1. Folie Title

Good afternoon and **welcome to the presentation** of my PhD thesis**. Thank you** very much for your nice introduction, Mr. X. **It’s a pleasure** to have you all here so numerously. As the thesis title indicates I was working with viruses, particularly parvoviruses, keeping the focus on the initiation of the release of the progeny virions.

1. Folie Contents

My talk is structured as indicated on this slide. The introduction is splitted into two parts. First, I’ll start with a general and brief introduction into the general nature of viruses. Secondly, I’ll introduce you into the field of parvoviruses focusing late steps in their life cycle.

1. Folie Nature of viruses

First, I’d like to give a very short introduction into the large field of virology. Viruses infect **all types of organisms** such as animals, plants, and microorganisms including bacteria and archaea. Therefore, they appear in **every ecosystem** on earth in a large variety. Basically, a virus is a set of genes, composed of either **DNA or RNA**, which is packaged in a **protein coat**. Some viruses also have an outer **lipid bilayer** membrane external to the coat which is called envelope. In general, viruses display a **vast diversity of morphologies**. Some of which are displayed here. There are **helical** viruses such as tobacco mosaic virus, **icosahedral** viruses as MVM (my model parvovirus which I’ll introduce later on), **enveloped** viruses with the lipid bilayer as for example HIV and more **complex** viruses such as bacteriophage T4 with a tail and a **prolate** head. Prolate means an icosahedon elongated at its 5-fold axis forming a cylinder with a cap at either end.

Quick digression from the nature of viruses to the history of virology.

The **first evidence of the existence of viruses** arose in the closing years of the 19th century when **Dimitry Ivanovsky** observed that extract from diseased tobacco plants remained infectious to healthy tobacco plants when passed through a filter that had pores small enough to retain bacteria. The **term “virus”** which means poison, venom, or infectious agent in latin was introduced by **Martinus Beijernick** a few years later. The first structure of a virus, the one of tobacco mosaic virus, was crystallized in 1935 by **Wendell Meredith Stanley**.

1. Folie Classification

In order to **organize the vast variety of different viruses**, the international committee on taxonomy of viruses introduced a virus taxonomy system. The classification is based on **phenotypic characteristics** such as morphology, the type of nucleic acid the virion contains, the mode of replication, the host range of a virus, and the type of disease it causes. The **Baltimore classification is a simplified classification** system that only considers the **mechanism of mRNA production of a virus**. As you already can see here in the table based on the ICTV classification, Parvoviruses are the only viruses in the known biosphere that have both single-stranded and linear DNA genomes.

1. Folie Size comparison

On this slide you can see that viruses can differ significantly in size. Very complex viruses, such as smallpox virus, have a size of 200 by 300nm. Also TMV can be 300 nm in length. Other viruses like poliovirus and parvovirus are much smaller having a size of only 25-30nm. The picture here I specifically put for the Polacek research group, as you can see, Parvoviruses, including MVM have a similar size as ribosomes. Hence, the prefix “parvum” which means small in latin. Pandoraviruses belong to the largest viruses and can have the size of a small bacterium.

1. Folie Parvoviruses WHY?

After the general introduction into the nature of viruses I’d like to keep the focus now on Parvoviruses. First of all, why is it interesting to study parvoviruses? There are several reasons to investigate the biology of parvoviruses. First of all, these small viruses include important human pathogens which can cause severe diseases. Known **human pathogenes** are the erythroparvovirus B19, the human bocaparvovirus, as well as the tertaparvovirus PARV4. AAV represents a further non-pathogenic human parvovirus which is used as a **vector for gene therapy**. AAV can site-specifically integrate into the chromosome 19 and its dominant phase of the life cycle is a persistent or latent state. Due to their simplicity parvoviruses strongly depend on their host cell. Therefore, they represent interesting tools to **study those immense virus-host interactions**. The latter point is in connection with their natural **oncolytic and oncotropic properties**. Due to their elevated proliferation, cancer cells offer a perfect environment to parvoviruses which hijack the cellular machinery for their own reproduction. It was observed that primary cell lines of normal origin resisted parvovirus infection, whereas their transformed counterparts were sensitive to the viral cytopathic effects. Finally, their compact structure and the fact that they are devoid of a lipid envelope make them highly resistant to physicochemical treatments. They resist lipid solvents, alcohol and ether treatment as well as incubations up to 60 °C. Furthermore they’re stable at a pH range of 3-10. Therefore, they are clearly more stable compared to most other, especially enveloped viruses. Because of their robustness, animal parvoviruses serve as **worst case models to challenge the inactivation or elimination procedures** under the most stringent conditions.

1. Folie Parvoviruses Taxonomy

Within the ***Parvoviridae*** family, there is a **vast genetic divergence** and extensive diversity. This might be the consequence of the high **mutation rates of parvoviruses which is similar to RNA viruses**. Depending on their host range, the members of the family Parvoviridae are subdivided into the **subfamilies Parvovirinae infecting vertebrates and Densovirinae** infecting insects and other arthropods, respectively. Parvoviridae are further subdivided into **8 genera** based on differences in transcription map, organization of their ITRs, the ability to replicate autonomously or with a helper virus, the sense of the single-stranded DNA, and sequence homology amongst the subfamily. **Minute virus of mice is the type species of the genera PROTOPARVOVIRUS** which belong to the subfamily Parvovirinae.

1. Folie Structure

The genome of MVM is organized in two ORFs. The left hand side encodes the non-structural proteins and the right hand side encodes the structural proteins. Both ORFs are under the control of separate promoters, P4 for the non-structural ORF and P38 for the structural ORF, respectively.

The viral capsid is made up of 60 copies of one single polypeptide sequence encoded by the structural ORF. It displays an icosahedral symmetry with the triangulation number 1. The virion contains structural proteins of three size classes which share the same C-terminal core structure. VP1 is the minor structural protein represented only by 10 copies per capsid whereas VP2 is the major structural protein. During entry, the virion undergoes endosomal proteolytic digestion which cleaves N-VP2 to generate VP3. VP3 has a truncation of approx. 25 aa at its N-terminus.

1. Folie Life cycle

Binding to the surface of the cell enables the first step of the infection. It is an important key parameter of tropism and pathogenesis. For MVM only the glycan component of the specific receptor is known. The primary attachment factor is Sialic Acid.

MVM enters the cell by receptor-mediated endocytosis. Within the endosomes MVM undergoes important structural transitions. These include the cleavage of the exposed N-VP2 termini and the externalization of the originally sequestered VP1u region.

The endosomal escape represents the major barrier for the subsequent steps of MVM infection. Most of the incoming viruses fail to enter the nucleus and end up in lysosomes. Therefore, MVM has a relatively high particle to infection ratio. It is suggested that the PLA2 activity within VP1u mediates endosomal escape of MVM. However, the site of endosomal escape still remains unknown.

MVM cannot diffuse freely through the cytoplasm. Free diffusion is heavily restricted in the lattice-like mesh of intermediate filaments and microtubules. It has been demonstrated that the transport of MVM depends on active dynein-mediated transport and requires a functional cytoskeleton. Additionally, the proteasome is essential for the infection of MVM although no particle ubiquitination was observed

Viral structural components need to enter the nucleus at two stages of their life cycle. Early in infection, the incoming virion delivers its genome and late in infection, viral structural proteins accumulate in the nucleus for self-assembly of progeny particles. Nuclear import of the incoming virion is highly controversial. Due to their small size, parvoviruses could enter the nucleus as intact particles but their presence in the nucleus has not yet been demonstrated. In general it’s accepted that MVM enters the nucleus through the NPC by exposing classical NLS sequences within VP1u. However, there is a report suggesting an NPC-independent mechanism which involves a caspase-3 mediated degradation of the nuclear lamina*. De novo* synthesized structural proteins pre-assemble in the cytoplasm to trimeric assembly intermediates which are imported into the nucleus where they assemble to empty capsids.

Due to their unique linear and single-stranded genome, parvoviruses adapted their exclusive replication strategy called rolling hairpin replication. NS1 and the hairpins are required to melt the DNA and to re-direct the replication fork along the genome.

My research basically focuses on the last step of the viral life cycle. Currently, it remains largely unknown how MVM is released from infected cells. The release of MVM has been for a long time considered a passive process which is associated with NS1-induced cellular lysis. However, there is growing evidence that an active nuclear export and egress precedes passive release of progeny virions.

1. Folie: Principle in Virology

These observations are in conflict with a basic principle of virology which considers the release of non-enveloped viruses as the bare consequence of cellular lysis. An active egress and non-lytic budding through the host cell membrane is only suggested for enveloped viruses. Evidence of an active egress of non-enveloped viruses came mainly from observations made in polarized cells where virus progeny was predominantly released at the apical surface. Examples are SV-40, Simian Rotavirus, and Poliovirus.

1. Folie: Mechanism of nuclear Export of MVM

The mechanism for nuclear export of MVM remains elusive. It has been suggested that genome packaging into the pre-assembled empty calsids triggers N-VP2 externalization. The phosphoserine-rich N-VP2 residues are supposed to mediate nuclear export of DNA filled capsids. In such a way, empty capsids remain incompetent for nuclear export.

1. Folie: Exocytosis

The exocytic pathway through the ER and Golgi has been proposed as the route for active egress of MVM. MVM progeny would become engulfed in COPII vesicles where they accumulate with dynamin in the perinuclear area. MVM egress induced gelsolin-mediated actin degradation resulting in cell rounding. However, microtubules were observed to be stabilized during an MVM infection.

1. Folie: Challenge

The documentation of an active egress is a difficult task. Passively released virions could be misinterpreted as actively egressing particles and they can even contribute to second rounds of infection. However, it is impossible to exclude that lysis of a few cells may passively release progeny virions. Parvoviruses, particularly MVM, are highly robust and can persist as intact particles in the lysosomes of infected cells. Moreover, the entry and the proposed egress route partially overlap, thus complicating the discrimination between incoming virions and progeny.

1. Folie: Chromatofocusing

Now I make a short digression to an experiment I was running for another project. I needed to separate viruses based on their IEP. After several unsuccessful trials to do so by isoelectric focusing in 0.8% agarose gels I decided to perform chromatofocusing through a MonoP column. In black you can see the continuous pH gradient that was applied. MVM and CPV (canine parvovirus) were properly separated and eluted according to their IEP. Since there was not enough material to measure absorbance I just quantified the viral genomes in the eluted fractions by qPCR.

When I measured the effect of a single point mutation on the very surface of the 3-fold spike I encountered a major surprise. Removal of one single charge on the surface of MVM resulted in a dramatic shift towards a more basic IEP. This shift can be explained by the symmetry of the viral capsid. One point mutation is repeated 60x with respect to the assembled capsid! Chromatography is a valuable tool to detect very minor changes on the surface of small non-enveloped viruses. Therefore, it might help to separate intracellular viruses according to their maturation stages and surface properties!

1. Folie: Aim

The aim of my PhD project was to confirm the existence of a process for nuclear export and active egress of MVM progeny. Furthermore, I wanted to understand the critical capsid maturation steps which trigger the active pre-lytic egress of MVM.

1. Folie: Strategy

Anion-exchange chromatography was performed in order to separate intracellular particles based on their surface properties. AEX has several advantages. In one experiment I can isolate, purify, and concentrate distinct intracellular maturation intermediates. Using standard biochemical and molecular biological methods, structural and functional characteristics of the isolated populations were investigated. By site-directed mutagenesis the role of different capsid regions in nuclear export and egress was studied.

1. Folie Two populations

Now I come to my results. Here you can see different anion-exchange chromatographs. For the first one, progeny particles released in the SN of infected cultures of A9 mouse fibroblasts were collected 8dpI when the cytopathic effect was complete. The collected particles were precleared and analyzed by AEX. The relative amounts of DNA-filled capsids were quantified by qPCR. Two distinct virus populations were separated (grey). In parallel, a second AEX-qPCR analysis was performed including both medium and cell lysate. By including the additional intracellular viruses the same two populations were detected but with altered proportions (black).

In order to verify the integrity of the two DNA containing populations, the particles were subjected to nucleolytic digestion. Both populations resist DNase I digestion, indicating that they represent assembled DNA containing particles.

Since capsid assembly occurs in the nucleus it was of interest to verify the presence of both virus populations in the nucleus at the onset of DNA packaging and assembly. As shown here, both DNA containing particles co-existed in the nucleus in similar amounts 18 hpI.

To verify the AEX profile of pre-assembled empty capsid precursors they were purified by CsCl gradient centrifugation and subjected to AEX. Dot-blot analysis using an ab against intact capsids revealed that their AEX profile resembled that of the FC-P1 population.

Since FC-P1 particles are pre-dominantly cell-associated and have a similar AEX profile as the EC precursors it is tempting to speculate that they represent immature particles without egress potential whereas FC-P2 display a further maturation step which enables active release.

1. Folie: Different N-VP2 conformation

To further characterize the two populations they were intensively purified by applying them to AEX twice. Following purification they were used to infect A9 cells in order to investigate their capacity to initiate the infection. As demonstrated here, both were able to reach the nucleus where their genomes were replicated efficiently. Therefore, both populations represent infectious virions.

As previously explained, DNA packaging has been suggested to trigger N-VP2 exposure. Therefore, we analyzed the surface conformation of N-VP2 in the two FC populations by IP with a specific antibody raised against this region. N-VP2 occupies a surface position in FC-P2 virions but is sequestered in FC-P1.

IF reveals that both virion progenies bound similarly to A9 cells. The N-VP2 of FC-P2 was detectable on the surface of the cells. However, N-VP2 was not accessible to specific antibodies when the cells were infected with FC-P1 particles.

1. Folie: N-VP2 Externalization of FC-P1 Can Be Enforced

CHT has been demonstrated to mimic the in vivo cleavage of N-VP2 that occurs in the endosomes during entry. EC do not expose N-VP2 and thus they cannot be cleaved. The purified capsid populations were separately subjected to proteolytic digestion by CHT under neutral or acidic conditions. FC-P2 particles were completely processed under all tested conditions. In contrast, the N-VP2 of FC-P1 was only marginally accessible to CHT under neutral conditions and required acidification to improve the cleavage rate. The substantial amount of VP2 that remained unprocessed originates from EC which elute in the same AEX fractions as the FC-P1 population but they are not cleavable!

The increased exposure of N-VP2 of FC-P1 under acidic conditions or also increased temperature was also confirmed by IP using the specific Ab against N-VP2.

When incubated with cells, N-VP2 of FC-P1 became exposed after internalization and a proportion remained detectable for several hpI. This indicates a slower processing of VP2 to VP3.

We next investigated whether the sequestered N-VP2 conformation of FC-P1 is responsible for its distinct AEX profile. Therefore, FC-P1 virions were exposed to acidic conditions to provoke N-VP2 externalization. When we analyze their AEX-qPCR it shows that despite the externalization of N-VP2 the AEX profile remained unchanged. Accordingly, the distinct N-VP2 conformation is not responsible for the different AEX profile.