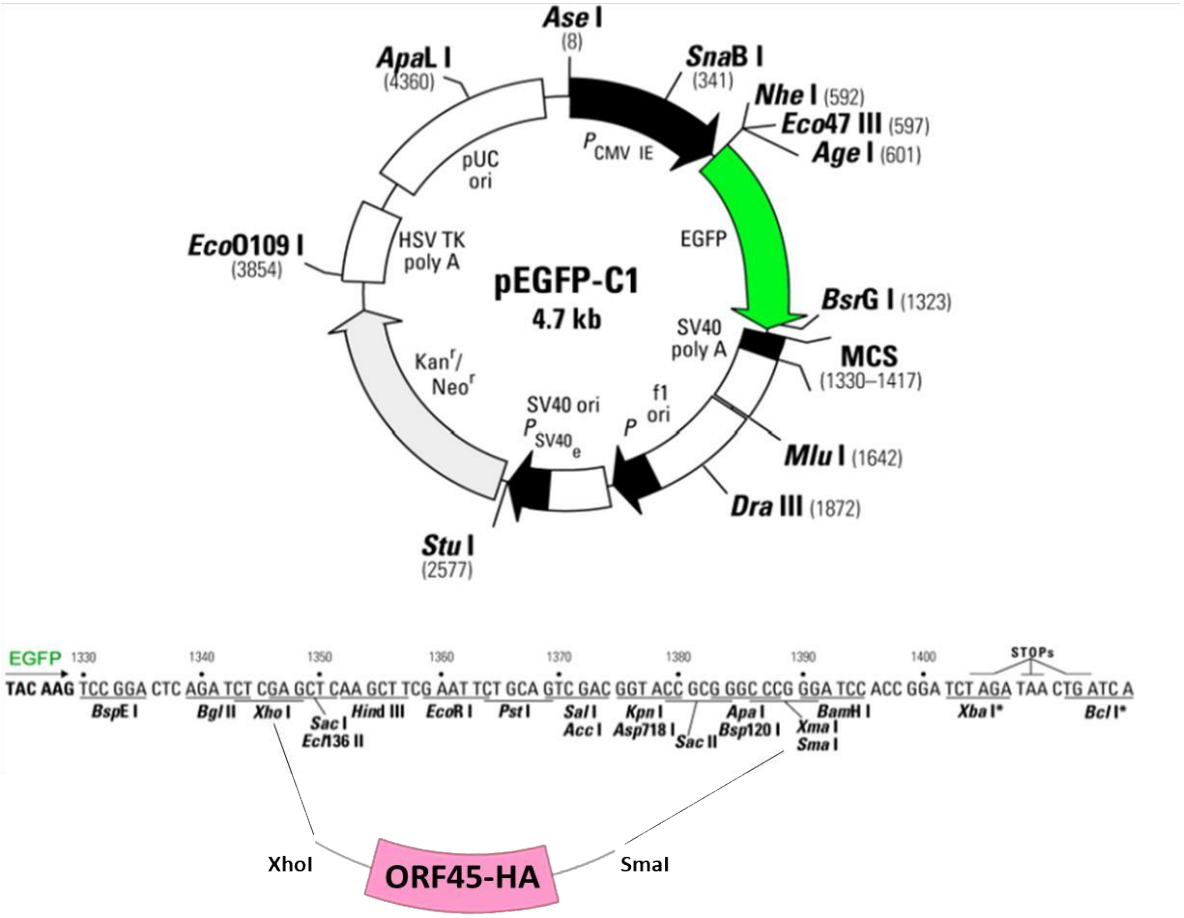
a. <u>p-CMV-ORF45-HA</u>:



b. <u>p-LEFT-ORF45-KANAGALK-RIGHT-ORF45</u>:



c. <u>p-EGFP-ORF45-HA</u>:



pEGFP-C1 map downloaded from site (Anon s.d.)

List of papers published during the PhD in Medical-Veterinary Science.

I. Capacity to Elicit Cytotoxic CD8 T Cell Activity Against Mycobacterium avium subsp. paratuberculosis Is Retained in a Vaccine Candidate 35 kDa Peptide Modified for Expression in Mammalian Cells. Front Immunol. 2019; 10: 2859.

II. Bovine Herpesvirus-4-Vectored Delivery of Nipah Virus Glycoproteins Enhances T Cell Immunogenicity in Pigs. Vaccines 2020.

III. Immunotargeting of the xCT cystine/glutamate antiporter potentiates the efficacy of HERS-target immunotherapies in breast cancer. Cancer Immunology.2020.

IV. A Simplified SARS-CoV-2 Pseudovirus Neutralization Assay. Vaccines. 2021.

V. Immunization With Bovine Herpesvirus-4-Based Vector Delivering PPRV-H Protein Protects Sheep from PPRV Challenge. Front Immunol. 2021; 12: 705539.

VI. Persistency of Mesenchymal Stromal/Stem Cells in Lungs. Front Cell Dev Biol. 2021; 9: 709225.

VII. Immune response to SARS-CoV-2 mRNA vaccination and booster dose in patients with multiple myeloma and monoclonal gammopathies: impact of Omicron variant on the humoral response. OncoImmunology- 06 Sep 2022.

VIII. Under review (Jan. 2023): Characterization of BoHV-4 ORF45.
Luca Russo, Emanuele Capra, Valentina Franceschi, Davide Cavazzini, Roberto Sala, Barbara Lazzari, Sandro Cavirani and Gaetano Donofrio.

Conferences.

75° SISVET CONGRESS IN VETERINARY MEDICINE
Lodi, from 15 June -18 June 2022
Oral presentation: BOHV-4 ORF45 IS AN INDISPENSABLE GENE FOR BOHV-4 LYTIC REPLICATION AND ITS PROTEIN PRODUCT IS ASSOCIATED TO THE VIRION.
Luca Russo, Valentina Franceschi, Gaetano Donofrio.

PhD Visiting: 1/10/22 al 31/12/22

INIA-CISA (**Madrid).** (Centro de Investigación en Sanidad Animal - Instituto Nacional de Investigación y Tecnología Agraria)**.**

Study title: Study of the in vivo immune response against various animal pathologies following immunization by viral vectors.

The INIA-CISA- INTERNAL VIROLOGY SEMINAR:

«Laboratory diagnosis, an essential tool for the control of African swine fever»
Dª. Marisa Arias Neira INIA-CISA (MADRID).

«Entry and formation of African swine fever virus»
D. Germán Andrés (CISA-INIA, CSIC) (MADRID).

Seminar.

Brian Nosek (University of Virginia and Executive Director of the Center for Open Science, US). The reproducibility crisis and a way forward.

Università di Parma e patrocinato dalla Società Italiana delle Scienze Veterinarie (S.I.S.Vet) e dalla Associazione Nazionale Veterinari Igienisti (A.I.V.I), dal titolo "Gestione della contaminazione microbica in impianti di macellazione suinicola: criticità e approcci innovativi".

Dip.to Scienze Chimiche della Vita e della Sostenibilità Ambientale Dottorato di ricerca in "Biotecnologie e Bioscienze" "Pubblicare su riviste scientifiche ad alto impatto"

Webinar Promega – PCR. Promega

Webinar Virbac – VACCINES. Virbac

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