Genetic analysis of the mouse cytomegalovirus nuclear egress complex

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Mirela Popa
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Erstgutachterin: Prof. Dr. Bettina Kempkes
Zweitgutachterin: Prof. Dr. Elisabeth Weiß
Sondergutachter: Prof. Dr. Ulrich Koszinowski

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1. Summary

Cytomegaloviruses represent a part of the beta herpesviruses subfamily characterized by a restricted host range and a slow replication cycle. DNA replication, late viral transcription, and viral nucleocapsid maturation occur in the replication compartments (RCs) within the nuclei of infected cells. During infection, RCs expand from small replication sites to large domains, disrupting the nuclear interior by compressing and marginalizing host chromatin which represents a natural barrier for the export of virus nucleocapsids to the cytoplasm. Two conserved essential murine cytomegalovirus (MCMV) proteins, M50 and M53, play a critical role in capsid export from nucleus to the cytosol where the final events of the virus maturation take place.

To understand more about the function of the M53 protein, we have analyzed a comprehensive set of loss-of-function mutants derived from a random genetic screen to identify the dominant negative (DN) alleles of the M53 gene. Mutations with inhibitory effect have accumulated within two conserved regions of M53. Recombinant MCMVs were constructed for conditional expression of these inhibitory mutants in order to analyze the phenotype they induced. Conditional expression of single inhibitory M53 mutants down-regulated virus replication up to one million folds. Studies on this DN phenotype revealed a complete block of nuclear egress and, unexpectedly, also an inhibition of viral capsids maturation.

This work proved that random mutagenesis followed by cis-complementation based genetic screens is a valuable strategy to identify viral DN mutants and provided the first evidence that DNA cleavage/packaging and capsid export are coupled during cytomegalovirus infection identifying a new function for egress protein M53.

2. Introduction

2.1 Herpesviridae: a general introduction

Herpesviruses are large enveloped viruses with linear double stranded DNA genome. Based on their biological and genetic properties the family of *Herpesviridae* is divided into three subfamilies: *alpha herpesvirinae*, *beta herpesvirinae,* and *gamma herpesvirinae* (Pellet and Roizman, Fields of Virology – fifth edition 2006; McGeoch *et. al*., 2006; van Regenmortel *et. al*., 2004; Davison, 2002).

Cytomegaloviruses (CMVs) represent the beta herpesvirus subfamily. CMVs infect mammals, show colinear genome organization, similar gene content, and are characterized by a restricted host range. All CMVs studied so far show a slow replication cycle and establish lifelong latency in their native host. A typical consequence of beta herpesvirus infection in vivo and in cell culture is that infected cells are frequently enlarged (cytomegalia) and exhibit cytoplasmic and nuclear inclusions (so called owl's eyes) (Rove *et. al*., 1956).

The human cytomegalovirus (HCMV) is detected with a prevalence of 50-90% in the human population. Both the primary and the latent CMV infections are mainly asymptomatic in normal individuals. However, CMV causes severe and fatal disease in immunocompromised patients and is responsible for the most common infection induced birth defects (Drew, 1988; Rubin, 1990). Murine cytomegalovirus (MCMV) infection serves as an animal model system for studies on CMV biology (Reddehase *et. al*., 2008).

In the following chapter the characteristics of the herpesvirus particle and the main steps of the lytic infection cycle will be reviewed with a special attention to the data available for CMVs. For consistency, the MCMV nomenclature will be

used to describe herpesvirus common gene or gene products even if the primary data were generated in another model system. To identify the homologues and to fit MCMV nomenclature to other herpesviruses the corresponding gene names used in the main model herpesviruses are listed in Table1.

Gene product		Homologues		Function
MCMV	**HSV-1**	**beta (HCMV)**	**gamma (EBV)**	
M115	UL1 (gL)	Yes (UL115)	Yes (BKRF2)	Envelope
No	UL4	No	No	?
M104	UL6 (Portal)	Yes (UL104)	Yes (BBRF1)	DNA Packaging
M103	UL7	Yes (UL103)	Yes (BBRF2)	Tegument
M100	UL10 (gM)	Yes (UL100)	Yes (BBRF3)	Envelope
M99	UL11	Yes (UL99)	Yes (BBLF1)	Tegument
M97	UL13 (VPK)	Yes (UL97)	Yes (BGLF4)	Tegument
M95	UL14	Yes (UL95)	Yes (BGLF3)	Tegument
M89	UL15 (TER1)	Yes (UL89)	Yes (BGRF1/BDRF1)	DNA packaging
M94	UL16	Yes (UL94)	Yes (BGLF2)	Tegument
M93	UL17 (CTTP)	Yes (UL93)	Yes (BGLF1)	DNA packaging
M46	UL18 (TRI2)	Yes (UL46)	Yes (BORF1)	Capsid
M86	UL19 (MCP)	Yes (UL86)	Yes (BCLF1?)	Capsid
No	UL20	No	No	Envelope?
M87	UL21	Yes (UL87)	Yes (BCRF1)	Tegument
M75	UL22 (gH)	Yes (UL75)	Yes (BXLF2)	Envelope
M76	UL24	Yes (UL76)	Yes?	Tegument
M77	UL25 (PCP)	Yes (UL77)	Yes (BVRF1)	DNA packaging
M80	UL26 (prePR)	Yes (UL80)	Yes (BVRF2/ECRF3)	Capsid
M80.5	UL26.5 (pAP)	Yes (UL80.5)	Yes (EC-RF3A)	Capsid
M55	UL27 (gB)	Yes (hUL55)	Yes (BALF4)	Envelope
M56	UL28 (TER2)	Yes (UL56)	Yes (BALF3)	DNA packaging
M53	**UL31**	**Yes (UL53)**	**Yes (BFLF2)**	**Nuclear egress**
M52	UL32 (CTNP)	Yes (UL52)	Yes (BFLF1)	DNA Packaging
M51	UL33	Yes (UL51)	Yes (Putative ORF)	DNA Packaging
M50	**UL34**	**Yes (UL50)**	**Yes (BFRF1)**	**Nuclear egress**
M48.2	UL35 (SCP)	Yes (UL48/49)	No	Capsid
M48	UL36 (VP1/2; LTP)	Yes (UL48)	Yes (BPLF1)	Tegument
M47	UL37 (LTPbp)	Yes (UL47)	Yes (BOLF1)	Tegument
M46	UL38 (TRI1)	Yes (UL46)	Yes (BORF1)	Capsid
No	UL41	No	No	Tegument
No	UL43	No	Yes (BMRF2)	Envelope?
No	UL44 (gC)	No	No	Envelope
No	UL45	No	No	Envelope
No	UL46 (VP11/12)	No	No	Tegument
No	UL47 (VP13/14)	No	No	Tegument
No	UL48 (VP16)	No	No	Tegument
M49	UL49 (VP22)	Yes (UL49)	Yes (BFRF2)	Tegument
M71	UL51	Yes (UL71)	Yes (BSRF1)	Tegument
No	UL53 (gK)	No	No	Envelope
M69	UL54 (ICP27)	Yes (UL69)	Yes (BMLF1/EB2)	Tegument?
No	UL56	No	No	Tegument?
No	US2	No	No	Tegument
No	US3	No	No	Tegument
No	US4 (gG)	No	No	Envelope
No	US5 (gJ)	No	No	Envelope?
No	US6 (gD)	No	No	Envelope
No	US7 (gI)	No	No	Envelope
No	US8 (gE)	No	No	Envelope
No	US9	No	No	Envelope
No	US10	No	No	Tegument
No	US11	No	No	Tegument

Table 1. Components of mature or maturing particles of herpesviruses and their putative function (from Mettenleiter, 2006/2004; McGeoch *et. al*., 1988; Chee *et. al*., 1990; Baer *et. al*., 1984). ?, indicates that the presence in virus particle/substructure is not unequivocally shown.

2.1.1 The structure of herpesvirus particles

The infectious herpesvirus particles share a common morphology with an icosahedral capsid (Grünewald and Cyrklaff, 2006; Grünewald *et. al*., 2003; Zhou *et. al*., 2000) surrounded by a tegument, which is a protein meshwork linking the capsid and envelope (Newcomb *et. al*., 2003; Gibson, 1996), which is a cell-derived lipid bilayer embedded with virally encoded glycoproteins.

Herpesvirus capsids package linear double strand DNA genomes ranging from a low of 110 kbp - VZV (Gray *et. al*., 2001) to 241 kbp – HCMV (Davison *et. al*., 2003) with a coding capacity of up to 200 proteins. These include structural components, virus-specific enzymes and factors involved in virus replication and modulation of host defense (Kalejta, 2008; Tang *et. al*, 2006; Zhu *et. al*., 2005; Britt and Boppana, 2004; Dolan *et. al*., 2004; Varnum *et. al*, 2004; Davison *et. al.*, 2003; Dunn *et. al.*, 2003; Murphy *et. al.*, 2003; Yu *et. al*., 2003; Rawlinson *et. al*, 1996; Roizman and Baines, 1991; Chee *et. al*., 1990). Herpesvirus capsids share well conserved icosahedral structures with 162 capsomers (150 hexamers and 12 pentamers) and triplex (TRI) structures (MCMV M46) connecting adjacent capsomers at the inner level of the shell (Adamson *et. al*., 2006; Grünewald *et. al*., 2003; Newcomb *et. al*., 2001/1996/1993; Trus *et. al*., 2001/1996/1995; Zhou *et. al*., 2000/1999/1998a and b/1995/1994; Booy *et. al*., 1996; Schrag *et. al*., 1989). The hexons, the capsomers having six neighbors, occupy the capsid edges and faces, while the pentons, the capsomers having five neighbors, are distributed at the vertices. All 150 hexons of MCMV are composed of both six dimers of the major (MCP – M86, 154 kDa) and small capsid protein (SCP - M48.2, 8.5 kDa). The majority of the pentons consist of pentamers of MCP, while one edge is formed by a cylindrical structure called the portal which is composed of 12-15 copies of the portal protein (PORT – MCMV M104) (Cardone *et. al*., 2007; Deng

et. al., 2007; Newcomb *et. al*., 2005/2001). The portal has a channel believed to serve as a tunnel for the genomic DNA during both DNA packaging in the process of virus maturation and DNA release following virus entry (Lebedev *et. al*., 2007; Yang, Homa and Baines, 2007; Oliveira *et. al*., 2006; Streblow *et. al*., 2006; Wills *et. al*., 2006; Dittmer *et. al*., 2005; Gibson, 1996).

The next layer of the herpesvirus particle is the tegument, which is progressively acquired, during maturation, in both nucleus and cytoplasm. Up to 20 tegument proteins have been identified in virion preparations, including the large tegument protein (LTP) and the LTP binding protein (LTPbp) (MCMV M48 and M47). Viral capsids, surrounded by tegument, bud into the Trans-Golgi network where they acquire a cell-derived lipid envelope containing many virally encoded glycoproteins (see Table 1), including gB (M55), gM (M100), gH (M75), gL (M115), gO (M74), gN (M73), gp48 (M4), gpTRL10 and the UL33 MCMV homolog (Heldwein and Krummenacher, 2008; Mettenleiter, 2006). In addition to viral proteins, a small number of cellular RNAs and proteins, including actin (Baldick and Shenk, 1996), amino peptidase N (Giugni *et. al*., 1996), annexins (Pietropaolo and Compton, 1997; Wright *et. al*., 1995), ß2-microglobulin (Stannard, 1989; Grundy *et. al*., 1987 a/b; McKeating *et. al*., 1987), protein phosphatase I (Michelson *et. al*., 1996), histone 2A and cofilin have been shown to associate with the HCMV virion (Kattenhorn *et. al*., 2004; Varnum *et*. *al*., 2004; Gibson, 1996). Although infected cells contain these cellular components in a great abundance, it is still unclear whether they are only associated with virion preparations (Baldick and Shenk, 1996; Wrigth *et. al*., 1995) or play a specific role in CMV infection.

2.1.2 Lytic replication of herpesviruses

2.1.2.1 Viral entry

Attachment and entry of herpesviruses occur either in one step by immediate receptor-mediated fusion of the viral envelope with the cellular plasma membrane at the surface of the host cell or in two steps consisting of a receptor mediated attachment followed by endocytosis. In the latter case, fusion happens only when the virions have reached a specific endosomal compartment. Released capsids with a remaining layer of tegument proteins are then transported through the cytoplasm via microtubules to the nucleus and the viral genome is transferred into the cell nucleus through the nuclear pore complex (see Figure 1).

Virus attachment involves multiple cell surface components interacting with glycoproteins of the viral envelope (Spear *et. al*., 2004; Spear and Longnecker, 2003). After binding to specific cellular receptors the viral envelope fuses with the cellular membranes. This process is under the control of essential herpesvirus envelope proteins such as gB and the gH/gL complex. In addition to these three conserved glycoproteins (gB, gH and gL), some herpesviruses require additional receptor binding proteins, such as glycoprotein D for herpes simplex virus 1 (Heldwein and Krummenacher, 2008; Ligas *et. al*., 1988; Johnson *et. al*., 1988), gp350/220 for Epstein-Barr virus entry (Spear and Longnecker, 2003), or either the gO (Huber and Compton, 1998) or the UL128-131 complex of HCMV (Adler *et. al*. 2006). The HCMV gB (homolog of MCMV M55), gH (homolog of MCMV M75) and gL (homolog of MCMV M115) are type I viral transmembrane glycoproteins. The proteolytically processed glycoprotein gB promotes attachment to the cell surface by binding to heparan sulfate proteoglycans (Kielian *et. al.*, 2006; Boyle and Compton, 1998; Compton *et. al.*, 1993).

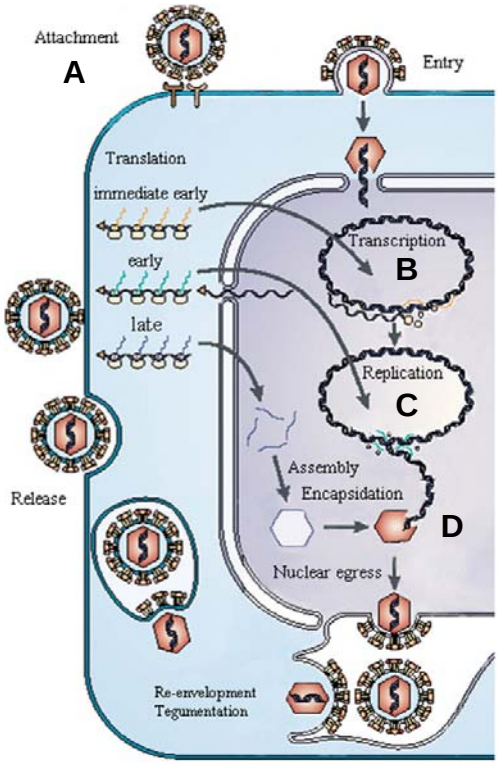

Figure 1. Herpesviruses life cycle (after Coen and Schaffer, 2003): A. *Attachment and entry.* Viral glycoproteins, members of the particle outer layer, are responsible for the envelope fusion with the plasma membrane and nucleocapsid (hexagons) delivery into the cytoplasm. Capsids are transported along the microtubule to the nuclear pores. Viral DNA is released into the nucleosol and circularizes. B. *Transcription*. Immediate-early proteins transcription relies on viral and cellular trans-activators. These proteins play a role in further transcription control (upper translation sites). C. *Replication*. The control of cellular environmental is exercised by early proteins (middle translation sites). Their synthesis requires new viral DNA molecules. D. *Assembly, encapsidation and nuclear egress.* Late proteins (lower translation sites) auto-assemble and generate capsids, which incorporate one unit length viral DNA genome. Nucleocapsid egress supposes a primary envelopment at the inner nuclear membrane and releases into the perinuclear space. After the fusion of the primary envelope with the outer nuclear membrane the new particles follow a complex maturation by re-envelopment. The mature virus particles reach exocytose vesicles and are released into the extracellular space.

gB also interacts with integrins or epidermal growth factor receptor (EGFR) (Compton, 2004; Feire *et. al*., 2004; Wang *et. al*., 2003). In addition, the gH/gL complex can also bind to integrins thereby aggregating the gB and gH/gL complexes (Wang *et. al*., 2003). CMV entry involves additional envelope glycoproteins, such as gO (HCMV homolog of M74) which binds to the gH/gL complex, important for HCMV entry in fibroblasts (Huber and Compton, 1998, Jiang *et. al*., 2008; Kinzler and Compton, 2005; Theiler and Compton, 2001). Infection of endothelial and epithelial cells, however, needs another complex build

by UL128, UL130 and UL131A to promote entry via endocytosis (Ryckman *et. al*., 2008; Adler *et. al*., 2006). After internalization herpesvirus particles exploit the cytosolic organelle transport system involving microtubules (Dohner *et. al*., 2006/2005/2002; Radtke *et. al*., 2006; Wolfstein *et. al*., 2006). The transport of capsids along microtubule to the microtubule-organizing center (Welte *et. al*., 2004; Ogawa-Goto *et. al*., 2003) depends on the dynein/dynactin motor complex (Dohner *et. al*., 2002; Sodeik *et. al*., 1997). When the capsids reach the nuclear pores, the capsid and tegument proteins facilitate the release of viral DNA into the nucleus (Ojala *et. al*., 2000; Sodeick *et. al*., 1997).

2.1.2.2 Kinetics of viral gene expression

During productive infection, there are three major phases of gene expression (see Figure 1). The products of so-called immediate-early (IE) genes are synthesized in the first few hours after the viral entry and depend mainly on host factors for their expression. These proteins together with some entering tegument proteins regulate the viral and cellular gene expression in order to optimize the cellular environment for the production of viral progeny and are required for the expression of early genes which lead to the second wave of viral transcription (White *et. al*., 2007; White and Spector, 2005; Goldmacher, 2004; Castillo and Kowalik, 2002; Fortunato *et. al.*, 2002/2000; Colberg-Poley, 1996; Stenberg, 1996). Early genes encode proteins which are involved in viral DNA replication and transactivate late genes (Spector, 1996/1990). Concurrent effects on cellular metabolism allow viral DNA synthesis. Finally, the expression of late genes (encoding mainly structural proteins) allows the assembly of new particles and release (Spector *et. al*., 1996).

2.1.2.3 Replication and gene expression

HCMV DNA replication begins at 14 to 24 h post infection (hpi), and the release of progeny starts between 36 and 48 hpi, reaching maximum levels between 72 and 96 hpi (Hertel and Mocarski, 2004).

CMV infection deregulates important cell cycle regulators, such as cyclins (Advani *et. al*., 2000; McElroy *et. al*., 2000; Bresnahan *et. al*., 1998; Jault *et. al*. 1995), cell cycle checkpoint proteins (p53 and pRb: Song *et. al*., 2005; Kalejta *et. al*., 2003; Jault *et. al*., 1995) and DNA replication effectors (Sanchez *et. al*., 2003; Pajovic *et. al*., 1997; Poma *et. al*., 1996). CMV appears to stimulate the generation of an intracellular environment similar to S phase (Biswas *et. al*., 2003; Wiebusch *et. al.*, 2003; Advani *et. al*., 2000a/b; Sinclair *et. al*., 2000; Secchiero *et. al*., 1998), while eliminating competition with the cellular genome synthesis and avoiding the onset of apoptosis (Reeves *et. al*., 2007; Andoniou *et. al*., 2006).

Lytic replication starts at specific sites on the viral genome, so-called origins of replication (ori_{Lyt}). Some viruses have one (HCMV), other two (VZV, EBV, KSHV) or three (HSV-1, HSV-2) origins. In many herpesviruses the ori_{Lyt} is found next to the single-strand DNA binding protein (SSB) ORF (open reading frame) and the initiation of DNA replication is controlled by the SSB in cis. All herpesviruses encode a core set of six enzymes involved in DNA synthesis – the replication fork machinery – which only is active during productive (lytic) infection: ori binding protein (OBP, MCMV M84; Colletti *et. al*., 2007/2005; Xu *et. al*., 2004), single-strand binding protein (SSB, MCMV M57), a viral catalytic subunit of DNA polymerase (POL, MCMV M54), associated polymerase processivity subunit (PPS, MCMV M44), heterodimeric helicase-primase (HP) consisting of an ATP-ase subunit (helicase, MCMV M105), a primase subunit (MCMV M70) and an accessory subunit (helicase associated factor, MCMV M102) (Iskenderian *et. al*.,

1996; Pari and Anders, 1993). The rolling cycle of DNA replication results in the production of multi-genomic concatemers, which is the substrate for cleavage/packaging reaction filling the preformed capsids with unit length genomes (see 2.1.2.4., for a review see Roizman and Knipe, 2001).

Alpha herpesviruses and non-CMV beta herpesviruses are controlling the DNA replication by interaction of the OBP complex with the SSB protein to unwind the dsDNA and set free the ori_{Lyt} sites for the replication machinery (Macao *et. al*., 2004; Boehmer and Lehman, 1997; see Figure 2). The control of CMV and gamma herpesvirus replication depends on DNA-binding trans-activators (Xu *et. al.*, 2004 a/b).

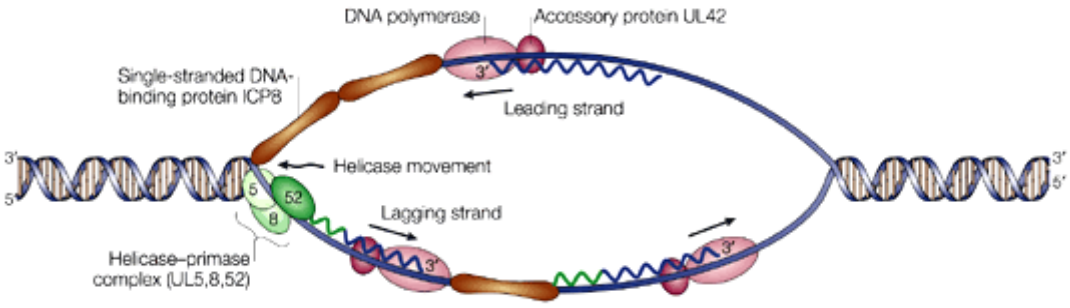

Figure 2. Herpesviruses DNA replication (after Coen and Schaffer, 2003). The elongation phase of herpesviruses DNA replication occurs by a bidirectional replication fork mechanism. On the left side of the replication fork, the helicase–primase complex (HCMV UL 70, UL 102 and UL 105 - see the HSV-1 UL 5, UL 8 and UL 52 bubbles, respectively) unwinds the double-stranded DNA, fact indicated by a wavy arrow. The primase subunit attaches on the lagging strand an RNA primer, represented as a wavy line starting at the 52 bubble. The lagging-strand primers, as well as the leading strand primer (depicted to the right and above), will be elongated into DNA (wavy lines along the strands) in the opposite directions by the catalytic subunit (HCMV UL 54) and the accessory protein (HCMV UL 44) of the DNA polymerase (see the HSV-1 DNA polymerase and accessory protein UL 42 bubbles). Single-stranded DNA fragments are protected by the single-stranded DNA-binding protein (HCMV UL 57 - see the flat bubbles: HSV-1 ICP8).

2.1.2.4 Herpesvirus assembly and egress

2.1.2.4.1 Nuclear events in herpesviruses morphogenesis

a) Capsid formation. The translocation of the major capsid protein (M86 in MCMV) into the nucleus requires its interaction with the assembly protein precursor (pAP; M80.5 in MCMV), which provide the nuclear leader sequence (Nguyen *et. al*., 2008; Plafker and Gibson, 1998; Wood *et al*., 1997). Capsid precursors (‘procapsids’) auto catalytically assembled by the interaction of M86 hexamers complexes with small capsid protein SCP (M48.2) and pentamers of M86 on a scaffold composed of the proteinase precursor (prePR; M80 in MCMV) and pAP (Singer *et. al*., 2005; Newcomb *et. al*., 2003; Walters *et. al*., 2003; Desai and Person, 1996; Liu and Roizman, 1991; Gibson and Roizman, 1974). During maturation the triplex complex (M46) is incorporated into the shell and the scaffold is cleaved by the viral protease (M80). These processes lead to structural changes through the shell which are required for the tight capsid formation (Rixon and McNab, 1999; Gibson, 1996; Gao *et. al*., 1994).

Apart from minor differences in the scaffolding protein (HSV homolog of pAP; Trus *et. al*., 1996), the procapsid has the same protein composition as the mature capsid but is differing in many morphological aspects: (i) the procapsid is spherical whereas the mature capsid is icosahedral; (ii) the procapsid has holes between capsomers whereas the mature capsid is sealed; (iii) in cross section procapsid hexons are oval whereas those of the mature capsid are hexagonal (Newcomb *et. al*., 1996; Trus *et. al*., 1995); and (iv) by incubation at + 4°C procapsids are disassembled whereas mature capsids are unaffected.

b) DNA cleavage and packaging. During DNA replication viral concatemeric structures are formed. Unit-length genomes are produced and packaged into the preformed capsids by a machinery resembling to that of bacteriophages (Wills *et.*

al., 2006) and involves at least seven gene products, namely the homologues of MCMV M51, M52, M56, M77, M89, M93, and M104. The current model of the herpesvirus DNA packaging is based on data mostly acquired by the study of alpha herpesviruses (Yang and Baines, 2008; Yang *et. al*., 2008; Yang, Homa and Baines, 2007; Wills *et. al*., 2006; Yang and Baines, 2006; Beard and Baines, 2004; Taus *et. al*., 1998). As the core of the cleavage/packaging reaction seems to be extremely conserved, a mechanistic analogy with DNA bacteriophage genome packaging has been assumed (Gibson *et. al*., 2008). Therefore, the terminology of the subunits follows that used for bacteriophages. The terminase is recognizing and binding to the genome terminal repeats. Interaction between the portal complex and terminase leads to cleavage of the first genome end and the DNA is concomitantly transported through the portal channel to the inside of the capsid. When the capsid is full the second genome end is recognized and cut. As a final step, the terminase dissociates from the portal which is then sealed by accessory proteins. The herpesvirus terminase (TER) comprises three conserved units (M89 – TER1; M56 – TER2; M51 – TER3) and docks at the portal vertex. The TER2 recognizes specific DNA sequences of concatemers and performs DNA cleavage, generating linear unit length genomes. TER1 contains a conserved P-loop ATP-ase motif and provide the necessary energy for encapsidation (Wills *et. al*., 2006; Thurlow *et. al*., 2005). The M51 homologues (TER3) stabilize the terminase at the docking site and are involved in translocation of cleaved genomic DNA into the capsid's interior (Beard and Baines, 2004).

DNA encapsidation needs additional factors, such as capsid transport nuclear protein (CTNP - M52 homologue; Borst *et. al*., 2008); viral protein kinase VPK (M97 homologue; Azzeh *et. al*., 2006); the homologues of M77 (Wagenaar *et.*

al., 2001; de Wind *et. al*., 1992) and also M93 (Klupp *et. al*., 2005; Oshima *et. al*., 1998; Nalwanga *et. al*., 1996), described as capsid maturation proteins.

c) The nuclear egress. After the packaging reaction is completed, the DNA filled capsids move along the actin microfilaments (Forest, Barnard and Baines, 2005) and contact the inner nuclear membrane (INM) (Buser *et. al.*, 2007; Darlington and Moss, 1968; see Figure 3). Intranuclear movement of herpesviruses requires a capsid maturation signal for transport. This signal is believed to be generated by the interaction of M93 and M77 (Klupp *et. al*., 2006; Wills *et. al*., 2006; Taus *et. al*., 1998).

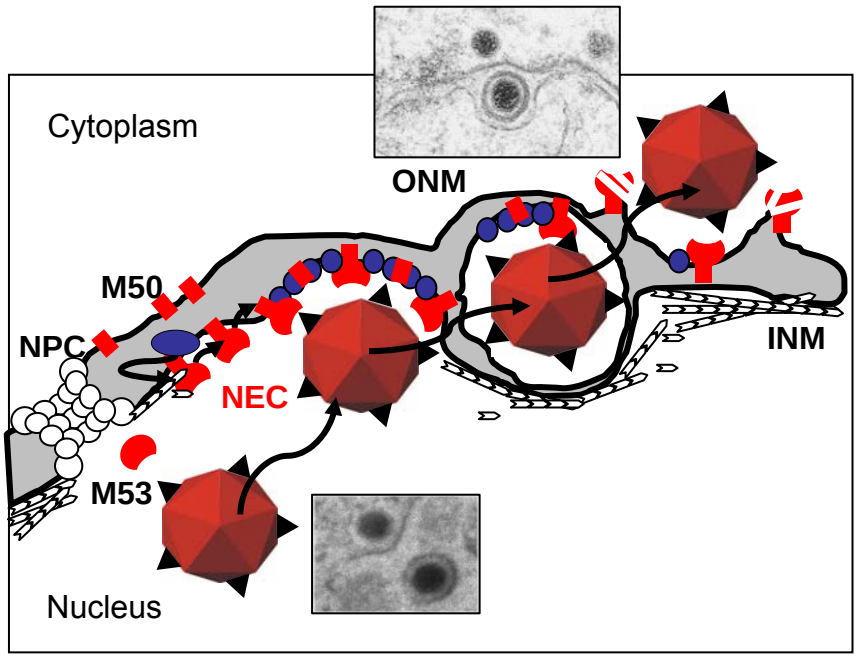

Figure 3. The envelopment-deenvelopment model of nuclear egress of herpesviruses. Nuclear egress complex (NEC) formation induces a cascade of events with Ca-dependent PKC recruitment, lamin phosphorylation and partial dismantle of the inner nuclear membrane (INM). Mature nucleocapsids (high density core) acquire a primary envelope and are translocated into the perinuclear space. Here, the primary envelope fuses with the outer nuclear membrane (ONM) releasing the capsid to cytoplasm. NPC, nuclear pore complex.

To achieve intimate contact with the inner nuclear membrane (INM), the nuclear matrix has to be partially disassembled. This requires at least two viral proteins, MCMV M53 and M50 which are conserved in all Herpesviridae (Rupp *et. al.*, 2007; Loetzerich *et. al.*, 2006; Schnee *et. al.*, 2006; Rupp *et. al.*, 2005;

Simpson-Holley *et. al*., 2005/2004; Bubeck *et. al.*, 2004; Reynolds, Liang and Baines, 2004; Fuchs *et. al*., 2002a; Muranyi *et. al.*, 2002; Klupp *et. al*., 2000; Roller *et. al*., 2000; Chang *et. al*., 1997).

M50, a member of the UL34 herpesvirus protein family, is a type II transmembrane protein found equally distributed in both nuclear leaflets and other membranous structures upon transfection (Muranyi *et. al*., 2002). MCMV M53, a member of the UL31 herpesvirus protein family, is translocated to the nucleus due to its nuclear leader sequence (9 aa NLS) within the N terminal variable region (Loetzerich *et. al*, 2006). All members of the UL31 family share 4 C-terminal conserved regions (CRs) but little is known about the viral functions associated with these domains. M53 CR1 (106-137) harbors the M50 binding site (Loetzerich *et. al*., 2006; Schnee *et. al*., 2006) and CR3 (168-233) could be involved in matrix retention (Lötzerich 2009, ms in preparation).

The interaction of M53 and M50 upon co-transfection or infection leads the relocalization of both proteins to the INM. The colocalization of M50 and M53 at the INM results in nuclear egress complex (NEC) formation (Rupp *et. al*., 2007; Lötzerich *et. al*., 2006; Schnee *et. al*., 2006; Gonella *et. al*., 2005; Rupp *et. al*., 2005; Bubeck *et. al*., 2004; Lake and Hutt-Fletcher, 2004; Liang *et. al*., 2004; Bjerke *et. al*., 2003; Fuchs *et. al*., 2002a; Mettenleiter, 2002; Reynolds *et. al*., 2001; Klupp *et. al*., 2000; Shiba *et. al*., 2000). This interaction is a prerequisite for primary envelopment. In the absence of either protein (HSV-1: Roller *et. al*., 2000; Chang *et. al*., 1997; PrV: Fuchs *et. al*., 2002; MCMV: Rupp *et. al*., 2007; Loetzerich *et. al*., 2006; Bubeck *et. al*., 2004; Muranyi *et. al*., 2002) the nuclear egress is severely affected.

It is believed that NEC interacts with several cellular and/or viral partners at the INM. The HSV-1 homolog of M53 is associated with the nuclear matrix upon

infection (Chang and Roizman, 1993) and binds to lamin A (Reynolds *et. al.*, 2004). In the case of CMV the NEC interacts with lamin B receptor (LBR) and p32 (Lötzerich 2009, ms in preparation).

The 20 to 80 nm deep orthogonal filamentous meshwork of the nuclear lamins is a barrier for capsid budding (Mou *et. al*., 2008). Herpesviruses have developed a strategy for its disorganization (Gonella *et. al*., 2005; Reynolds, Liang and Baines, 2004; Muranyi *et. al*., 2002) by recruiting the cellular protein kinase C (Ca^{2+} dependent PKC) at the INM, which phosphorylates the lamins (A/C and/or B) (Park and Baines, 2006; Muranyi *et. al*., 2002). This induces a local dissolution of the lamin A network (Mou *et. al*., 2008; Reynolds *et. al*., 2002), redistribution of lamin B receptor (LBR) or lamin associated protein 2β (LAP2β) (Muranyi *et. al*., 2002; Reynolds *et. al*., 2002; Scott and O'Hare, 2001) and partial reorganization of chromatin (Simpson-Holley *et. al.*, 2005; Muranyi *et. al*., 2002). These modifications allow the capsids access to the INM. The HSV1 homolog of M50 induces a dissociation of the inner and outer nuclear membranes rather than alterations of the nuclear lamin, suggesting an additional mechanism. Deletion of M53 or M50 homologues resulted in the accumulation of capsids in the nucleus (Granato *et. al*., 2008; Rupp *et. al.*, 2007; Reynolds *et. al*., 2002; Mettenleiter, 2002; Klupp *et. al*., 2000; Roller *et. al.*, 2000; Chang *et. al.*, 1997) with a severe impairment of nuclear egress.

Some tegument proteins – LTP and LTPbp - may also modulate the nuclear egress of pseudorabies virus (Luxton *et. al*., 2006). Besides the protein kinase C, no other cellular protein has been found to be involved in this morphogenesis step. In wild type virus infections the DNA-filled capsids are preferentially enveloped, possibly due to the M77 homologues maturation signaling (Klupp *et. al.*, 2006; Stow, 2001).

By immuno-electron microscopy, it has been shown that the primary enveloped virions - found in the perinuclear cleft – are different from the mature virus particles regarding the morphology and protein content (Miranda-Saksena *et. al.,* 2002; Granzow *et. al*., 2001). HSV homologues of M50 and M53 proteins (Liang *et. al*., 2004) were detected as present at the surface of perinuclear particles but absent from the mature virions. In contrast, major tegument proteins of the mature virions are absent or found only in small quantities in primary enveloped virions (Naldinho-Souto, Browne and Minson, 2006). Regarding the viral envelope glycoproteins, it is disputed whether they are a part of the virion in the perinuclear cleft (Miranda-Saksena *et. al.*, 2002) because several of them have been detected at the inner nuclear membrane (Meyer and Radsak, 2000).

Capsids gain access to the cytoplasm by fusion of their primary envelope with the outer nuclear membrane (ONM). Recently, it has been shown in the HSV1 model that gB/gH complex is involved in perinuclear fusion of the primary enveloped particles (Farnsworth *et. al*., 2007). Its mechanism, however, is still unclear and the data cannot be confirmed by the very close related PRV system (Klupp *et. al.* 2007).

2.1.2.4.2 Cytoplasmic maturation of herpesvirus particles

After translocation to the cytoplasm, new protein–protein interactions allow the late tegumentation and secondary envelopment. The tegumentation is initiated at the capsid and Trans-Golgi network, resulting in two subassemblies that combine to produce a mature virion.

The herpesvirus capsid proximal tegument contains the conserved M48 – LTP (Kattenhorn *et. al*., 2005; Fuchs *et. al*., 2004) and M47 - LTPbp (Hyun *et. al*., 1999), and presumably also the conserved M93/M77 complex (Wills *et. al*., 2006).

Proteins of the inner tegument remain associated with the incoming capsids until they dock at the nuclear pore (Granzow, Klupp and Mettenleiter, 2005; Luxton *et. al*., 2005), and are involved in the intracytoplasmic transport of the capsids during entry and exit (Luxton *et. al*., 2006; Wolfstein *et. al*., 2006). It has been suggested that the HSV1 homologue of M48 is directly anchored to the capsid (Zhou *et. al*., 1999) at the pentonal sites, which are not covered by the small capsid protein. At the final envelopment site in the Trans-Golgi network, the viral glycoproteins are associated with a subset of tegument proteins (Turcotte, Letellier and Lippé, 2005). At least two conserved proteins play important roles in this process: glycoprotein M (M100 - Krzyzaniak *et. al*., 2007; Shen *et. al*., 2007) involved in glycoproteins retention and M99 responsible to direct the tegument proteins to the envelopment site (Loomis, Courtney and Wills, 2003; Bowzard *et. al*., 2000).

Taken together, there is evidence that after the nuclear egress the intracytoplasmic capsids are decorated by M48 and M47 homologues and transported to the sites of the secondary envelopment marked by the glycoproteins M and M99, which are responsible for targeting the major viral glycoprotein complexes (Leege *et. al*., 2009; Kopp *et. al*., 2004). Although it is not sure which protein(s) form(s) the bridge between the viral glycoprotein complexes and the tegumented capsids, the biochemical and morphological data are indicating that in several herpesviruses M49 or its homologues are necessary for this step of the secondary envelopment. However, it is clear that other factors also need to be involved at least in alpha herpesviruses, where M94 homologues are not essential for the virus growth (May *et. al*., 2005; Vittone *et. al*., 2005; Dorange *et. al*., 2002; Fuchs *et. al*., 2002).

The net result of the secondary envelopment, similar to the acquisition of a double membrane by wrapping of a cisterna around the capsid, is a mature

herpesviruses particle within a cellular vesicle. This structure is then transported to the plasma membrane and after fusion of the vesicle and plasma membrane the herpes virion is released. This process mimics the release of secreted proteins and exosomes from the cell.

2.2 Dominant negative mutants of cellular and viral proteins

Classical approaches to identify the function of an essential protein include deletion or temperature sensitive (ts) mutants. For the identification of individual properties of the multifunctional viral proteins these approaches have major limitations or are not easily applicable, e.g. deletion of essential herpesvirus proteins.

Another option in genetics of diploid organisms is to design mutations of the gene of interest that abolishes only one feature and inhibits the function(s) of the wild type (wt) allele after the simultaneous expression. Loss-of-function mutations induce the production of a gene product with less (attenuated) or no function (null allele). The respective mutants, so called dominant negative mutants (DNs), are altered molecules that are acting antagonistically to the wt allele. Therefore, this approach tends to be more valuable for the proteins that become functional only as a member of a complex (Herkowitz, 1987).

DN mutants of cellular genes have been proven to be very useful to dissect complex cellular pathways. Knowledge on protein structure and functions or sequence motifs aids the design of DN mutants. For the last decade they represented an additional opportunity to unveil new regulatory mechanisms (mitotic spindle assembly – Boleti *et. al*., 2000; apoptosis: cyclin dependent protein kinases – Meikrantz and Schlegel, 1996; DNA replication: IRF3 DN alternative splicing and carcinogenesis – Young *et. al*., 2003; lamin B and replication centers -

Ellis *et. al*., 1997), signaling pathways (ECs activation: c-jun induces AP-1 transcription factor activation for ICAM-1 expression - Wang *et. al*., 2001), or even therapeutic approaches (cancer therapies: transforming growth factor type II receptor DN – suppression of mammary tumor formation – Gorska *et. al*., 2003; inhibitor-κB of NFkB transcription factor nuclear factor - Duffey *et. al*., 1999; anti viral therapies: cyclin T1 DN induce proteasome degradation of HIV1 TAT – Jadlowsky *et. al*., 2008; ICP0 DN mutant of HSV1 inhibit HSV1 and HIV1 replication - Weber *et. al*., 1992).

Yet, the existing information on most viral proteins does not provide sufficient data to design DN mutants. A systematic screen for DN alleles needs to consider that all viruses, with exception of retroviruses, possess only one allele of its genes. However, single DNs of viral genes were detected for single genes (HIV1 protease DN – Miklossy *et. al*., 2008; EBV LMP1 DN – Adriaenssens *et. al*., 2004; HSV1 ICP0 DN – Weber *et. al*., 1992). In some cases, fusion of wt allele with a tag (MCMV SCP-GFP- Rupp *et. al*., 2005) or another protein (HBV VP22-core protein – Yi *et. al*., 2005; duck hepatitis B polymerase-core protein – von Weizsäcker *et. al*., 1999) resulted in an inhibitory-phenotype of the fusion protein.

2.3 Aims and concepts

Functional mechanisms of the nuclear egress of herpesviruses are still poorly understood. The current knowledge is mainly based on studies using deletion or temperature sensitive (ts) mutants. Recently in our laboratory we described a new approach to screen for DN mutants of herpesvirus genes combining random mutagenesis and a specially designed genetic screen in the virus context. This has been exemplified by the genetic analysis of M50 (Rupp *et. al*., 2007)

The primary aim of this study was to set up an inhibitory screen for the second MCMV NEC protein M53. A comprehensive transposon insertion library was available and a set of non-complementing mutants were identified (Loetzerich *et. al*., 2006).

The major aim of this project was to characterize the MCMV M53/p38 protein by a genetic analysis that allows definition of new functional domains responsible for specific steps of the MCMV intranuclear morphogenesis. Until now no structural knowledge of the NEC proteins is available. Therefore, large randomly generated libraries of M53 and M50 mutants were used to map essential sites of these viral proteins (see Figure. 4; M53 - Loetzerich *et. al*., 2006). To gain functional information the phenotype induced by the inhibitory mutants needs to be analyzed in the virus context. In order to do that, a viral conditional expression system is required. This system allows the expression of the inhibitory mutant on demand (Rupp *et. al*., 2007/2005). The second major target of this study was to analyze the phenotype of the inhibitory mutants of M53, in case they are identified.

Completing this study should confirm the general applicability of the genetic screen based on random mutagenesis to identify DN mutants of gene products where structure or function is not well understood.

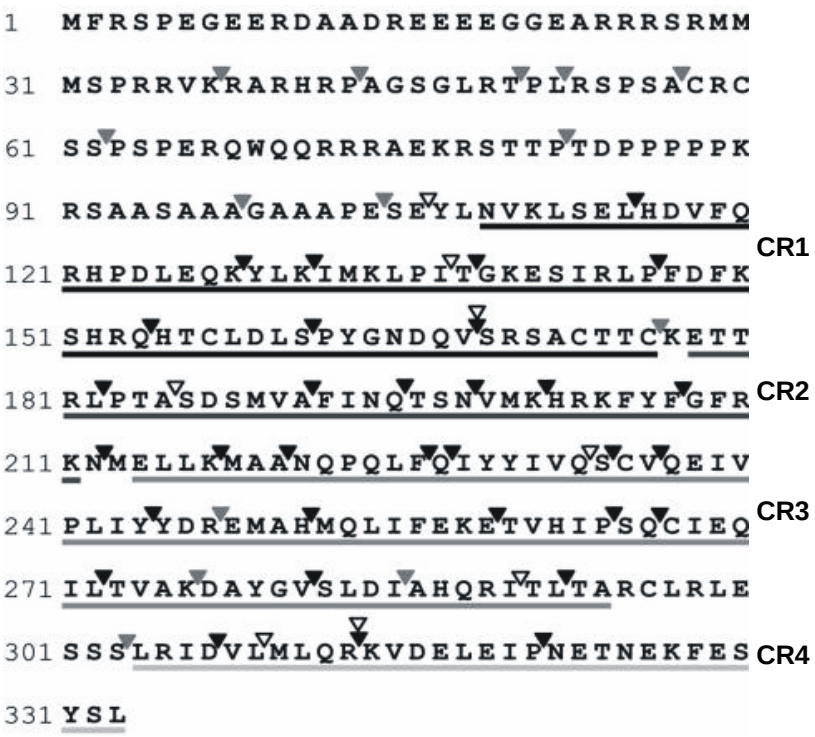

Figure 4. M53 mutants and their ability to rescue the ΔM53 MCMV null phenotype (after Loetzerich *et. al.*, 2006). The sequence displayed is the amino acid sequence of M53/p38. Arrowheads indicate transposon insertion sites. Open arrowheads indicate insertions leading to a stop codon. Light gray arrowheads indicate in-frame insertion mutations that rescued the ΔM53 phenotype. Black arrowheads indicate in-frame insertion mutations that were not able to rescue the ΔM53 null phenotype. Underlined parts of the M53 amino acid sequence indicate the conserved regions (CR1 to CR4).

At the beginning of this study the following specific information was available for the MCMV NEC: (i) there is a conserved subfamily specific interaction between M50 and M53 homologues at the INM (Schnee *et. al*., 2006; Fuchs *et. al*., 2002; Muranyi *et. al*., 2002; Neubauer *et. al*., 2002; Reynolds *et. al*., 2002/2001; Klupp *et. al*., 2000; Roller *et. al*., 2000); (ii) lack of each protein is lethal for the productive infection (M50 - Rupp *et. al*., 2007; Bubeck *et. al*., 2004; M53 - Loetzerich *et. al*., 2006); (iii) NEC formation is responsible for Ca^{2+}-dependent PKC recruitment to the nuclear envelope and induction of cellular lamina phosphorylation with destabilization of the nuclear matrix (Muranyi *et. al*., 2002); (iv) nuclear egress of different herpesviruses is the result of viral proteins interactions with nuclear envelope components, especially the LBR complex (Loetzerich, PhD thesis LMU Munich 2007; Marschall *et. al*., 2005); (v) the M53

binding site of M50 is docked to its N terminal part - this is also most likely true for the other UL34 family members (Rupp *et. al*., 2007; Schnee et. *al*., 2006; Bubeck *et. al*., 2004); (vi) the M50 binding site of M53 is located at the conserved region 1 - CR1 (Loetzerich *et. al*., 2006).

Therefore, by studying the inhibitory phenotype of the M53 mutants we aimed to understand more about the process of MCMV nuclear egress.

3. Materials

3.1 Devices

- Bacterial shaker (ISF-1-W) Kühner, Swiss;
- Centrifuges, Germany: Avanti J-20xp Beckman Coulter GmbH; L8-55M ultracentrifuge Beckman Coulter GmbH; Multifuge 3S-R Heraeus Instruments; Biofuge Stratos Heraeus Instruments; 5417 R Eppendorf;
- Confocal microscope Axiovert 200M Zeiss, Germany;
- Developing-machine Optimax TR MS Laborgeräte, Germany;
- Fluorescence microscope (1x71) Olympus, Germany;
- Gel drying system (model 583) Bio-Rad, Germany;
- Gene Pulser Bio-Rad, Germany;
- Incubators (B5050E, BB16CU) Heraeus Instruments, Germany;
- Light-microscope Axiovert 25 Zeiss, Germany;
- Mini-Protean 3 Cell Bio-Rad, Germany;
- PCR systems: GeneAmp®PCR system 9700, Applied Biosystems, USA; T- Gradient, Biometra, Germany;
- Photo documentation apparatus (Eagle Eye) Bio-Rad, Germany;
- Roler mixer SRT Stuart, Staffordshire, UK;
- Semi-dry-transfer cell (Trans-blot-SD) Bio-Rad, Germany;
- Thermo shaker 5436 Eppendorf, Germany;
- Vortex-mixer Bender/Hobein AG, Swiss;
- Water bath F10 Julabo, Germany.

3.2 Consumables

- Cell culture dishes, Becton Dickinson, Germany;
- Cell culture plates (6-, 12-, 24-, 48-, 96 well), Becton Dickinson, Germany;
- Cell scratcher (25-, 39 cm), Costar, Germany;

- Cuvettes, Germany:
- Condensor cuvettes (2 mm) Bio-Rad;
- Unique cuvettes Brand;
- Dishes (ø 9 cm for agar plates) Grainer, Germany;
- Eppendorf reaction tubes (1.5 ml & 2 ml) Eppendorf, Germany;
- Falcon reaction tubes (15 ml & 50 ml), Becton Dickinson, Germany;
- Spin cup columns and tubes (Handee), Pierce, USA;
- Ultracentrifugation tubes (25 x 89 mm), Beckman Coulter, Germany;
- Whatman paper (Blotting paper), Macherey & Nagel, Germany.

3.3 Kits and reagents

- Amersham Biosciences (Germany): GFX Micro Plasmid Prep kit - purification from 1-1.5 ml of culture; GFX PCR DNA and Gel Band Purification kit - Purification of DNA from gel band;

- Dianova (Germany): horseradish peroxidase (HRP) coupled donkey anti-rabbit (IgG, H+L fragments) antibodies; HRP coupled goat anti-mouse (IgG, H+L fragments) antibodies; HRP coupled goat anti-rat (IgG, H+L fragments) antibodies; Cy3 coupled goat anti-mouse (IgG F_{ab} fragment) secondary antibodies;

- Fermentas (Germany): SphI (PaeI isoenzyme) endonuclease;

- GATC (Germany): pcDNA3.1-RP/1 primer;

- Gibco (Germany) reagents: D-PBS (-Ca, -Mg); DMEM +4500 mg/L glucose + L-glutamine –pyruvate; fetal calf serum (FCS); 100x glutamine; 10x MEM; 100x MEM non-essential amino acids; newborn calf serum (NCS); penicillin/streptomycin (P/S 10000 U – 10000 µg/ml); RPMI 1640 + L-glutamine; sodium bicarbonate (7,5% w/v); trypsin-EDTA (0,25 % - 1 mM);

- GE Healthcare (UK): Amersham ECL Plus Western Blotting Detection System; Amersham Hybond-N, nylon membranes optimized for nucleic acid transfer; Amersham Hybond-P PVDF Membrane, optimized for protein transfer; Amersham Hyperfilm ECL, High Performance chemiluminescence film;

- Invitrogen (Germany): DH10B and PIR1 - Escherichia coli strains; Monomeric Cyanine Nucleic Acid stains, TO-PRO-3;

- Macherey Nagel (Germany): NucleoBond AX100 - Low copy and high copy plasmid purification (Midi AX100 columns);

- Metabion (Germany): all used oligonucleotide for genetic manipulation and sequencing (see Tabel 2: Primers sequences);

Primer name	Type	Size (base pair)	Tm (°C)	Sequence
s309for	cloning	31	77.6	5' GCG CAT GCA TCC ATG TTT AGG AGC CCG GAG G 3'
s309rev	cloning	29	73.2	5' GCG CCT CGA GTT AAA CAA GCA TCA GCA CG 3'
T309for	cloning	30	72.1	5' AAA AGG CGC GCC AAG CTT GGT ACC ATG TTT 3'
T309rev	cloning	29	71.8	5' GGG AAA AAG CC ACCT TCC TCT GCA GCA TT 3'
H262S for1	cloning	25	69.5	5' GGG GGG TAC CAT GTT TAG GAG CCC G 3'
H262S rev1	cloning	29	68.1	5' CCC CAT CGA TAC CGT CTC CTT CTC GAA AA 3'
H262Sfor2	cloning	26	71.1	5' GGG GAT CGA TCC CGT CCC AGT GCA TC 3'
H262S rev2	cloning	27	68.0	5' GGG GGC ATC AGG CGA AAT TGT AAA CGG 3'
D303for1	cloning	29	72.3	5' GGG GAA AAC ACA CGT GCC TGG ACC TCT CC 3'
D303rev1	cloning	30	73.6	5' GGG GAT GCA TCA GCT CGA CTC TAG CCG CAG 3'
D321rev1	cloning	30	72.2	5' GGG GAT GCA TCA GAT CTC GAG CTC GTC CAC 3'
D327rev1	cloning	30	72.2	5' GGG GAT GCA TCA CTC GTT CGT CTC GTT GGG 3'
BioHA1	oligo cloning	117	90.7	5' CAT GAG CGG CCT GAA CGA CAT CTT CGA GGC CCA GAA GAT CGA GTG GCA CGA GGG CAG CTA CCC CTA CGA CGT GCC CGA CTA CGC TAG CCC GGA GGG AGA GGA ACG GGA CGC CGC CGA 3'
BioHA2	oligo cloning	124	91.2	5' CAT GGT ACT CGC CGG ACT TGC TGT AGA AGC TCC GGG TCT TCT AGC TCA CCG TGC TCC CGT CGA TGG GGA TGC TGC ACG GGC TGA TGC GAT CGG GCC TCC CTC TCC TTG CCC TGC GGC GGC TGG C 3'
Sak1for	cloning	30	75.0	5' GGG GAC CGG TCG AGA AGA GGA GGA AGG AGG 3'
Sak1rev	cloning	30	70.9	5' GGG GAC CGG TGC TCC TAA ACT TAT CGT CGT 3'
SakFlag for	cloning		79.9	5' 3'
BioHAfor	cloning	115	89.7	5' GCT TGG TAC CAT GAG CGG CCT GAA CGA CAT CTT CGA GGC CCA GAA GAT CGA GTG GCA CGA GGG CAG CTA CCC CTA CGA CGT GCC CGA CTA CGC TAG CCC GGA GGG AGA GGA ACG G 3'
BioHArev	cloning	20	65.5	5' GCG GCT GGT TAG CGG CCA TC 3'
BioHA repfor	cloning	55	80.6	5' AGC TTG GTA CCA TGA GCG GCC TGA ACG ACA TCT TCG AGG CCC AGA AGA TCG AGT G 3'
SVTinsfor	cloning	38	84.0	5' GGG GGG GGC GCG CCA AGC TTG GTA CCA TGT TTA GGA GC 3'
CMVSak	cloning	19	67.4	5' GCC CAA CGA CCC CCG CCC A 3'
CMVfor	seq	21	65.7	5' CGC AAA TGG GCG GTA GGC GTG 3'
BGHrev	seq	18	56	5' TAG AAG GCA CAG TCG AGG 3'
M53for1	seq	19	61.0	5' CAC ACG TGC CTG GAC CTC T 3'
M53rev1	seq	19	61.0	5' AGA GGT CCA GGC ACG TGT G 3'
TnR	seq	20	61.4	5' CTC TCA TCA ACC GTG GCT CC 3'
TnR3	seq	21	59.8	5' ACT CGC TAC CTT AGG ACC GTT 3'
TnL	seq	20	55.3	5' GAA TAT GGC TCA TAA CAC CC 3'
Pack1for	probe	20	67.0	5' TGC ATC GAC GGT CCC AGC CA 3'
Pack1rev	probe	20	65.0	5' CCG CGG TGG TCC CCA TTG TG 3'

Table 2. Primers' sequences. PCR and sequencing primers, used during this study, were produced by Metabion, Germany (for, forward primer; rev, reverse primer; rep, repaired sequence; seq, sequencing primer; probe, probe useful for Southern-blot technique).

- Micro Tech Lab (Austria): Confocal Matrix;

- Miltenyi Biotec (Germany): FcR Blocking Reagent;

- MoBiTec (Germany): Alexa 488 goat anti-rat (IgG, H+L fragments) antibodies; Alexa 555 goat anti-mouse (IgG, H+L fragments) antibodies; Alexa 555 goat anti-rabbit (H+L fragments) antibodies; Alexa 633 goat anti-rabbit (IgG, H+L fragments) antibodies;

- New England Biolabs (Germany): Acc65I, AscI, BamHI, BsiWI, BspEI, BspMI, EarI, EcoRI, HindIII, HpaI, KpnI, NotI, NsiI, PmeI, PstI, PvuII, SacII, SpeI, XhoI, T4DNA ligase, T4DNA ligase buffer, specific enzymes buffers, 1 kbp and 100 bp ladders;

- Novagen (USA): Benzonase Nuclease;

- Pierce (England): Handee-Spin;

- QIAGEN (Germany): QIAquick Spin - QIAquick PCR Purification kit protocol; SuperFect Transfection Reagent - Protocol for Transient Transfection of Adherent Cells/protocol with specific modifications for primary cells; DNAeasy Blood & Tissue kit – protocol for cell culture;

- Roche (Germany): Anti-HA High Affinity (3F10), rat monoclonal IgG; DIG Easy Hyb Granules; Expand High Fidelity PCR System; PCR DIG Probe Synthesis Kit; DIG Luminescent Detection kit; PIC (Proteinase inhibitor cocktail, 100x);

- Sigma (Germany): acetic acid, 1:29 bis/acrylamide, agarose, ampicillin, APS, anti-actins rabbit antibodies; anti-Flag (M2) HRP-antibodies, Bacto-tryptone, Bacto-yeast, β-mercaptoethanol, bromphenolbleu, boric acid, BSA, carboxymethylcellulose, chloramphenicol, DTT, $EDTANa_2$, ethanol, ethidium bromide, fish-gelatin, glycerol, glycine, HCl, hygromycin, isopropanol, L arabinose, methanol, milk pulver, sodium acetate, sodium chloride, sodium citrate, paraformaldehyde, phenol, phenol-red, potassium chloride, SDS, sucrose, TEMED, TRIS, TRIS-HCl, TRITON X-100, Tween 20, urea, zeocin.

3.4 Buffers and solutions

a. agarose gel running buffers:

- TAE 50x (for 2 liters buffer: 484.6 g TRIS (2 M), 37.2 g EDTA Na_2 (0.05 M), 41 g sodium acetate (0.25 M), pH at 7.3 with glacial acetic acid);

- TBE 10x (for 1 liter buffer: 108 g TRIS, 55 g boric acid, 40 ml EDTA Na_2 (0.5 M), pH 8.0);

b. cells buffers/solutions:

- cells washing buffer: PBS (saline phosphate buffer: - $CaCl_2$, -$MgCl_2$);

- cells detaching solution: 0.25% trypsin – 1 mM EDTA;

c. viruses stock buffers:

- VSB (Virus standard buffer): dissolve 6.055 g TRIS/HCl (0.05 M), 0.895 g KCl (0.012 M), 1.86 g EDTA-Na_2 (0.005 M) in 800 ml autoclaved water; adjust the pH to 7.8 with HCl; add water for a 1000 ml final volume; autoclave;

- VSB, supplemented with 15% sucrose: put in 500 ml flask 75 g sucrose, add 500 ml VSB, autoclave;

d. Western-blot buffers:

- total lysis buffer, TLB (62.5 mM Tris pH 6.8; 2% (v/v) SDS; 10% (v/v) glycerin; 6 M urea; 0.01% bromphenolbleu; 0.01% (w/v) phenol red; always add fresh β-mercaptoethanol for a 5% (v/v) end concentration);

- high salt lysis buffer LYS-450 (450 mM NaCl; 20 mM Tris/HCl, pH 8.0; 1% Triton X 100);

- low salt lysis buffer LYS-150 (150 mM NaCl; 20 mM Tris/HCl, pH 8.0; 1% Triton X 100);

- 4x sample buffer (6% (v/v) SDS; 40% (v/v) glycerol; 0.5 M Tris pH 6.8; 4% (v/v) β-mercaptoethanol);

- 10x SDS-PAGE electrophoresis buffer (Laemmli: 288 g glycine; 60.6 g Tris; 20 g SDS; add water to 2 liters end volume, pH 8.3 without adjustment);

- SDS-PAGE 4x stacking gel buffer (1.5 M Tris; 0.4% (v/v) SDS; adjust the pH at 6.8 with HCl);

- SDS-PAGE 4x separation gel buffer (0.5 M Tris; 0.4% (v/v) SDS; adjust the pH at 8.8 with HCl);

- blotting buffer (Tris 6 g; 28.8 g glycine; 400 ml methanol; add water to 2 liters end volume);

- TBST (10 mM Tris HCl pH 8.0; 150 mM NaCl; 0.05% (v/v) Tween 20);

- blocking buffer (5% (w/v) milk pulver in TBST buffer);

e. Southern-blot buffers:

- 0.25 M HCl (978.5 ml distilled water; 21.5 ml 36% HCl or 20.8 ml 37% HCl);

- gel denaturation buffer (1.5 M NaCl; 0.5 M NaOH);

- gel neutralization buffer (1 M Tris; 1.5 M NaCl; adjust the pH at 7.4 with HCl);

- blotting buffer (20x SSC: 3 M NaCl; 0.3 M Na citrate);

- membrane washing buffer 2% SSC (v/v) (for 1 liter buffer: 100 ml 20x SSC, 10 ml 10% SDS);

- membrane washing buffer 0.5% SSC (v/v) (for 1 liter buffer: 25 ml 20x SSC, 10 ml 10% SDS);

- 10x maleic acid buffer (1 M maleic acid; 1.5 M NaCl; adjust the pH at 7.5 with HCl).

3.5 Media

- DMEM + 4500 mg/L glucose + L-glutamine -pyruvate: supplemented with 10% fetal calf serum (FCS) or new born calf serum (NCS) and 5 ml penicillin/streptomycin (P/S, 10000 U – 10000 µg/ml);
- LB agar: in a flask for 500 ml add 7.5 g agar and mix with 500 ml LB medium. Autoclave! Cool down at room temperature (RT) and under 50^{o}C could add the antibiotics. Use 20-25 ml agar for each plate;
- LB medium: mix 950 ml ultra pure water, 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 8-10 g NaCl; adjust the pH at 7 with 5 N NaOH and complete the volume at 1 liter with ultra pure water. Immediately autoclave;
- LB-zeo-agar: in a flask for 500 ml add 7.5 g agar and mix with 500 ml LB-zeo medium. Autoclave! Cool down at room temperature (RT) and under 50^{o}C could add the antibiotics. Zeocin is light and salt sensitive. Use 20-25 ml agar for each plate;
- LB-zeo medium: mix 950 ml ultra pure water, 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 4-5 g NaCl; adjust the pH at 7 with 5 N NaOH and complete the volume at 1 liter with ultra pure water. Immediately autoclave;
- Methylcellulose buffer: dissolve ON 3.75 g carboxymethylcellulose in 388 ml autoclaved water, autoclave the solution; add 25 ml FCS, 50 ml 10x MEM, 5 ml glutamine 100x, 2.5 ml 100x MEM non-essential amino acids, 5 ml P/S (10000 U – 10000 µg/ml), 24.7 ml 7.5% sodium bicarbonate; if everything was done correctly the color of medium should turn from yellow to purple-red;
- RPMI 1640 + L-glutamine: supplemented with 10% FCS and 5 ml P/S (10000 U – 10000 µg/ml).

3.6 Bacteria and cell lines

In order to propagate the M53 ORFs carrier plasmids and the MCMV BACs two Escherichia coli strains were used: PIR1 [F^{-} Δlac169 rpoS (Am) robA1 creC510 hsdR514 endA recA1 uidA (ΔMluI):pir-116] and DH10B cells [F^{-} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara, leu)7697 galU galKλ^{-} rpsL nupG] respectively, carrying or not the FLP recombinase expression plasmid, pCP20. The propagation was conditioned by the antibiotic resistance derived from the carried constructs (chloramphenicol of BACs, zeocin of carrier plasmid, and ampicillin of helper plasmid). For virus production a large variety of mammalian cells was used. BALB/c murine embryonic fibroblasts (MEFs; Serrano *et. al.*, 1997), M2-10B4 (stroma-cell-line from bone marrow of BALB/c-mouse, ATCC CRL-1972), mouse lymphoid endothelial cells immortalized by simian virus 40 (SVEC 4-10 endothelial

cells; ATCC CRL-2181), and NIH 3T3 fibroblasts (contact inhibited fibroblasts of NIH Swiss-mouse, ATCC CRL-1658) were prepared and treated as described previously (Adler *et. al*., 2006; Menard *et. al*., 2003). M53 transcomplementing cell line (M53tTA - Ruzsics Z.) was propagated in Dulbecco modified Eagle medium supplemented with 5% neonatal calf serum and hygromycin. Mouse mammary epithelial C127 cells (ATCC CRL-1616) and 293 cells (adenovirus transformed human kidney-carcinoma cells, ATCC CRL-1573) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Rupp *et. al*., 2007).

3.7 DNA

3.7.1 BACs

- ∆M53FRT (Loetzerich, 2006): wt MCMV BAC with an integrated m16-m17 FRT site, chloramphenicol resistance and M53 deleted gene at endogenous position;

- ∆M53FRT carrying different non-tagged or tagged M53 mutants at the m16-m17 FRT site: ∆M53FRT-H262S (5,6); -s294 (2,3); -i303 (1,2); -i304 (1,3); -i324 (2,4); -i325 (3,5); -i326 (4,5); -s331 (2,5); or tagged versions: ∆M53FRT-FlagM53 (2) and -Flag309 (2,4,5);

- pSMfr3 16-17FRT (Bubeck *et. al*., 2004): wt MCMV BAC with an integrated m16-m17 FRT site, chloramphenicol resistance; construct used under the C3xFRT name during this study;

- C3xFRT KA3 (Loetzerich *et. al.*, 2006): attenuated mutant, 6 weeks reconstitution;

- C3xFRT carrying different non-tagged M53 mutants at the m16-m17 FRT site: C3xFRT-i115 (2,3); -i128 (4,6); -i131 (3,4); -i138 (1,5); -i146 (2,8); -i154 (4,5); -i161 (4,5); -i168 (3,4); -s168 (2,3); -i182 (2,5); -s185 (5,9); -i191 (1,4); -i195 (4,9); -i198 (4,5); -i201 (1,2); -i207 (3,4); -i212 (1,2); -i217 (2,4); -i220 (1,2); -i227 (5,10); -s233 (8,16); -i234 (3,5); -i236 (2,3); -i244 (1,2); -i251 (3,4); -i259 (1,5); -H262S (5,7); -i264 (7,8); -i266 (1,2); -i272 (1,2); -i281 (1,3); -s290 (1,9); -i292 (1,2); -s294 (1,2); -s303 (4,5); -i304 (8); -i307 (1,5); -s309 (2,3); -i313 (3,7); -i321 (1,2); -s321 (1,3); -i324 (2,3); -i325 (1,5); -i326 (2,3); -s327 (3,6); -s331 (1,5); or tagged ones: C3xFRT-BioHAM53 (2,5); -BioHA309 (1,3); -FlagM53 (1,2); -Flags309 (2,3); -Flag∆Sak309 (8,10);

- C3xFRT carrying different conditional expressed M53 mutants at the m16-m17 FRT site: -SVTM53 (3,4,5); -SVTi207 (1,2); -SVTs309 (6,7,8,9); -SVTi313 (2,3); -SVTi321 (2,3); -SVTFlagM53 (1,2); -SVTFlag309 (1,2); -SVTFlag∆Sak309 (8,10); -SVTs309i128 (2,8).

3.7.2 Plasmids

- pCP20 (Cherepanov *et. al*., 1995): ampicillin resistant/temperature sensitive helper plasmid;

- po6k-ie (Bubeck *et. al*., 2004): zeocin resistant entry vector carrying an FRT site;

- pok-ie-wtM53 (Loetzerich *et. al*., 2006): zeocin resistance, FRT site;

- rescue plasmids based on po6k-ie carrying M53 mutants, zeocin resistance and FRT site: i115, i128, i131, i138, i146, i154, i161, i/s168, i182, s185, i191, i195, i198, i201, i207, i212, i217, i220, i227, s233, i234, i236, i244, i251, i259, i264, i266, i272, i281, s290, i292, s294, i303, i304, i307, s309, i324, i325, i326, s331 (Mark Lötzerich - mutants generated by Tn-7 random mutagenesis);

- rescue plasmids based on po6k-ie carrying M53 mutants, zeocin resistance and FRT site: M53, i313, i321, s309i128 (mutants generated by cloning);

- rescue plasmids based on po6k-ie carrying M53 mutants, zeocin resistance and FRT site: H262S (aa exchange, clone 5), s303 (10), s321 (4, 7), s327 (5) (mutants generated by PCR);

- pL- wtM53, i313, i321 (Loetzerich *et. al*., 2006): ampicillin resistance, Litmus vector;

- po6k-SVTe (Rupp *et. al*., 2005): po6k-ie carrying the SV40 simian virus promoter/enhancer, zeocin resistance FRT entry vector;

- po6k-SVT-M53 clone 17(Rupp *et. al*., 2005): zeocin resistance, FRT site;

- po6k-ie-SVT-s309/repl.2, -i313/1-5, -i321/6-2, -i207/1, -s309i128/2: zeocin resistance, FRT site;

- TAP-tag M53 mutants based on po6k-ie, zeocin resistance and FRT site (generated by PCR): BioHAM53 (clone 10) and BioHA309 (clone 3);

- Flag-tag M53 mutants based on po6k-ie, zeocin resistance and FRT site (generated by PCR): FlagM53 (Loetzerich M., PhD thesis), Flag309 (clone 3-10), FlagΔSak309 (clone 2/15), SVTFlagM53 (clones 1 old and 10 new), SVTFlag309 (clones 1/15 and 3/14), SVFlagΔSak309 (clones 2 and 3);

- po6k-ie-M50 (Bubeck *et. al*., 2004): zeocin resistance, FRT site;

- po6k-ie-M50HA (Lemnitzer F., unpublished data): zeocin resistance, FRT site.

3.8 Viruses

a) derived from MEFs tranfection with the following BACs:

- controls: C3xFRT; ΔM53FRT;

- C3xFRT-i115 (2,3); -i128 (4,6); -i131 (3,4); -i138 (1,5); -i146 (2,8); -i154 (4,5); -i161 (4,5); -i168 (3,4); -s168 (2,3); -i182 (2,5); -s185 (5,9); -i191 (1,4); -i195 (4,9); -i198 (4,5); -i201 (1,2); -i207 (3,4); -i212

(1,2); -i217 (2,4); -i220 (1,2); -i227 (5,10); -s233 (8,16); -i234 (3,5); -i236 (2,3); -i244 (1,2); -i251 (3,4); -i259 (1,5); -H262S (5,7); -i264 (7,8); -i266 (1,2); -i272 (1,2); -i281 (1,3); -s290 (1,9); -i292 (1,2); -s294 (1,2); -s303 (4,5); -i304 (8); -i307 (1,5); -s309 (2,3); -i313 (3,7); -i321 (1,2); -s321 (1,3); -i324 (2,3); -i325 (1,5); -i326 (2,3); -s327 (3,6); -s331 (1,5); -SVTM53 (3,4); -SVTi207 (1,2); -SVTs309 (6,7,8,9); -SVTi313 (2,3); -SVTi321 (2,3); -SVTs309i128 (2); -BioHAM53 (2,5); - BioHA309 (1,3); -FlagM53 (1,2); -Flag309 (2,3); -SVTFlagM53 (1,2); -SVTFlag309 (1,2); -SVTFlagΔSak309 (8,10);

- ΔM53FRT-H262S (5, 6);

b) derived from M2-10B4 or NIH3T3 cells infection:

- control: C3xFRT; ΔM53tTA MCMV (carry the promoter activator of M53 ORF in trans; Ruzsics Z.);

- C3xFRT-SVTM53 (4); -SVTs309 (9); -SVTi313 (2); -SVTi321 (2); -SVTi207 (1, 2); -SVTFlagM53 (1, 2); -SVTFlag309 (1, 2); -SVTFlagΔSak309 (8, 10); -SVTs309i128 (2).

4. Methods

4.1 PCRs

All PCR products were generated by the TD72 touch-down protocol comprising 5 min. 95°C initial denaturation; 18 cycles of 30 sec. 95°C denaturation, touch-down from 62°C to 45°C for 2 min. annealing and 2 min. 72°C elongation; 17 cycles of 30 sec. 95°C denaturation, 2 min. 45°C annealing and 2 min. 72°C elongation; 7 min. 72°C final elongation - performed with Biometra or Applied Biosystems instruments. Specific primers (see Table 4) were designed by the Vector NTI 7.0 program (Invitrogen). All PCR was carried out by using High Fidelity Expand PCR System (Roche) in 100 µl end volume (see Table 3). The characteristics of the PCR products propagated in this study are listed in the Table 4.

Components	Stock conc.	Master mix conc.
H2O	--	variable
PCR buffer 10x	10x	1x
dNTPs mix	1.5 mM	2.0 µM
Forward primer (for)	10 µM	0.4 µM
Reverse primer (rev)	10 µM	0.4 µM
Expand polymerase	50 U/ml	2.5 U/ml
Template DNA	variable	10 ng/ml

Table 3. Master Mix composition.

PCR product (size)	Template (size)	Primers	
		forward	reverse
PCR s309 (961bp)	po6k-ie-s309 (3452 bp)	s309for	s309rev
PCR s309T (994bp)	po6k-ie-s309 (3452 bp)	T309for	T309rev
PCR H262S 1 (802bp)	po6k-ie-s309 (3452 bp)	H262Sfor1	H262Srev1
PCR H262S 2 (724bp)	po6k-ie-s309 (3452 bp)	H262Sfor2	H262Srev2
PCR 303 (464bp)	po6k-ie-s309 (3452 bp)	D303for1	D303rev1
PCR 321 (518bp)	po6k-ie-s309 (3452 bp)	D303for1	D321rev1
PCR 327 (536bp)	po6k-ie-s309 (3452 bp)	D303for1	D327rev1
BioHA327(1085bp)	po6k-ie-M53 (3473 bp)	BioHAfor	D327rev1
BioHArepM53 (1046bp)	po6k-ie-BioHA327(3578 bp)	BioHArepfor	D321rev1
FlagΔSak 1 (589bp)	po6k-ie-Flag309 (3497 bp)	CMVfor	Sakrev
FlagΔSak 2 (870bp)	po6k-ie-Flag309 (3497 bp)	Sakfor	D303rev1
SVTFlag309 (955bp)	po6k-ie-Flag309 (3497 bp)	SakFlagfor	D303rev1
SVTFlagΔSak309 (933bp)	po6k-ie-FlagΔSak309 (3512 bp)	SakFlagfor	D303rev1
PCR i128 (940bp)	po6k-ie-i128 (3496 bp)	SVTinsfor	D303rev1
Pack1 (425bp)	Wt MCMV BAC	Pack1for	Pack1rev

Table 4. PCR products and the respective primers.

4.2 Cloning steps

- The **po6k-SVT-s309$_{pre}$** ligation product (5250 bp) was obtained by ligation of the 4295 bp fragment - derived from the AscI/HpaI digested po6k-SVTe(4304 bp) vector - with the 955 bp fragment - derived from the AscI/PmeI digested PCR s309T (994 bp) PCR product. The construct was checked for correct alignment by enzymatic restriction [KpnI: po6k-SVTs309 (2293 bp, 1673 bp, 1284 bp) and po6k-SVTe vector control (2631 bp, 1673 bp)] and sequencing (M53for1, M53rev1 primers – for s309 ORF insertion; SV40for and BGHrev primers – for the right sense orientation of the insertion into the vector). *Note: because the po6k-SVT-s309 had one deletion in the N terminal part of the M53 protein a supplementary step was necessary:*

- The **po6k-SVT-s309** (5250 bp) was obtained by ligation of 4399 bp fragment - derived from AscI/BsiWI digested po6k-SVT-s309$_{pre}$ (<5250 bp) vector - with the 851 bp fragment - derived from AscI/BsiWI digested po6k-SVT-M53 (5350 bp) plasmid. The construct was checked for correct alignment by enzymatic restriction [KpnI: po6k-SVTs309 (2293 bp, 1673 bp, 1284 bp) and by po6k-SVTe vector control (2631 bp, 1673 bp)] and sequencing (M53for1, M53rev1 primers – for s309 ORF insertion; SV40for and BGHrev primers – for the right sense orientation of the insertion into the vector).

- The **po6k-SVT-i313** clones 1-5 (5399 bp):

1. The pL-i313 intermediate ligation product (3793 bp) was obtained by ligation of the 2967 bp fragment - derived from the BamHI/BspEI digested pL-wtM53 (3778 bp) vector - with the 826 bp fragment - derived from the BamHI/BspEI digested po6k-ie-i313 (3488 bp) plasmid. The construct was checked for correct alignment by enzymatic restriction [PmeI/SnaBI: pL-i313 (2817 bp, 976 bp) and by pL-wtM53 vector control (only SnaBI restriction site: 3778 bp)] and by sequencing (M13FP and M13RP primers – for the right sense orientation of the insertion into the vector).

2. The po6k-SVT-i313 final ligation product (5399 bp) was obtained by ligation of the 4295 bp fragment - derived from the AscI/HpaI digested po6k-SVTe (4304 bp) vector - with the 1089 bp fragment - derived from the AscI/StuI digested pL-i313 (3793 bp) plasmid. The construct was checked for correct alignment by sequencing (M53for1, M53rev1 primers – for i313 ORF insertion; SV40for and BGHrev primers – for the right sense orientation of the insertion into the vector).

- The **po6k-SVT-i321** clone 6-2 (5399 bp):

1. The pL-i321 intermediate ligation product (3793 bp) was obtained by ligation of the 2967 bp fragment - derived from the BamHI/BspEI digested pL-wtM53 (3778 bp) vector - with the 826 bp

fragment - derived from the BamHI/BspEI digested po6k-ie-i321 (3473 bp) plasmid. The construct was checked for correct alignment by enzymatic restriction [PmeI/SnaBI: pL-i321 (2789 bp, 1004 bp) and pL-wtM53 vector control (only SnaBI restriction site: 3778 bp)] and by sequencing (M13FP and M13RP primers – for the right sense orientation of the insertion into the vector).

2. The po6k-SVT-i321 final ligation product (5399 bp) was obtained by ligation of the 4295 bp fragment - derived from the AscI/HpaI digested po6k-SVTe (4304 bp) vector - with the 1089 bp fragment - derived from the AscI/StuI digested pL-i321 (3793 bp) plasmid. The construct was checked for correct alignment by sequencing (M53for1, M53rev1 primers – for i321 ORF insertion; and SV40for and BGHrev primers – for the right sense orientation of the insertion into the vector).

- The **po6k-ie-H262S**, clone 5 (3909 bp)) was obtained by ligation of 2383 bp fragment - derived from ApaI/KpnI digested po6k-ie-M53 (3473 bp) vector - with 785 bp fragment - derived from the ClaI/KpnI digested “PCR1 H262S 1” PCR product (802 bp) and 305 bp fragment – derived from the ApaI/ClaI digested “PCR2 H262S 2” PCR product (724 bp). The construct was check for correct alignment by enzymatic restriction [ClaI: po6k-ie-H262S (3909 bp) and po6k-ie-M53 vector control (uncut)] and sequencing (M53for1, M53rev1 – for M53 H262S ORF insertion; CMVfor and pcDNA3.1-RP/1 of GATC company – for the right sense of insertion into the vector).

- The **po6k-ie-s303**, clone 10 (3308 bp) was obtained by ligation of 2927 bp fragment - derived from BspMI/NsiI digested po6k-ie-M53 (3473 bp) vector - with 381 bp fragment - derived from the BspMI/NsiI digested “PCR 303” PCR product (464 bp). The construct was check for correct alignment by sequencing (M53for1, M53rev1 – for s303 ORF insertion; CMVfor and pcDNA3.1-RP/1 of GATC Company – for the right sense of insertion into the vector).

- The **po6k-ie-s321**, clones 4 and 7 (3362 bp) were obtained by ligation of 2927 bp fragment - derived from BspMI/NsiI digested po6k-ie-M53 (3473 bp) vector - with 435bp fragment - derived from the BspMI/NsiI digested “PCR 321” PCR product (518 bp). The construct was check for correct alignment by sequencing (M53for1, M53rev1 – for s321 ORF insertion; CMVfor and pcDNA3.1-RP/1 of GATC Company – for the right sense of insertion into the vector).

- The **po6k-ie-s327**, clone 5 (3380 bp) was obtained by ligation of 2927 bp fragment - derived from BspMI/NsiI digested po6k-ie-M53 (3473 bp) vector - with 453 bp fragment - derived from the BspMI/NsiI digested “PCR 327” PCR product (536 bp). The construct was check for correct alignment by sequencing (M53for1, M53rev1 – for s327 ORF insertion; CMVfor and pcDNA3.1-RP/1 primers – for the right sense of insertion into the vector).

- The **po6k-ie-Flag309**, clone 3-10 (3526 bp) was obtained by ligation of 2951 bp fragment - derived from BspMI/NsiI digested po6k-ie-FlagM53 (3497 bp) vector - with the 575 bp fragment - derived from BspMI/NsiI digested po6k-ie-s309 (3502 bp) plasmid. The construct was check for correct alignment by enzymatic restriction [AccI: po6k-ie-Flag309 (1810 bp, 1716 bp), po6k-ie-FlagM53 vector control (1810 bp, 1687 bp); NcoI – po6k-ie-Flag309 (1728 bp, 1509 bp, and 289 bp) and po6k-ie-FlagM53 vector control (1699 bp, 1509 bp, 289 bp)] and sequencing (M53for1, M53rev1 primers – for Flag309 ORF insertion; CMVfor and BGHrev primers – for the right sense of insertion into the vector).

- po6k-ie-FlagΔSak309, clone 2/15 (3512 bp) was obtained by ligation of 2564 bp fragment - derived from NdeI/BspMI digested po6k-ie-Flag309 (3526 bp) vector - with the 453 bp fragment - derived from NdeI/AgeI digested FlagΔSak 1 PCR product (589 bp) and the 495 bp fragment - derived from AgeI/BspMI digested FlagΔSak 2 PCR product (870 bp). The construct was check for correct alignment by enzymatic restriction [AgeI: po6k-ie-FlagΔSak309 (3512 bp), po6k-ie-Flag309 vector control (uncut)] and sequencing (M53for1, M53rev1 primers – for FlagΔSak309 ORF insertion; CMVfor and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-SVTFlag309**, clones 1/15 and 3/14 (5274 bp) were obtained by ligation of 4700 bp fragment - derived from AscI/BspMI digested po6k-ie-SVTs309 (5250 bp) vector - with the 574 bp fragment - derived from AscI/BspMI digested SVTFlag309 PCR product (955 bp). The construct was check for correct alignment by enzymatic restriction [AscI: po6k-ie-SVTFlag309 (5274 bp) and po6k-ie-SVTs309 vector control (5250 bp)] and sequencing (M53for1, M53rev1 primers – for Flags309ORF insertion; SV40for and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-SVTFlagM53**, clones 1old and 10new (5414 bp) were obtained by ligation of 4459 bp fragment - derived from AscI/BspMI digested po6k-ie-SVTM53 (5384 bp) vector - with the 574 bp fragment - derived from AscI/BspMI digested SVTFlag309 PCR product (955 bp). The construct was check for correct alignment by enzymatic restriction [HindIII: po6-ie-SVTFlagM53 (2086 bp, 1633 bp, 1047 bp, 634 bp, 14 bp) and po6k-ie-SVTM53 vector control (2086 bp, 1633 bp, 1031 bp, 634 bp)] and sequencing (M53for1, M53rev1 primers – for FlagM53 ORF insertion; SV40for and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-SVTFlagΔSak309**, clones 2 and 3 (5250 bp) were obtained by ligation of 4700 bp fragment - derived from AscI/BspMI digested po6k-ie-SVTs309 (5250 bp) vector - with the 550 bp fragment - derived from AscI/BspMI digested SVTFlagΔSak309 PCR product (933bp). The construct was check for correct alignment by enzymatic restriction [AgeI: po6k-ie-SVTFlagΔSak309 (3994 bp,

1256bp); po6k-ie-SVTs309 (5250 bp))] and sequencing (M53for1, M53rev1 primers – for FlagΔSak309 ORF insertion; SV40for and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-BioHAM53**, clone 10 (3458 bp):

1. po6k-ie-BioHA327, clone 11(3578 bp) was obtained by ligation of 2970 bp fragment - derived from BspMI/KpnI digested po6k-ie-M53 (3473 bp) vector - with the 608 bp fragment - derived from BspMI/KpnI digested BioHA327 PCR product (1058 bp). The construct was check for correct alignment by sequencing (M53for1, M53rev1 primers – for BioHA327 ORF insertion; CMVfor and BGHrev primers – for the right sense of insertion into the vector).

2. The po6k-ie-BioHAM53 was obtained by ligation of 2936 bp fragment - derived from Acc65I/BspMI digested po6k-ie-M53 (3473 bp) vector - with the 612 bp fragment - derived from Acc65I/BspMI digested BioHArepM53 PCR product (1046 bp). The construct was check for correct alignment by enzymatic restriction [Acc65I/BspMI: po6k-ie-BioHAM53 (2936 bp, 612 bp) and vector control po6k-ie-M53 (2936 bp and 537 bp)] and sequencing (M53for1, M53rev1 primers – for BioHAM53 ORF insertion; CMVfor and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-BioHA309**, clone 3 (3421bp) was obtained by ligation of 2965bp fragment - derived from Acc65I/BspMI digested po6k-ie-s309 (3502bp) vector - with the 612 bp fragment - derived from Acc65I/BspMI digested BioHArepM53 PCR product (1046bp). The construct was check for correct alignment by enzymatic restriction [Acc65I/BspMI: po6k-ie-BioHA309 (2965 bp, 612 bp) and vector control po6k-ie-M53 (2936 bp and 537 bp)] and sequencing (M53for1, M53rev1 primers – for BioHA309 ORF insertion; CMVfor and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-SVTi207**, clone 1 (5399 bp) was obtained by ligation of 5083 bp fragment - derived from BspMI/BsiWI digested po6k-ie-SVTM53 (5384 bp) vector - with the 316 bp fragment - derived from BspMI/BsiWI digested po6k-ie-i207 (3496 bp) plasmid. The construct was check for correct alignment by enzymatic restriction [PmeI: po6k-ie-SVTi207 (2249 bp, 2013 bp, 653 bp, 484 bp) and vector control po6k-ie-SVTM53 (2249 bp, 2013 bp, 1122 bp)] and sequencing (M53for1, M53rev1 primers – for i207 ORF insertion; SV40for and BGHrev primers – for the right sense of insertion into the vector).

- The **po6k-ie-SVTs309i128**, clones 2 (5265 bp) was obtained by ligation of 4700 bp fragment - derived from Acc65I/BspMI digested po6k-ie-SVTs309 repl.2 (5250 bp) vector - with the 565 bp fragment - derived from Acc65I/BspMI digested “PCR 128” PCR product (940 bp). The construct was

check for correct alignment by enzymatic restriction [PmeI: po6k-ie-SVTs309-i128 (2249 bp, 2013 bp, 582 bp, 421 bp) and vector control po6k-ieSVTs309 (2249 bp, 2013 bp, 988 bp)] and sequencing (M53for1, M53rev1 primers – for s309i128 ORF insertion; SV40for and BGHrev primers – for the right sense of insertion into the vector).

4.3 Propagation of electro-competent Escherichia coli cells

- inoculate over night (ON) at 37^{o}C or 30^{o}C Escherichia coli strains (E. coli: DH10B or PIR1) which carry or not the pCP20 temperature sensitive ampicillin resistant plasmid (touch the glycerol stock with a sterile tip and resuspend the cells in 2 ml prewarmed LB buffer);
- inoculate few microliters from the ON culture in approximately 200 ml prewarmed buffer and cultivate to a 0.3-0.5 O.D. 600;
- keep on ice 10 min. to stop the multiplication of bacteria;
- centrifuge the cells 10 min. at 6000-8000 rpm;
- resuspend and wash cells in prechilled 10% sterile glycerol for 10 min. at 6000-8000 rpm; repeat this step 2-3x;
- resuspend cells in the rest of glycerol or in 800-1000 μl 10% glycerol;
- aliquot 60-100 μl cells in 0.5-1.5 ml Eppendorf tubes;
- snap-freeze cells in liquid nitrogen;
- store for long term at - 80^{o}C (steps 5, 6 and 7 on ice).

(Applications: PIR1, DH10B, -ΔM53FRT/pCP20; -C3xFRT/pCP20.)

4.4 Electro-transformation of Escherichia coli strains

- thaw on ice for 10 min. to achieve electro-competent cells (with or without the wt MCMV BAC, chloramphenicol resistant) in approximately 60-100 μl of the 50% glycerol stock solution;
- pipette plasmid DNA over bacterial cells and keep on ice 30 min;
- put samples at 42-43^{o}C for 1-1.5 min.;
- remove samples from the heating block and let them again on ice 2 min.;
- add 1 ml LB medium;
- shake the bacterial cells at 30^{o}C or 37^{o}C, 1400 rpm for 1 h (the temperature depends on the plasmid sensitivity);

- spin down the culture 1 min. at 6000 rpm;

- remove the supernatant keeping the last 100 μl of LB;

- resuspend the cells in the rest of LB and plate out few micro liters on LB-agar plates supplemented with the necessary antibiotics.

(Applications: on DH10B ΔM53FRT/pCP20; C3xFRT/pCP20-/pKD46.)

4.5 Flp recombination

- thaw on ice electrocompetent cells which carry wt MCMV BAC (chloramphenicol resistant) and pCP20 plasmid (ampicillin resistant), approximately 60-100 μl of the 50% glycerol solution;

- add 20 ng DNA for each rescue-plasmid (zeocin resistant) which carry the M53 mutant of interest for the flip-in at an ectopic position of the wt MCMV BAC; (first dilute each plasmid maxi prep to 10 ng/μl final concentration).

- electroporate cells for 2500 KV (Gene Pulser, Bio Rad);

- add 1 ml LB-Zeo medium to the cells with at least 4.5 Ω resistances and mix by pipetting;

- mix samples by shaking (1400 rpm) at 30^{o}C for 1 h;

- plate 50 μl cells culture on chloramphenicol/zeocin (cam. 25 μg/ml - zeo.15 μg/ml) LB-agar plates and incubate ON at 43^{o}C;

- pick single colonies 24 h later and inoculate ON at 37^{o}C in LB medium (cam. 25 μg/ml, zeo. 15 μg/ml final concentrations).

Note: Modifications of the original protocol were necessary to reduce the incidence of double or multiple insertions in the ectopic position for the M53 mutants. The total amount of plasmid DNA is reduced from 80 to 20 ng. Shaking is optional because the temperature of 30^{o}C helps pCP20 to express more FLP recombinase, the enzyme which recognizes the FRT sites and facilitates the recombination of the marked fragments. The plated volume of the electroporated cells culture is diminished from 100 to 50 μl.

(Application: flip-in of M53 mutants on the ΔM53FRT, C3xFRT, Δ1-16FRT BACs.)

4.6 Preparation of glycerol stocks for Escherichia coli cells

- inoculate in LB medium ON at 37°C/30°C E. coli strains which carry different ts/antibiotics resistant plasmids;

- mix in 1.5 ml Eppendorf tubes 400 μl 60% sterile glycerol with 600 μl ON culture - and store for long term at - 80°C.

(Application: for all plasmids and BACs mentioned in the Materials chapter 3.7.)

4.7 Low copy plasmids mini preps

- inoculate ON Escherichia coli strains which carry BACs or low copy plasmids (touch the glycerol stock with a sterile tip and resuspend the cells in 10 ml prewarmed LB buffer);

- aliquot 8 ml culture in 15 ml Falcon tubes;

- spin down cells 15 min. at 3500 rpm;

- resuspend cells in 300 μl prechilled P1 buffer (Macherey Nagel) and transfer in 2 ml tubes;

- add 300 μl P2 buffer prewarmed at RT, mix by inversion 6-8 times, and keep 5-10 min. at RT until the suspension becomes transparent which means that the lysis already started;

- add 300 μl prechilled P3 buffer, mix by inversion 6-8 times, and keep 10-15 min. on ice until the proteins will precipitate;

- centrifuge samples 10-12 min. at 14000 rpm, RT; transfer the supernatants in a new 2 ml tubes;

- add 1 ml phenol, mix by inversion at least 80 times and centrifuge for 5 min. at 14000 rpm, RT;

- transfer the upper part in a 2 ml Eppendorf tube, add 900 μl isopropanol and centrifuge 30 min. at 14000 rpm, RT;

- discard the supernatant carefully to do not lose the white pellet, add 500 μl 70% ethanol and centrifuge 30 min. at 14000 rpm, RT;

- discard the supernatant, dry the pellet 5-10 min. at RT and resuspend in 100 μl sterile and double distilled water;

- keep the BAC and the low copy mini preps at + 4°C.

(Application: for all plasmids and BACs mentioned in the Materials chapter 3.7.)

4.8 Low copy plasmid maxi preps

- inoculate ON E. coli strains which carry BACs or low copy plasmids (touch the glycerol stock with a sterile tip and resuspend cells in 200 ml prewarmed LB buffer);
- keep on ice 10 min. to stop the multiplication of bacteria;
- spin down the cells 10 min. at 6000-8000 rpm;
- resuspend the cells in 8 ml prechilled S1 buffer (NucleoBond AX100/Macherey Nagel);
- add 8 ml S2 buffer prewarmed at RT, mix by inversion 6-8 times, and keep for 5-10 min. at RT until the suspension will become transparent which means the lysis already started;
- add 8 ml prechilled S3 buffer, mix by inversion 6-8 times, and keep 10-15 min. on ice until in the suspension will appear a white precipitate;
- simultaneously preequilibrate the AX100 columns with 2.5 ml N2 buffer and prepare the systems for precipitate filtration;
- filtrate the precipitate with Whatmann paper and add the liquid to the columns;
- wash the columns with 12 ml N3 buffer (1 or 2 times);
- discard the solution and add the 5 ml N5 buffer – prewarmed at 50^{o}C - to the column (the filtrate will contain DNA, be carefully!)
- measure the final volume, add to the 5 ml final filtrate 3.5 ml of isopropanol, when a white precipitate appears at the liquid interface mix by inversion carefully;
- centrifuge the samples 30 min. at 12000 rpm; discard carefully the supernatant in order not to loose the pellet;
- wash the pellet with 2 ml 70% ethanol and centrifuge 10 min. at 12000 rpm;
- dry the pellet 5-10 min. at RT and resuspend in 100 μl sterile and double distilled water;
- keep the BAC maxi at + 4^{o}C and the low copy maxi-preps at - 20^{o}C.

(Application: for all plasmids and BACs mentioned in the Materials chapter 3.7.)

4.9 Screening for M53 single insertion mutants at the FRT site of wt MCMV BAC

- thaw at RT the mini or maxi preps;
- prepare 500 μg of each BAC DNA;
- NotI or SpeI digestion in 50 μl finale volume for each BAC DNA, + 4 h at 37^{o}C;

Note: use as positive control wt MCMV BAC without insertion in the ectopic position and as negative control wt MCMV BAC lacking gene M53 in the authentic position.

- run ethidium bromide 0.8% agarose gels in fresh prepared TBE 1x at 60 V, 400 mA, for 19 h;
- expose the gel to UV and take pictures;
- chose clones carrying a single copy of M53 mutated ORF;
- save glycerol stock for all single insertion clones.

(Application: for all BACs mentioned in the Materials chapter 3.7.1.)

4.10 Transfection of MEFs

- thaw new MEFs (mouse embryonic fibroblasts – primary cells) in a water bath at 37^{o}C for few minutes (1 Eppendorf tube for a 10 cm plate);
- wash by pipetting cells of 1 Eppendorf tube into 25 ml DMEM 4500 mg glucose - supplemented with 10% FCS (fetal calf serum, inactivated 10 min. at 45^{o}C) and 5 ml P/S (penicillin/streptomycin 10000 U – 10000 µg/ml);
- centrifuge cells 5 min. at 1200 rpm;
- discard media, resuspend cells in 10 ml fresh medium and incubate at 37^{o}C in a 10 cm plate;

Note: MEFs need 2 days to reach 100% confluence.

- split the MEF 1:3.5:

1. remove the spent medium;
2. wash with 10 ml PBS (saline phosphate buffer);
3. remove the PBS and add 1 ml 0,25% trypsin – 1 mM EDTA; wait for cells detach;
4. add 10 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml), wash the dish surface at least 5 times;
5. split cells from one 10 cm plate to seven 6 cm plates (1:3.5);
6. incubate MEFs ON at 37^{o}C;

- use 1500 ng BAC DNA of each M53 mutant:

1. dilute 1500 ng from each BAC DNA - carrying a mutant in ectopic position – in 150 µl simple DMEM and add 10 µl Superfect reagent;
2. mix by tapping, let 10-15 min. to RT;

3. add 1 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml) and mix by inversion;

- transfection of MEFs:

1. remove spent medium, wash with 5 ml PBS;
2. remove PBS, add medium with BAC-Superfect;
3. incubate at least 2.5 h at 37^{o}C;
4. remove the medium with BAC DNA, add 5 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml);
5. incubate ON at 37^{o}C;

- split transfected MEFs 1:3.5:

1. remove spent medium, wash with 5 ml PBS;
2. remove the PBS, add 500 µl 0,25% trypsin – 1 mM EDTA; wait for cells detach;
3. add 5 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml), wash the surface of the plate at least 5 times;
4. split cells from one 6 cm plate to one 10 cm plates (1:3.5);
5. incubate MEFs at 37^{o}C , wait until first plaques appear;

- split 1:2 the transfected plates without plaques;
- replace medium in negative plates after 10 days or when the medium changes pH;
- wait for the total lysis in positive plates;
- take the supernatant of positive plates;
- store virus supernatants at – 80^{o}C.

(Application: for all BACs mentioned in the Material chapter 3.7.1.)

4. 11 Infection of M2-10B4 cells

- split the uninfected M2-10B4 cells 1:4:

1. remove medium;
2. wash with 20 ml PBS (saline phosphate buffer);
3. remove PBS and add 2 ml 0,25% trypsin – 1 mM EDTA; wait for cells detach;

4. add 20 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q/ glutamine 100x), wash the surface of the plate at least 5 times;
5. split cells from one 14.5 cm plate into four 14.5 cm plates;
6. incubate M2-10B4 cells ON at 37^{o}C;

(Note: M2-10B4cells need 2 days to reach 40-80% confluence.)

- infection of M2-10B4 cells:

1. add 2 ml of MEFs virus supernatant;
2. incubate ON at 37^{o}C;

(Note: the virus will need 1-2 weeks to lyse the entire culture).

- split the infected M2-10B4 cells 1:2:

1. take the M2-10B4 supernatant, approximately 20 ml;
2. wash with 20 ml PBS;
3. remove PBS and add 2 ml 0,25% trypsin – 1 mM EDTA; wait for cells detach;
4. add 20 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q 100x), wash the surface of the plate at least 5 times;
5. split cells from one 14.5 cm plate to two 14.5 cm plates;
6. incubate M2-10B cells at 37^{o}C , wait for lysis of the entire culture;

- store virus containing supernatants for further procedures at – 80^{o}C.

(Application: for all viruses mentioned in the Materials chapter 3.8a.)

4.12 Preparation of virus inoculums

- split the uninfected M2-10B4 cells 1:6:

1. remove medium, add 20 ml PBS;
2. remove PBS, add 2 ml 0,25% trypsin – 1 mM EDTA; wait for cells detach;
3. add 20 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q 100x), wash plates at least 5 times;
4. split cells from one 14.5 cm plate to six 14.5 cm plates;

5. incubate M2-10B4 cells 2-3 days at 37°C;

- Infection of M2-10B4 cells:

1. add 2 ml M2-10B4 virus supernatant to the medium;

(Note: for each virus mutant infect three 14.5 cm plates)

2. incubate M2-10B4 cells at 37°C , wait for the lysis of the entire culture;
3. store virus containing supernatants for further procedures at – 80°C.

(Application: for all virus mutants mentioned in the Materials chapter 3.8 b).

4.13 Semi-quantitative conditional expression

- split the M2-10B4 cells 1:4:

1. remove medium;
2. wash with 20 ml PBS;
3. remove PBS and add 2 ml 0,25% trypsin – 1 mM EDTA; wait for cells detach;
4. add 20 ml fresh medium (DMEM, 10%FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q 100x), wash plates at least 5 times;
5. split cells 1:4; add 5 ml cells suspensions to a final volume of 25 ml medium (DMEM, 10%FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q 100x - 24 wells plates);

- incubate M2-10B4 cells ON at 37°C;
- then add the M2-10B4 virus;
- incubate the cells for 5 days at 37°C;
- check plates for cytopathic effects (CPE).

Note: The results are read as +/- reaction by plaques formation at different virus dilution. The CPE is expressed as percentages of monolayer lysis.

(Application: for all mutants mentioned in the Materials chapter 3.8b.)

4.14. Virus titration - on MEFs (inoculum and stock)

- split cells 1:2 from 10 cm dish to two 48 wells plates ;
- incubate plates for 1 day at 37°C;

- prepare dilutions: put 5 μl of viral sample into 500 μl of medium in the first row (10^{-2} dilution);

- dilute serially 1:10 the samples on 48 wells plates (easier than using Eppendorf tubes) (50 μl -> 450 μl), 5 times;

- prepare MEFs: vacuum aspire supernatants from 48 well plates with MEF cells;

- transfer 200 μl of diluted samples onto plates with cells (in quadruplicate); go from high to low dilution with one set of tips;

- incubate 1 h at 37°C;

- aspirate supernatant with virus (from high to low dilution);

- add 300-500 μl of methylcellulose to each well with 10 ml plastic pipette;

- incubate 4 days at 37°C;

- read plates at suitable concentration (between 5-50 plaques per well);

- calculate PFU/ml – plaques forming units/ml (formula: plaques x dilution x 5)

(Application: for mutants mentioned in the Materials chapter 3.8 b).

4.15 Viral growth kinetics

MEFs should be seeded into 48 well microtiterplates the day before infection (d_{-1}). Therefore, the cells of one 10 cm dish are trypsinized, diluted in 50 ml medium, and seeded in 2x48 well microtiterplates with 0.5 ml per well (do not forget: two more wells for cell count);

- day of infection = d_o

1. cell count: two wells are trypsinized, centrifuged (5000 rpm, RT, 5 min.) and resuspended in a small volume (about 80 μl - aspirate the medium of the trypsinization step until it is about 80 μl left);

Cell number : wells(2) : number of squares counted x 10 x volume of left medium

(For example 80μl) = n x 10^4 cells per well

2. virus master mix (MMix)preparation for an moi of 0.1 (for example, n x 10^3 PFU/ml);

- moi (multiplicity of infection), number of viral particles (PFU) per number of cells;

- virus master mix, viral prep used for cells infection;

- calculate the viral prep dilution corresponding to the moi of 0.1 when use in total 1 ml of 10 times concentrated MMix (10x MMix; for example n x 10^4 PFU/ml) for all 4 days of experiment, when each sample was analyzed in duplicate in two independent experiments;

3. MEFs infection: aspirate the medium; add 100 μl the 10x MMix to each well/8 wells per virus; incubation 1h at 37°C; aspirate the MMix; add 0.6 ml fresh medium/well +/- 1 μg/ml doxycycline, dox (per day calculate two samples without dox (-dox) and two with dox (+dox)); about 200 μl of MMix are kept and stored at – 80°C as "d_o samples" for titration;

- day 1: harvest "day_1 samples" (4 samples for each virus: 2/-dox and 2/+dox); transfer the medium in an Eppendorf tube; centrifuge (5000 rpm, RT, 5 min.) and transfer the clear supernatant in a new Eppendorf tube; store at – 80°C for titration;

- day 3:

1. harvest "day_3 samples" (4 samples for each virus: 2/-dox and 2/+dox) - see day 1;

2. to the remaining samples of +dox experiments add for a second time 0.5 μl of 1 μg/ml doxycycline per well;

- day 5: harvest "day_5 samples" (4 samples for each virus: 2/-dox and 2/+dox): see day 1.

(Application: for multi step and one step growth curves of all viruses mentioned in the Materials chapter 3.8 b).

4.16 Preparation of virus stocks

- plate out M2-10B4 cells in 14.5 cm dishes at 60% confluence (20 plates or more); alternatively use MEFs (not older then passage 4); confluence can be as high as 90% after 24 h;

- infect cells with ≅ 0.5 ml inoculums per plate (totally ≅ 10 ml supernatant);

- incubate cells for 3-5 days at 37°C;

Note: Complete monolayer with few to any infected cells should be split at a ratio of 1:2.

- harvest cells and supernatants (SN) when cells detach;

Note: All steps need to be done on ice.!

- scrape undetached cells with a cell scraper (Policemen) and collect materials in sterile 250 or 500 ml bottles;

- centrifuge cells and SN (6000 rpm, 15 min.);

- collect SN and put on ice;

- dounce the cell pellet in 4 ml of medium 20 times, in a prechilled douncer;

- centrifuge the resuspended pellet (12000 rpm, 10 min.) and collect the SN;

- pool the SNs from the last two steps;

Note: Skip this step if a pure virus stock is wanted (less cells debris).

- centrifuge SN (13000 rpm, 3 h); now, the virus particles will be in the pellet;

- throw away SN; keep the pellet with a little bit of medium on ice at + 4^{o}C ON;

- dounce the pellet in ≅ 3 ml medium, 20 times, in a prechilled douncer;

- add 10 ml cold 15% sucrose-virus separation buffer (VSB) into a SWB28-rotor tube (autoclaved);

- put virus on the top of sucrose-VSB cushion very carefully!!!

- add cold VSB on the top of viruses and balance tubes;

- ultracentrifuge at least 1 h at 20000 rpm, + 4^{o}C;

- discard the SN, the virus particles are in the pellet;

- add 1-3 ml VSB;

- incubate at + 4^{o}C until the pellet starts to dissolve (*can be left ON*);

- resuspend pellet thoroughly (1 ml blue sterile tips);

- alternative: centrifuge 3 min. at 3000 rpm in an Eppendorf centrifuge to remove residual cells debris;

- aliquot in 100 μl aliquots (few also in 50 μl);

(Application: mutants mentioned in the Materials chapter 3.8b and Table 5).

MCMV viruses	PFU/ml
wt	$1.1x10^{8}$
M53R	$5.5x10^{7}$
s309R	$5.5x10^{8}$
i207R	$1,35x10^{7}$
i313R	$2.2x10^{7}$
i321R	$1.87x10^{8}$
FlagM53R	$6x10^{9}$
Flag309R	$1,85x10^{7}$
FlagΔSak309R	$3.9x10^{8}$
s309i128R	$1,25x10^{8}$

Table 5. Virus stock preps (by sucrose cushion method).

4.17 293 cells transfection

- split cells 1:4:

1. remove medium;
2. wash with 10 ml PBS (saline phosphate buffer), with care, because 293 cells easily detach from the plate;
3. remove PBS and add 1ml 0,25% trypsin – 1 mM EDTA/10cm dish (or 0.5 ml/6 cm dish); wait for cells detach;
4. add 10 ml fresh medium (DMEM, 10%,FCS, 5 ml P/S 10000 U – 10000 μg/ml, 5 ml Q 100x), rinse plates at least 5 times;
5. split cells from one 10 cm plate to eight 6 cm plates (1:4);

6. incubate 293 cells ON at 37°C;

- use 5000 ng DNA in total per transfection (total DNA amount of all plasmids transfected on the same plate):

1. dilute 5000 (single plasmid) or 2500 ng DNA (each plasmid for co-transfection) in 150 µl DMEM and add 30 µl Superfect reagent;
2. mix by tapping, let 10-15 min. to RT;
3. add 1 ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q 100x) and mix by inversion;

- transfection of 293 cells:

1. remove medium, wash with 5 ml PBS;
2. remove PBS, add medium with plasmid-Superfect;
3. incubate at least 2.5-3 h at 37°C;
4. remove plasmid-Superfect, add 5ml fresh medium (DMEM, 10% FCS, 5 ml P/S 10000 U – 10000 µg/ml, 5 ml Q 100x);
5. incubate ON at 37°C;

- scrape cells with a cell scraper (Policemen) and collect them in sterile 2 ml Eppendorf tube*;*
- centrifuge cells (8000 rpm, 2 min.);
- discard the SN and keep the pellet;
- wash cells with 1 ml sterile PBS (8000 rpm, 10 min.);
- store at – 80°C.

(Application: protein expression studies – po6k-ie, po6k-ie-M50HA, -M53, -s309, -i128, -s309i128, -FlagM53, -Flags309, -FlagΔSak309)

4.18 Infection of NIH3T3 cells

- split the NIH3T3 cells 1:4:

1. remove media;
2. wash with 10 ml PBS (saline phosphate buffer);
3. remove PBS and add 1 ml 0,25% trypsin – 1 mM EDTA; wait for cells detach;

4. add 10 ml fresh medium (DMEM, 10%NCS, 5 ml P/S 10000 U – 10000 µg/ml), rinse plate at least 5 times;
5. split cells from one 10 cm plate to eight 6 cm plates;
6. incubate NIH3T3 cells ON at 37°C;

- Infection of NIH3T3 cells with virus stock at an MOI of 1:

1. add the virus stock to the medium;
2. incubate ON at 37°C;

- collect cells 24 h post infection and store them at – 80°C.

(Application: protein expression of M53 derivates - M53R, s309R, i313R, i321R, i207R, s309i128R, FlagM53R, FlagΔSak309R.)

4.19 SDS-PAGE

Gels and buffers:

- SDS-PAGE 4.5% stacking gel fraction (2.5 ml 1:29 bis/acrylamide 30%; 3.75 ml 4x stacking gel buffer pH 6.8; 9.75 ml distilled water; 100 µl 10% APS; 20 µl TEMED);
- SDS-PAGE separation gel fraction (2.5 ml 4x separation gel buffer pH 8.8; 100 µl 10% APS; 3.3 µl TEMED; the 1:29 bis/acrylamide 30% and distilled water amounts are depending on gel concentration);
- running buffer: (Laemmli: 288 g glycine; 60.6 g Tris; 20 g SDS; add water to 2 liters end volume, pH 8.3 without adjustment);
- 4x sample buffer (6% (v/v) SDS; 40% (v/v) glycerol; 0.5 M Tris pH 6.8; 4 % (v/v) β-mercaptoethanol);

Conditions: RT, 160 V, ≈ 1 h ± 30 min. (depends on the protein size).

(Application: protein separation after 293 cells transfection and NIH3T3 cells infection.)

4.20 Western-blot analysis

- membrane activation: 5 min. submersion in methanol, 5 min. submersion in water;
- transfer the gel from casting glace to the membrane and blot the DNA for 30 min. at 18 V using blotting buffer (25 mM TRIS, 190 mM glycine, 20% methanol);
- block at least 2 h (ON is recommended) at + 4°C; the unspecific binding for antibodies is blocked with 5% milk powder in TBST buffer (10 mM TRIS pH 8.0, 150 mM NaCl, 0.02% Tween 20);

- wash blot with water at least 2 times (removal of the milk solution);

- wash blot with TBST 2 times;

- incubate blot with primary antibodies (see Table 6) diluted in TBST for 2 h at + 4°C;

Antibodies	Anti-actin	Chroma 101	Saj 1	Sak 1	M50	M2	Anti-HA
Target	actins	IE2	MCP	M53	M50	Flag-tag	HA-tag
Host	rabbit	Mouse	rat	rat	rabbit	mouse	rat
Dilution	1:10000	1:4000	1:2000	1:2000	1:2000	1:4000	1:4000

Table 6. Specificity of the WB primary antibodies.

- wash blot with TBST 2 times;

- wash blot 1 h 30 min. with TBST, changing the buffer every 10 min.; the nonspecifically bound antibodies should be removed;

- incubate blot with secondary horseradish peroxidase coupled goat antibodies (see Table 7) diluted in TBST (1:7500) for 1 h at RT;

Target	actin	IE2	MCP	M53	M50	Flag-tag	HA-tag
Anti - species	Anti-rabbit	Anti-mouse	Anti-rat	Anti-rat	Anti-rabbit	Anti-mouse	Anti-rat

Table 7. Specificity of the WB secondary antibodies.

- wash blot with TBST 2 times;

- wash blot 1 h 30 min. with TBST, changing the buffer every 10 min.; the nonspecific bound secondary antibodies should be removed;

- wash blot 2 times with water;

- develop blot with Amersham ECL Plus Western Blotting Detection System (GE Healthcare);

- the chemiluminescent signal is read out using Amersham Hyperfilm ECL ki and High Performance chemiluminescence film (GE Healthcare);

- scan the film.

(Application: gene products detection for M50HA (≅ 35 kDa), M53 (38 kDa), i128 (38 kDa), s309 (34-35 kDa), s309i128 (≅ 34-35 kDa), FlagM53 (40 kDa), Flag309 (≅ 34-35 kDa), FlagΔSak309 (32-33 kDa), pp89 (IE2, 89 kDa), pp86 (MCP, 150 kDa) gene products.)

4.21 HA-tag pulldown

Note: this protocol was optimized to prepare protein complexes from LYS-450 supernatants of MCMV infected NIH3T3 cells or from M50HA-M53 transfected 293 cells; for others cells or sub cellular compartments then nucleus, one should optimized the salt concentration of the used lysis buffer.

- cell lysis (work on ice, takes around 1 h 30 min.):

1. infect or transfect cells in 6 cm dishes (semi-confluent cells);
2. remove the medium, collect the cells and proceed immediately for lysis or snap freeze;
3. wash cells with 2 ml prechilled PBS;
4. pellet the cells (6000 rpm, + 4°C, 3 min.), remove the supernatant and lyse cells with 600 µl of LYS-450 in presence of Benzonase (Novagen) on ice for 90 min. (keep 10% of the cells for protein expression analysis);
5. remove cells debris by spinning the lysates at 14000 rpm (full speed) for 20 min. at + 4°C;

- binding of proteins to the HA affinity matrix (work at + 4°C and on ice!):

1. transfer the supernatant to new Eppendorf tubes, add 120 µl HA High Affinity matrix (Roche) and incubate by rolling at + 4°C for 90 min.;
2. wash the matrix 5 times with 1000 µl high salt concentration buffer (450 mM NaCl) and 3 times with 1000 µl low salt concentration buffer (150 mM NaCl) by 3 min. centrifugation at 6000 rpm for each step;
3. resuspend the matrix in 120 µl 4x sample buffer and freeze at - 80°C;

- protein complex analysis: load 50 µl HA matrix beads per well of a SDS-PAGE after denaturation at 95°C for 10 min.

Note: Original HA-tag reference - Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems; Terpe K.; Applied Microbiology Biotechnology; 2003 Jan; 60(5):523-33.

(Application: protein complex detection after co-transfection of 293cells with po6k-ie-M50HA and po6k-ie-M53, -s309, -i128, -s309i128, -FlagM53, or -FlagΔSak309.)

4.22 Southern-blot analysis

- split 100% confluent NIH3T3 cells 1:3.5;
- after 3 h infect the cells at an MOI of 2;
- harvest the cells after 48 hpi;
- extract viral DNA using the cell culture protocol of the DNA Tissue & Blood kit (QIAGEN);
- quantify the total viral DNA;

- SphI (Pae I, Fermentas) digestion of 3 µg viral DNA or 1 µg wild type MCMV BAC for 48 h at 37°C;

- separate the DNA fragments in a 0.8% agarose gel at 70 V for 16 h;

- transfer DNA fragments from the agarose gel to a positive charged nylon membrane (Amersham Hybond-N+, nylon membranes optimized for nucleic acid transfer):

1. incubate the gel with 0.25 M HCl for 10 min., two times (DNA depurination!);
2. wash the gel with distilled water;
3. incubate the gel with denaturation buffer for 45 min. (improves the negatively charged DNA binding to the positively charged membrane and destroys any residual RNA!);
4. wash the gel with distilled water;
5. incubate the gel with neutralization buffer for 30 min.;
6. repeat step 5 for 10 min.;
7. transfer ON the DNA from gel to the nylon membrane by capillary action of the SSC 20x buffer through a paper tower (2 Whatmann paper sheets soaked in SSC 20x buffer which have the ends hanging into SSC 20x buffer tanks; gel; nylon membrane - avoid air bubbles!; 2 SSC 20x soaked Whatmann paper sheets, on the gel size; 2 dry Whatmann paper sheets, on the gel size; many dry towel paper sheets - carefully arranged to maintain an equal pressure above the gel and membrane!; weight - equal distributed).

- Dig labeled probe (PACK1) synthesis by PCR amplification (PCR DIG Probe Synthesis Kit, Roche);

Note: The PACK1 probe is specific for a unique left terminal fragment of the MCMV genome.

- cross-linking of the DNA to the nylon membrane by 0.125 Joule UV;

Note: A picture of the dried gel is informative for the quality of the DNA transfer.

- wash the membrane with 2x SSC 0.1% SDS buffer for 5 min., two times, at RT;

- wash the membrane with 0.5x SSC 0.1% SDS buffer for 5 min., two times, at 45°C;

Note: 45°C is the optimized hybridization temperature (T_m) of the Dig labeled PCR probe.

T_m = 49.82 + 0.41(%G+C) – (600/l), l = length of hybrid in base pairs; T_{opt} = T_m – 20°C to 25°C.

- incubate the membrane with hybridization buffer (DIG Easy Hybridization Granules, Roche) for 2-3 h at 45°C;

- denaturate the probe for 10 min. at 95°C;

Note: Use about 50 ng probe/20 ml hybridization buffer.

- incubate the membrane with PACK1/hybridization buffer ON at 45°C;

Note: the PACK1/hybridization buffer can be re-used 3-4 times when stored at - 20°C.

- develop the blot with the DIG Luminescent Detection kit (Roche);

Note: Keeping the membrane in CPSD chemiluminescent buffer at 37°C increases signal strength.

- monitor the chemiluminescent signal using the Amersham Hyperfilm ECL, High Performance chemiluminescence film (GE Healthcare).

(Applications: detection of concatemeric and unit length genomes at 48 h post infection of NIH3T3 cells with wt MCMV, M53R, s309R, i313R, i321R, i207R, FlagM53R, FlagΔSak309R, s309i128R viruses and 24 h post transfection of 293 cells with the wt MCMV BAC.)

4.23 Confocal microscopy and indirect immunofluorescent staining

- NIH3T3 cells infection:

1. prepare 12 wells plates with sterile cover slips, one cover slip per well;
2. split 100% confluent cells 1:3.5 on 7 plates of 12 wells;
3. after 24 h in culture infect the cells at an MOI of 0.5 or 0.2;

- cell staining:

1. rinse cells with PBS;
2. fix cells with 3% paraformaldehyde (PFA) in PBS for 15 min. at RT;

   Note: PFA is very toxic!

3. rinse cells with PBS and quench them with NH4Cl solution for 10 min. at RT;
4. rinse cells with PBS and treat them with 0.3% Triton X 100 in PBS for 10 min. at RT for permeabilization;
5. rinse cells with PBS and block unspecific binding of antibodies:

a. for primary antibody with 0.2% fish gelatin in PBS for 10 min. at RT;

b. for secondary antibody with goat serum (1:100 in PBS) for 10 min. at RT;

- incubation of primary *antibodies* (*see Table 8*) for 1 h at 37°C;

Name	Sak 1	αM50	M2
Dilution/moi 0.2	1:300	1:300	1:500
Dilution/moi 0.5	1:150	1:150	1:300

Table 8. Dilution of the primary antibodies used for immunofluorescence analysis

Note: Antibodies dilutions depend on the MOI and specific-reactivity. The ones used for NEC intranuclear localization were an M53 specific rat polyclonal antiserum (Sak 1), an M50 specific rabbit monoclonal antiserum (αM50) and a Flag specific mouse monoclonal antiserum (M2, Sigma).

- wash cells in a large volume of PBS for 10 min. at RT under moderate shaking conditions;

- incubate secondary fluorochrome coupled antibodies (goat IgG) and iodide (TOPO-PRO-3) for 1 h at 37^{o}C (see Table 9);

- rinse cells with distilled water and absolute ethanol;

- dry the cover slips;

- embedding (Histogel, Linaris) of the cells at + 4^{o}C ON in the dark room;

Note: Alexa flurochromes are light sensitive!

- scan stained cells using the LSM510 program with an Axiovert confocal microscope from Zeiss;

- keep slides up to 1 month at + 4^{o}C or store at - 20^{o}C in the dark room.

Type of antibody	H+L	H+L	H+L	F_{ab}	None - iodide -
Anti-species	rat	rabbit	mouse	mouse	-
Target	M53	M50	Flag-tag	Flag-tag	Genomic DNA
Dilution/moi 0.2	1:300	1:300	1:500	1:300	1:1000
Dilution/moi 0.5	1:300	1:300	1:800	1:500	1:1000
Fluorochrom	Alexa 488	Alexa 633 or Alexa 555	Alexa 555	Cy3	Cyanine
Laser	Ar 488	He-Ne 633 or 543	He-Ne 543	Ar 514	He-Ne 633
Excitation	495	632 or 553	553	550	642
Emission	519	647 or 568	568	570	660
Filter	BP475-525	BP505-550	BP505-550	BP505-550	LP560
Assigned color	green	red	white	white	blue

Table 9. Secondary antibodies and iodide used for immunofluorescence analysis.

(Applications: analysis of intracellular distribution of the cellular/viral DNA (TO-PRO-3, iodide) and viral proteins, such as M50, M53, FlagM53, FlagΔSak309, s309i128 after 24 h or 48 h post infection of NIH3T3 cells.)

5. Results

5.1 Genetic screen for inhibitory M53 mutants

Nuclear egress of herpesvirus nucleocapsids is governed by a protein complex that is formed by members of the UL34 and UL31 families and called the nuclear egress complex (NEC). In MCMV, these are represented by the M50 and M53 proteins. Both are interacting at the inner nuclear membrane (INM) of infected cells and promote a partial disassembly of the lamin meshwork and reorganization of the nuclear envelope that leads to the primary envelopment of the nucleocapsids. The mechanism of the NEC action and the crosstalk between the viral and cellular components involved in the nuclear export of the capsid are not fully understood.

In order to investigate the M53 and M50 gene products, we applied a genetic screen based cis-complementation of BAC based null mutants to functionally map the important sites and domains (Loetzerich *et. al*., 2006; Bubeck *et. al.*, 2004). The genetic complementation procedure was stepwise executed in E. coli and is exemplified by the analysis depicted for M53 in Figure 5A. Deletion of the endogenous M53 copy induced a null-phenotype which could be rescued by ectopic expression of the wild type (wt) allele. This proved that M53 is essential for MCMV replication (Loetzerich *et. al*., 2006).

Next, the whole ORF was subjected to a Tn7 random mutagenesis procedure generating 5 aa insertion mutants and stop codon formation. The mutants were introduced into the BAC lacking native M53 by FRT/Flp recombination (Bubeck *et. al*., 2004). Single copy insertion clones were selected by enzymatic restriction with NotI (see Figure 6) and viruses were reconstituted. The comprehensive mutational analysis of the M53 gene (Loetzerich *et. al.*, 2006) identified a large number of non-

viable mutants which have accumulated at the C terminal part of the protein, where no important functions have been mapped.

In a second step, the non functional mutants, that may not compensate for the lack of the native M53 gene, have been reintroduced one by one into the wild type MCMV BAC to screen whether any of them had inhibitory features in the presence of the native M53 gene (see Figure 5B). We expected that the majority of the mutants will not interfere with viral progeny production. Yet, potential dominant negative mutants should inhibit the virus reconstitution.

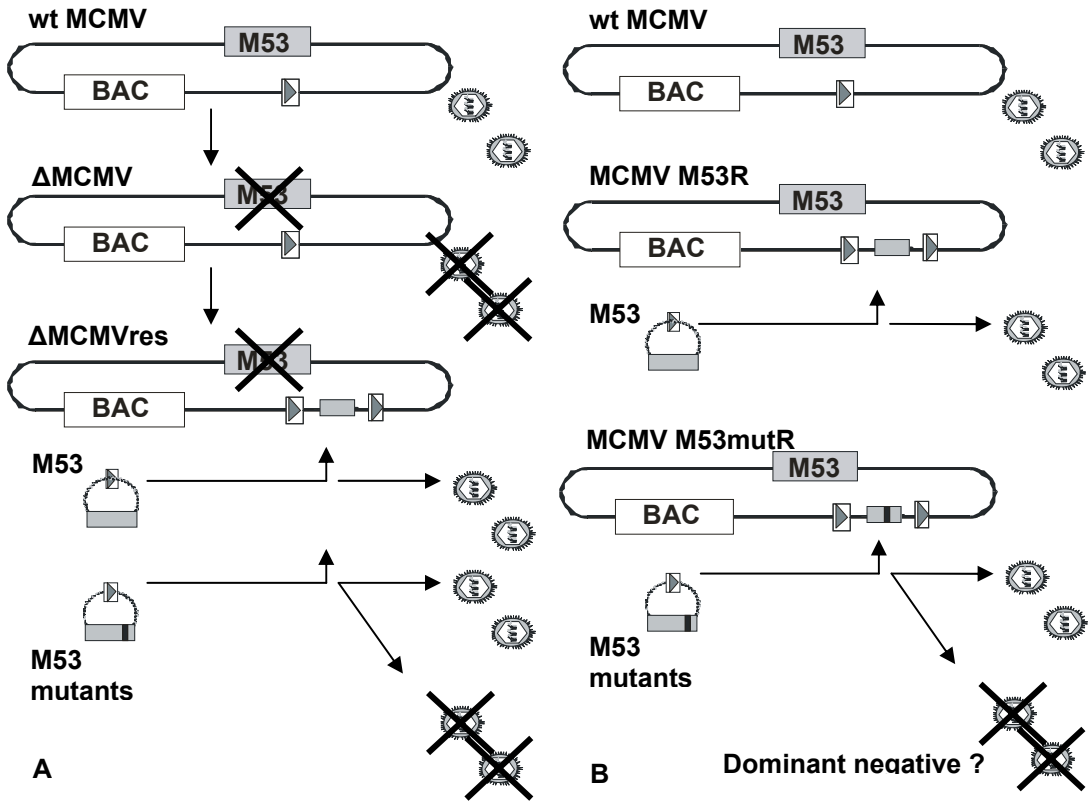

Figure 5. Genetic analysis of the MCMV genes in the virus context. A. Cis-complementation assay (modified after Bubeck *et. al*., 2004). DNA of different MCMV BACs was extracted from the DH10B bacterial cells and used to transfect mouse embryonic fibroblasts (MEFs). Wild type M53 was deleted by ET cloning (homologous recombination catalyzed by RecE and RecT recombinases) from the endogenous position of an FRT-MCMV BAC (wt MCMV) generating a null-phenotype. Reintroduction of the wt M53 ORF into the M53 deletion BAC (ΔMCMV) at the m16-m17 FRT ectopic position, via an FRT/Flp recombination system, rescued the virus reconstitution (ΔMCMVres), visualized by plaque assay. Insertion of different M53 mutants, produced by 5 aa insertions into M53 ORF, was screened by virus reconstitution. B. Inhibitory mutants screen. All non-viable mutants were screened in a second complementation assay. This time the whole wt MCMV genome was used as recipient (wt MCMV). Wild type M53 allele expression from the m16-m17 FRT ectopic position of the BAC (MCMV M53R) had no effect on virus reconstitution. M53 mutants that inhibited virus reconstitution in the presence of the endogenous expressed M53 (MCMV M53mutR) represent the potential dominant negative mutants (DNs).

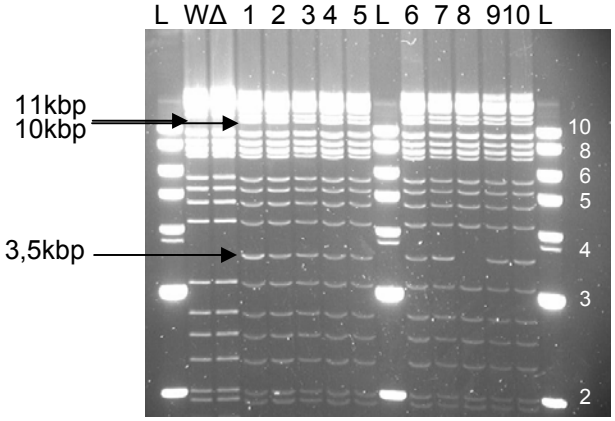

Figure 6. Selection of recombinant MCMV BACs carrying a single M53 copy as ectopic insertion. After flip-in of the M53 mutants at the m16-m17FRT site (11 kbp arrow) into the wild type MCMV BAC (W) the single ectopic insertion mutants (10 kbp arrow) were selected.Single insertions can be discriminated from double or multiple insertions (3.5 kbp arrow) by digestion with NotI endonuclease. A representative example for stop mutant 233 (s233) is the clone number 8, the selected candidate for virus reconstitution analysis. L – 1 kbp DNA ladder; Δ – MCMV BAC with M53 deletion from the endogenous position; numbers 1-10 – clones of the MCMV BAC carrying single or multiple ectopic insertions of s233 mutant.

In order to identify the dominant negative mutants of M53, a total of 47 mutants, spread along all four C conserved regions (CR) of the M53 sequence, were individually tested for virus reconstitution in the presence of the native M53 gene.

Viable insertion mutants covering all four conserved regions (i115, i128, i131, i138, i146, i154, i161, i/s168, s185, i201, i212, i227, s233, i234, i236, i244, i251, H262S, i264, i266, s290, s294, i302, i303, s331) were reconstituted after 5 days post transfection and lysed the whole cells monolayer as the wt allele did (see Tab. 10, column “Viable”). The mutants which had blocked the virus reconstitution accumulated in the C terminal conserved regions 3 and 4, and are represented by a group of 4 stop (s303, s309, s321, s327) and 9 insertion mutants (i259, i272, i292, i307, i313, i321, i324, i325, i326) (see Tab. 10, column “Complete inhibitory”). In total, a group of 10 stop mutants, one point mutation (H262S) and 36 insertion mutants were tested for the capacity to interfere with the virus reconstitution.

A block of 7 insertion mutants, located to the CR2 (i182, i191, i195, i198, i207, i217, i220) and one from the third conserved region (i281) showed an intermediate

phenotype (see Table 10, column “Partial inhibitory”). In this case the reconstitution resulted in severely delayed plaque formation.

Nr.	Mutant	Conserved region (CR)	Viable	Partial inhibitory	Complete inhibitory
1.	i115	CR1	√		
2.	i128	CR1	√		
3.	i131	CR1	√		
4.	i138	CR1	√		
5.	i146	CR1	√		
6.	i154	CR1	√		
7.	i161	CR1	√		
8.	i168	CR1	√		
9.	s168	CR1	√		
10.	i182	CR1-CR2 loop		√	
11.	s185	CR1-CR2 loop	√		
12.	i191	CR2		√	
13.	i195	CR2		√	
14.	i198	CR2		√	
15.	i201	CR2	√		
16.	i207	CR2		√	
17.	i212	CR2	√		
18.	i217	CR2-CR3 loop		√	
19.	i220	CR2-CR3 loop		√	
20.	i227	CR3	√		
21.	s233	CR3	√		
22.	i234	CR3	√		
23.	i236	CR3	√		
24.	i244	CR3	√		
25.	i251	CR3	√		
26.	i259	CR3			√
27.	H262S	CR3	√		
28.	i264	CR3	√		
29.	i266	CR3	√		
30.	i272	CR3			√
31.	i281	CR3-CR4 loop		√	
32.	s290	CR3	√		
33.	i292	CR3			√
34.	s294	CR3	√		
35.	i302	CR4	√		
36.	i303	CR4	√		
37.	s303	CR4			√
38.	i307	CR4			√
39.	s309	CR4			√
40.	i313	CR4			√
41.	i321	CR4			√
42.	s321	CR4			√
43.	i324	CR4			√
44.	i325	CR4			√
45.	i326	CR4			√
46.	s327	CR4			√
47.	s331	CR4			√

Table 10. The M53 dominant negative mutants screen. Mutants were selected from the non-viable mutant pool of the cis-complementation screen (Loetzerich *et. al*., 2006). Four mutants were created by the site directed mutagenesis, via PCR (one point mutation – H262S and 3 truncated versions by insertion of a stop codon - s303, s321 and s327). After inhibitory screen, mutants were separated into three groups: viable, partial and complete inhibitory. i, insertion of 5 aa at the indicated position (numbers); s, stop codon formation at the indicated position (numbers).

5.2 Conditional expression of the inhibitory M53 mutants

Constitutive expression of inhibitory M53 alleles induced a complete block of virus reconstitution. To study the inhibitory phenotype in the context of virus replication conditional expression of the mutants was required. Recently, Brigitte Rupp and collaborators established a system that allows the doxycycline (dox) regulated expression of genes from the cytomegalovirus genome, independent of the viral replication program (Rupp *et. al*., 2005). The principle of the conditional expression approach is depicted in Figure 7. In this system both the regulator and the regulated transcription unit are located in one cassette containing the mutant gene of interest (see Figure 7A). The cassette was inserted at a predefined intergenic region between the non-essential genes m16 and m17 of the MCMV genome, a position that reacted neutral to the in vitro and in vivo insertion by FRT/Flp recombination (see Figure 7B; Rupp *et. al*., 2007/2005).

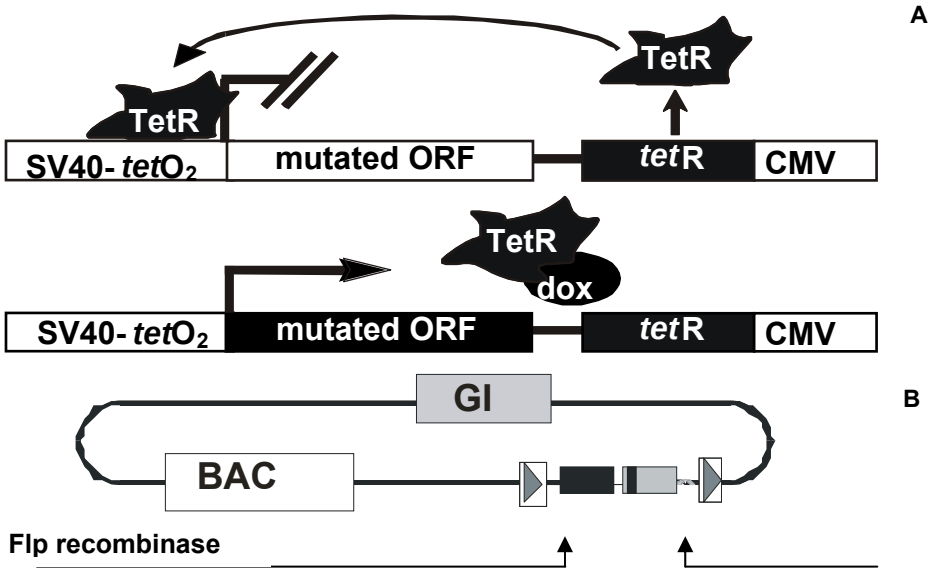

Figure 7. A. Conditional expression cassette (after Rupp *et. al*., 2005). The gene of interest (M53 ORF) is located downstream of an HCMV immediately-early promoter containing 2 minimal tetracycline operator sequences ($tetO_2$) inserted at a 10 bp distance of the TATA box and controlled by the SV40 early-enhancer (SV40). Access of the transcriptional machinery to the mutated ORF is controlled by the tetracycline repressor (TetR). Regulator expression is driven by HCMV immediately-early promoter-enhancer (CMV). Adding of doxycycline (dox) to the system allows the transcription of the gene of interest, due to TetR arrest in a complex with dox. B. Flp recombination (after Rupp *et. al.*, 2005). The regulation cassette can be delivered to the wt MCMV BAC by an FRT/Flp recombination performed in DH10B cells when both constructs carry an FRT site (BAC, wt MCMV BAC; white box with gray triangle, FRT sites; black box, zeocin resistance cassette of conditional cassette plasmid carrier; gray box with a black line, conditional expression cassette carrying the gene of interest).

This conditional expression system was used to study the s309, i313, i321 and i207 inhibitory mutants' phenotype. As a control it was constructed a dox-inducible expression cassette for the wt M53 gene. Individual constructs were integrated into the MCMV genome by site specific recombination at the FRT site between the m16-m17 genes. The recombinant viruses (s309R, i313R, i321R, i207R and M53R) were reconstituted by transfection of mouse embryonic fibroblasts (MEFs) in the absence of dox and propagated in M2-10B4 cells.

A semi-quantitative conditional expression assay was performed for each inoculum for selection of clones with a tight regulation. Constructs controlled by the SV40 early-enhancer allowed viral particles production after 5 days post infection (dpi) in the absence of dox (see Figure 8). Recombinant virus (R) expressing a second copy of the wt M53 allele (M53R) induced the formation of the plaque irrespective of the absence or presence of dox. Dox induction of s309 mutant expression resulted in the almost complete block of plaque formation irrespective of inoculums' dilution (see Figure 8).

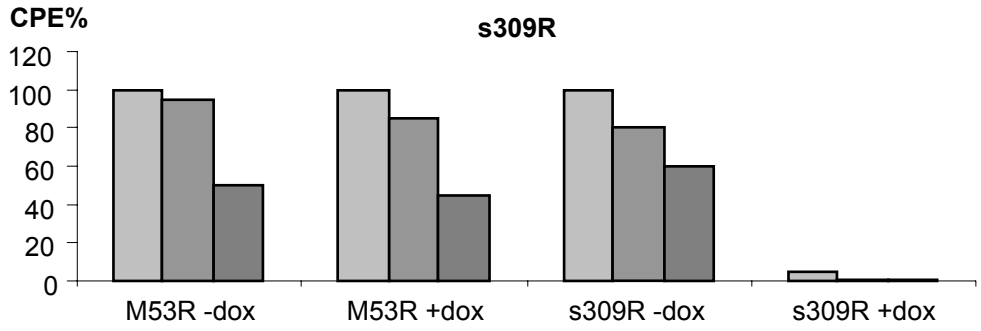

Figure 8. Semi-quantitative analysis of the s309 inhibitory mutant (s309R) effect on the production of viral progeny. M2-10B4 cells were infected for 5 days with 3 virus doses in either the absence (- dox) or presence (+ dox) of 1 µg/ml doxycycline (dox). The shading of gray represents the titration of viral inoculum: light – 1:1, medium - 1:10 and dark gray - 1:100. The virus expressing the wt M53 allele (M53R) represented the control. Cell supernatants were collected o and the cytopathic effects (CPE, expressed as percentages) were determined by titration on MEFs. The bar diagram shows the regulation of s309R virus growth in response to dox administration. Each mutant was analyzed in duplicate in at least two independent experiments.

The conditional effect of the s309R recombinant was compared with that of the CR4 insertion mutants (i313 and i321) and of CR2 (i207). The M53 CR4 and CR2 mutants inhibited virus growth in the presence of dox similar to s309 (see Figure 9) but i207 had a weaker effect.

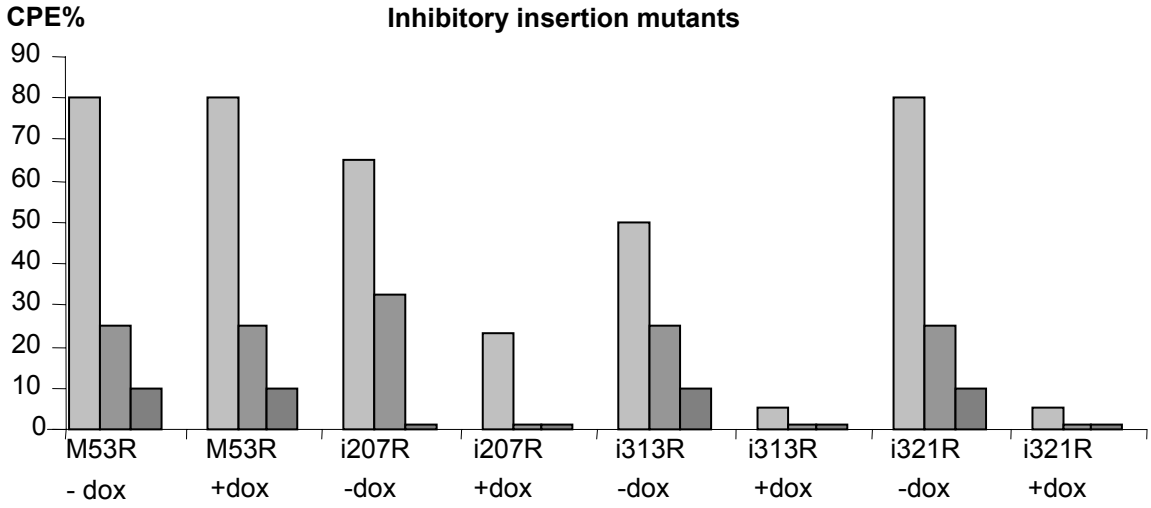

Figure 9. Semi-quantitative analysis of the M53 inhibitory mutants' effect on the production of viral progeny. M2-10B4 cells were infected for 5 days with 3 virus doses in either the absence (- dox) or presence (+ dox) of 1 µg/ml doxycycline (dox). The shading of gray represents the titration of the viral inoculum: light – 1:1, medium - 1:10 and dark gray - 1:100. The virus expressing the wt M53 allele (M53R) represented the control. Cell supernatants were collected and the cytopathic effects (CPE, expressed as percentages) were determined by titration on MEFs. The bar diagram shows the regulation of virus growth in response to dox administration after infection with the indicated recombinant viruses (R). Each mutant was analyzed in duplicate in at least two independent experiments.

Therefore, the growth kinetics of all recombinants were reevaluated under multi-step growth conditions by M2-10B4 cells infection at an MOI of 0.1 in the absence and presence of dox. Wt MCMV served as a positive control for infection and as negative control for general dox toxicity (see Figure 10A). Expression of a second copy of the wt M53 (see Figure 10B) had no effect on the virus replication. In contrast to the wt protein, s309 mutant blocked virus replication with a power of 6 to 7 orders of magnitude (see Figure 10C). Also the i313R and i321R constructs showed a similar inhibitory effect (see Figures 10D and 10E). Notably, the CR2 i207 mutant inhibited plaque formation only by 2 orders of magnitude (see Figure 10F).

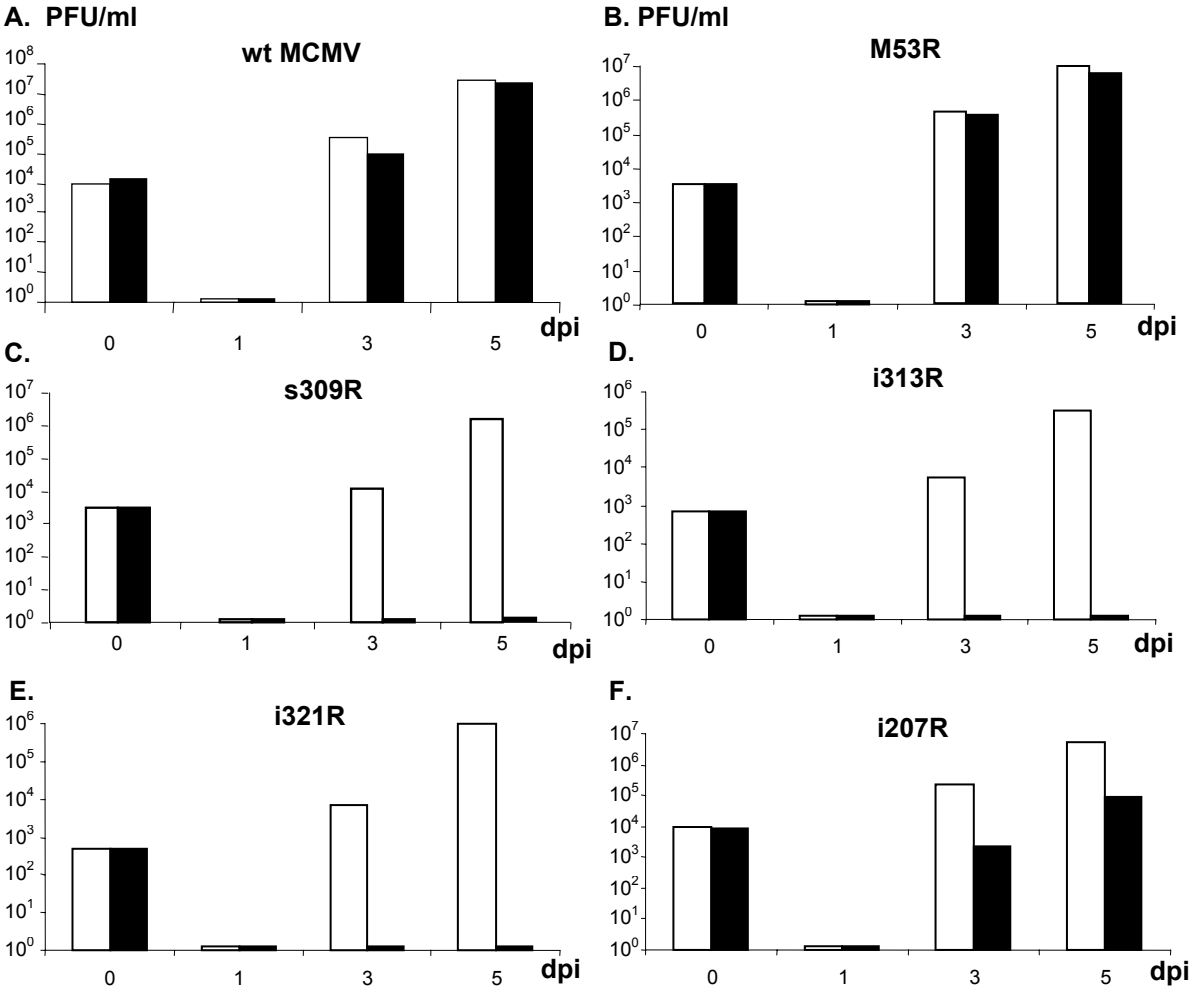

Figure 10. Effects of the M53 inhibitory mutants on the production of viral progeny. M2-10B4 cells were infected for 5 days at an MOI of 0.1 in either the absence (white bars) or in the presence (black bars) of 1 µg/ml dox. Wt MCMV was used as a positive control for infection and a negative control for general dox toxicity. Cell supernatants were collected on the indicated day's post infection (dpi), and the released infectious units (PFU, plaque forming unit) were determined by titration on MEFs. The bar diagram shows the regulation of virus growth in response to dox administration 3 days and 5 days after infection with the indicated recombinant viruses (R). The titer reduction is shown as the difference between the titer in the absence of dox and the titer in the presence of dox. Only titer reductions of >10-fold were considered significant. Each mutant was analyzed in duplicate in at least two independent experiments.

5.3 The stability of the inhibitory effect in different cell types

Previously, we had noticed that DN mutants of viral proteins are acting at a different degree in different cell types (Rupp *et. al*., 2007). In order to study the cell type specificity of the DN s309 we chose three different cell lines: SVEC4-10 mouse lymphoid endothelial cells, NIH3T3 contact inhibited murine fibroblasts and mouse mammary epithelial C127 cells. The cells were infected at an MOI of 0.1 in the absence and presence of dox with M53R and s309R and the virus production was

quantified. The M53R titer at day 5 post infection was about 10^4-10^5 PFU/ml irrespective of dox (see Figure 11 A, C and E). Thus, all three types of cells are less productive than M2-10B4 cells (10^6-10^7 PFU/ml, see Figure 10A).

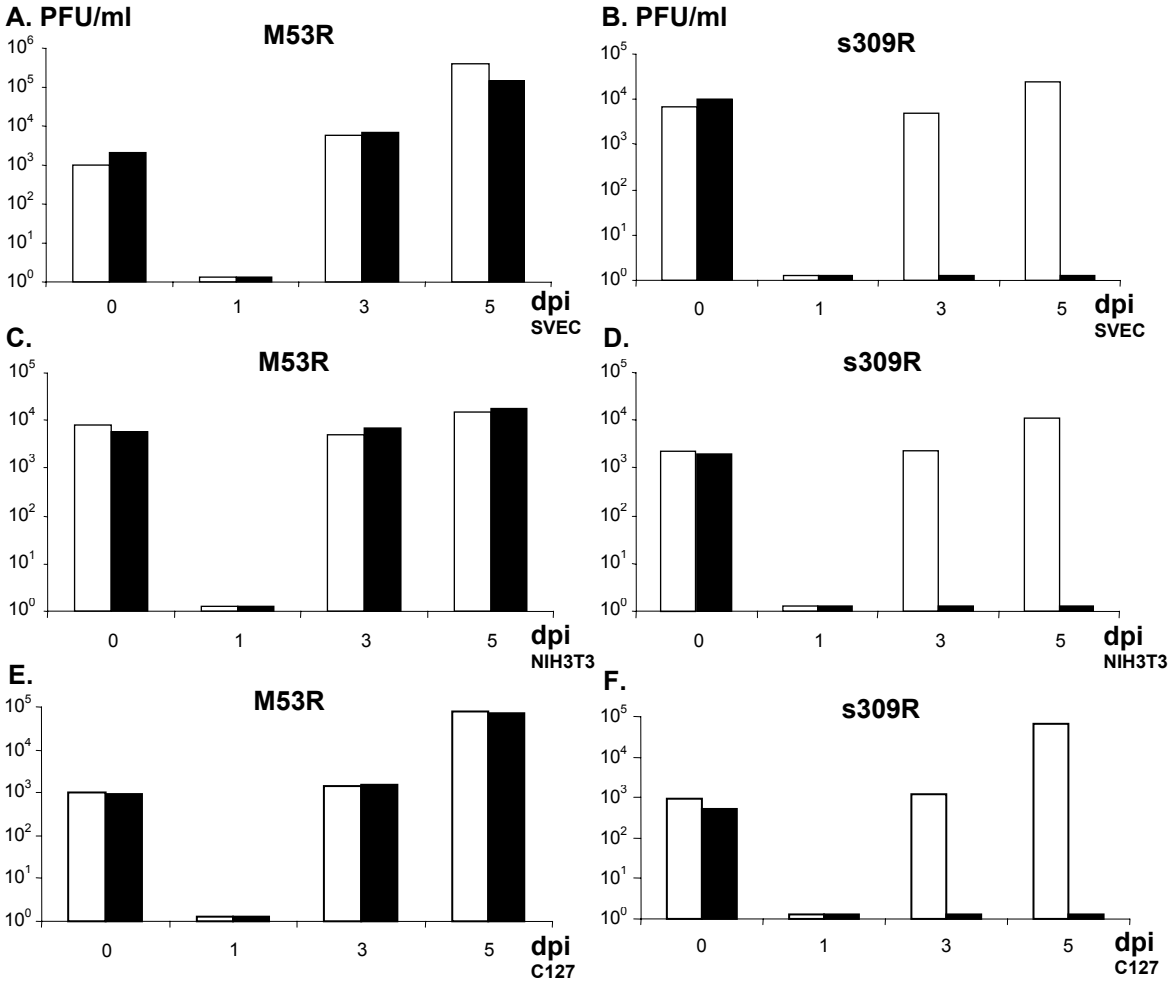

Figure 11. s309R inhibitory phenotype in different cell types. SVEC, NIH3T3 and C127 cells were infected for 5 days at an MOI of 0.1 in either the absence (white bars) or in the presence (black bars) of 1 µg/ml dox. The virus expressing the wt M53 allele (M53R) represented the control for M53 overexpression. Cell supernatants were collected and the released infectious units (PFU, particle per infectious unit) were determined by titration on MEFs. The bar diagram shows the regulation of virus growth in response to dox administration 3 days and 5 days after infection with the s309 recombinant virus (s309R). The titer reduction is shown as the difference between the titer in the absence of dox and the titer in the presence of dox. Only titer reductions of >10-fold were considered significant. Each mutant was analyzed in duplicate in at least two independent experiments. Dpi, day post infection.

The s309R recombinant revealed a similar pattern. Absence of dox allowed productive infection in the same range as M53R (10^4-10^5 PFU/ml). Yet, strong dox dependent inhibition of virus growth of the s309 mutant was comparable in all tested cell lines (see Figure 11 B, D and F).

5.4 The stability of the inhibitory phenotype at high virus load

In order to estimate the power of inhibition we analyzed the viral growth after a single replication cycle. Therefore, two cell types (M2-10B4 and C127) were infected at an MOI of 1 in the absence and presence of dox. The quantification of viral infectious units in the supernatant was done up to 3 days post infection.

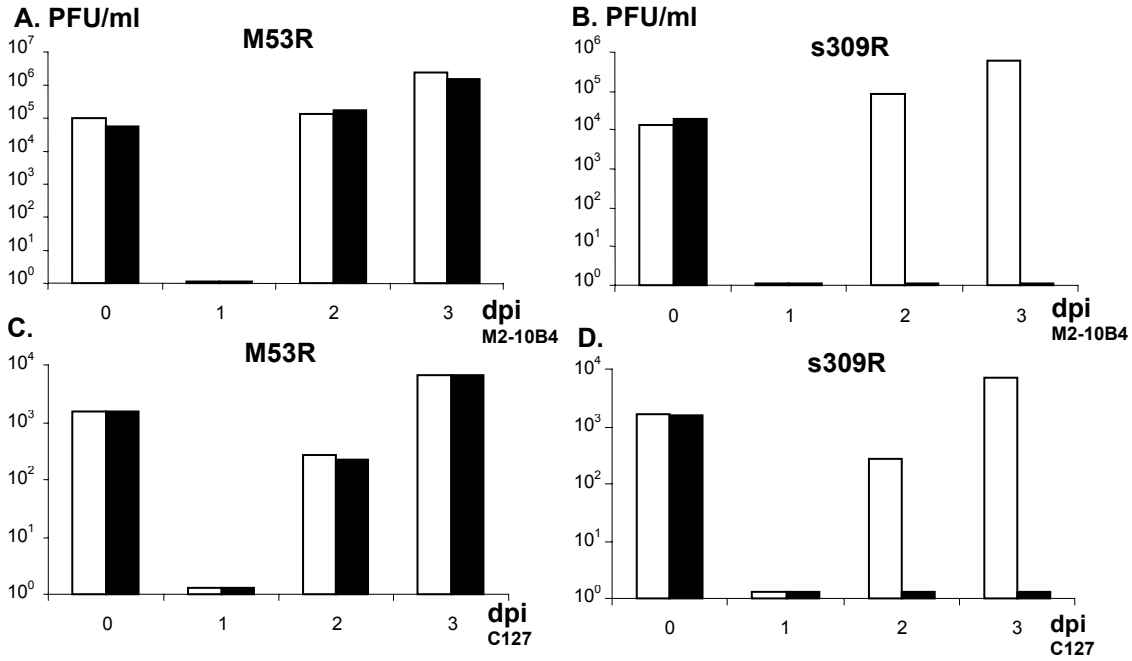

Figure 12. Stability of the s309 inhibitory phenotype. M2-10B4 and C127 cells were infected for 5 days at an MOI of 1 in either the absence (white bars) or the presence (black bars) of 1 µg/ml dox. The virus expressing the wt M53 allele (M53R) represented the negative control for M53 overexpression toxicity at high load. Cell supernatants were collected and the released infectious units (PFU, particle per infectious unit) were determined by titration on MEFs. The bar diagram shows the regulation of virus growth in response to dox administration 3 days and 5 days after infection with the s309 recombinant virus (s309R). The titer reduction is shown as the difference between the titer in the absence of dox and the titer in the presence of dox. Only titer reductions of >10-fold were considered significant. Each mutant was analyzed in duplicate in at least two independent experiments. Dpi, day post infection.

M53R infection of M2-10B4 cells was highly productive, with a titer of 10^6 PFU/ml irrespective of dox (see Figure 12A). In C127 endothelial cells the titer hardly reached 10^4 PFU/ml (see Figure 12C). Induction of s309 resulted in a reduction of viral growth by almost 6 orders of magnitude in M2-10B4 cells (see Figure 12B) and 4 orders of magnitude in C127 (see Figure 12D) cells. Comparing these values with the growth data obtained by multi-step replication of the same viruses (see Figures

10 B/C and 11 E/F) we concluded that the DN s309 mutant blocked virus production in a cell type independent manner.

5.5 Synthesis of the inhibitory protein

The conditional expression of inhibitory mutants of M53 confirmed the qualitative results of the first inhibitory screen. It was of interest to connect the phenotype with the synthesis of the inhibitory protein. Therefore, the production of the wt and the inhibitory proteins was studied by Western-blot.

First, we tested whether the deletion of CR4 affected the capacity of s309 protein to bind to M50, its NEC partner (see Figure 13A). 293 cells were co-transfected with plasmids expressing either the C terminal HA tagged M50 ORF (M50HA, lanes 2 to 5) or variants of M53, namely: wt protein (lane 3), s309 (lane 4) and i128 (lane 5) mutants. i128 lacks M50 binding capacity due to the insertion of 5 aa (Loetzerich *et. al*., 2006) into the M53 binding site and served as control for the loss of binding. The empty carrier vector (po6k-ie, lane 1) represented the negative control for protein expression.

At 24 h post transfection the cells were lysed by a high salt concentration buffer and an HA pulldown was performed. Proteins were separated on a 12% SDS-PAGE and detected by Western Blot. Next, the protein expression was checked in the total lysates (see Figure 13A) and equal lysate load was tested by the level of cellular actin (42 kDa). The s309 protein shifted to 34-35 kDa due of the deletion of the C-terminus of the protein (24 aa).

After HA pulldown similar amounts of M50HA could be detected, irrespective of the binding partners the wt M53 and s309. As expected, i128 mutant (38 kDa) lacking the binding site gave a weak signal. In conclusion, deletion of the CR4 domain did not affect the M50 binding capacity of DN s309.

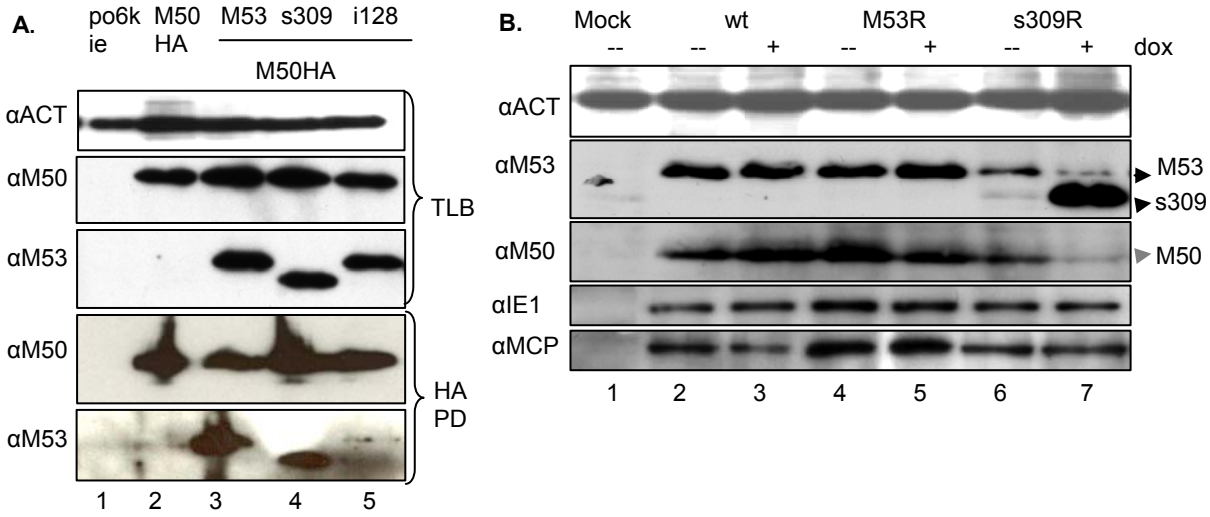

Figure 13 A. The DN s309 binds to M50. 293 cells were transfected with the empty vector pori6-ie (po6kie; lane 1) or the HA-tagged M50 ORF (lane 2) and co-transfected with plasmids expressing HA-tagged M50 (lanes 3 to 5) and the indicated M53 derivatives: wild type M53 (lane 3), s309 (lane 4), and i128 (lane 5). Transfected cells were lysed in total lysis buffer (TLB), and one-third of these lysates were loaded (upper two blots, indicated by TLB) and Western blotted with rabbit anti-M50 antiserum (αM50), rat anti-M53 antiserum (αM53), and rabbit anti-actin antiserum (αACT), respectively. The remaining 70% of the lysates were processed by an HA pulldown. The eluates from the HA columns were split 1:9 and served for Western blot with rabbit anti-M50 antiserum and rat anti-M53 antiserum, respectively (lower two blots, indicated by HA PD). The expected molecular mass of the M50 protein is 35 kDa; the wt M53 and the mutant i128 protein migrate at about 38 kDa and s309 at about 34 to 35 kDa. B. Analysis of viral protein amounts upon induction of DN s309 overexpression. NIH 3T3 cells were mock treated (lane 1) or infected at an MOI of 0.5 with wt MCMV (lanes 2 and 3), with MCMV mutants expressing a second M53 wt allele (M53R; lanes 4 and 5), or DN s309 (s309R; lanes 6 and 7) in the absence (–; lanes 1, 2, 4, and 6) and in the presence (+; lanes 3, 5, and 7) of 1 µg/ml dox. Protein expression was analyzed 24 h after infection by Western blotting with specific antibodies against pp89 (immediately-early protein, 89 kDa; αIE1), M86 (late protein, 150 kDa; αMCP – major capsid protein), M53 (38 kDa), and M50 (35 kDa) (NEC proteins). The dox-induced s309 (34-35 kDa; lower black arrowhead) expression decreased the native M53 signal (upper black arrowhead) and also reduced levels of M50 (gray arrowhead).

Second, we analyzed whether expression of DN s309 would affect the viral protein expression (see Figure 13B). Therefore, NIH3T3 cells were infected for 24 h at an MOI of 0.5 in the absence (lanes 2, 4 and 6) and in the presence of dox (lanes 3, 5 and 7) with M53R (lanes 4 and 5) and s309R (lanes 6 and 7). Uninfected cells (lane 1) served as negative and wt MCMV infection (lanes 2 and 3) as positive control. The load was tested at the cellular actin level.

The IE1 and MCP gene products served as markers for viral immediately-early and late gene expression. The presence of dox had no effect on these proteins for all three viruses: wt MCMV, M53R and s309R (see Figure 13B). Also the detection

levels of M53 and M50 proteins showed no difference in the absence or presence of dox in wt MCMV and M53R infected cells. In case of s309R a clear shift was noticed between M53 (38 kDa) and truncated mutant s309 (34-35 kDa).

The DN protein was already slightly expressed in the absence of dox and DN induction by dox was associated with low levels of M53 and M50. Thus, the inhibitory effect of the s309 mutant on MCMV growth could already be explained by a destabilization of the wt NEC members.

5.6 EM analysis of MCMV recombinant expressing the DN s309

In order to visualize the dominant negative phenotype electron microscopy (EM) studies were performed in collaboration with Christopher Buser at the EM facility (Zentrale Einrichtung Elektronenmikroskopie) of the University of Ulm.

Virus infection of NIH3T3 cells at an MOI of 3 was carried out in the absence and presence of dox. After M53R infection mainly mature C capsids were observed in the nucleus and cytoplasm, irrespective of dox (see Figure 23 A).

In contrast, dox induced s309 overexpression determined a nuclear accumulation of different capsid forms associated with a complete block of nuclear egress (see Figure 23 B to D). The block of nuclear egress was in concordance with the reduction of the NEC members, while the different capsid forms suggested an additional effect on nucleocapsid maturation.

5.7 Tagging of the M53 ORF

5.7.1 Flag-tagged M53 constructs hold the inhibitory feature

To further investigate the s309 effect, we wanted to label the DN protein in order to differentiate between the mutant and the wt gene product. Initially, a TAP-tagged s309 construct was checked for inhibitory feature on the virus context. This mutant had lost the DN effect (data not shown) and we looked for another tag which would preserve function. Therefore, the short Flag-tag (8 aa: Asp, Tyr, Lys, Asp, Asp, Asp, Asp, Lys; Terpe, 2003) was inserted at the N terminus of the s309 ORF (Flag309). To assess non-specific effects of the Flag-tag insertion, the wt M53 ORF was modified accordingly (FlagM53, see Figure 14). In order to discriminate between FlagM53 and Flag309 in the MCMV context the M53 antibody (Sak) binding motif of s309 ORF was deleted by PCR, resulting in FlagΔSak309 (see Figure 14).

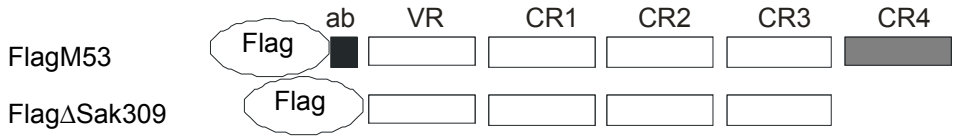

Figure 14. Schematic representation of the Flag-tagged M53 derivates. Depicted are the FlagM53 and FlagΔSak309 ORFs' domains. The N-terminal site (ab) recognized by the M53-specific serum (Sak), the variable region (VR), and the C-terminal four conserved regions (CR1 to CR4) are indicated. The N-terminal end of the s309 mutant was replaced by a Flag-tag (Flag circle). This construct (FlagΔSak309) is not recognized by the anti-M53 serum.

First, the Flag-tagged proteins expression was checked (see Figure 15A). 293 cells were co-transfected with vector plasmids carrying either Flag-tagged proteins (FlagM53 – lane 3, Flag309 – lane 4 and FlagΔSak309 – lane 5) or the C terminal HA tagged M50 (M50HA, lanes 2 to 5). The po6k-ie empty vector served as negative control (lane 1). The load was tested by the cellular actin level. As expected, anti-M53 antibodies (Sak, αM53) allowed M53 detection (FlagM53 – 40 kDa, Flag309 – 34-35 kDa), whereas FlagΔSak309 (32-33 kDa) was identified by anti-Flag antibody (Figure 15A). Rabbit polyclonal anti-M50 antibodies (αM50) detected the NEC

partner. The Flag-tag had no effect on protein expression. An HA pulldown was performed and the eluted samples were probed for Flag-tagged proteins (see Figure 15B). Deletion of M53 antibody epitope and Flag-tagging of DN s309 did not influence the M50 binding capacity of FlagΔSak309 but the Flag-tag was affecting the anti-M53 antibody binding in case of Flag309 (compare Figure 13A lane 4 and Figure 15B lane 4). Next, MCMV BACs expressing the Flag-tagged ORFs were constructed and checked for virus reconstitution. FlagM53 and FlagΔSak309 mutants induced comparable effects to the untagged proteins. FlagM53 MCMV BAC reconstituted after 5 days and lysed a complete MEFs monolayer in 2 weeks post transfection whereas FlagΔSak309 MCMV BAC blocked virus reconstitution (data not shown).

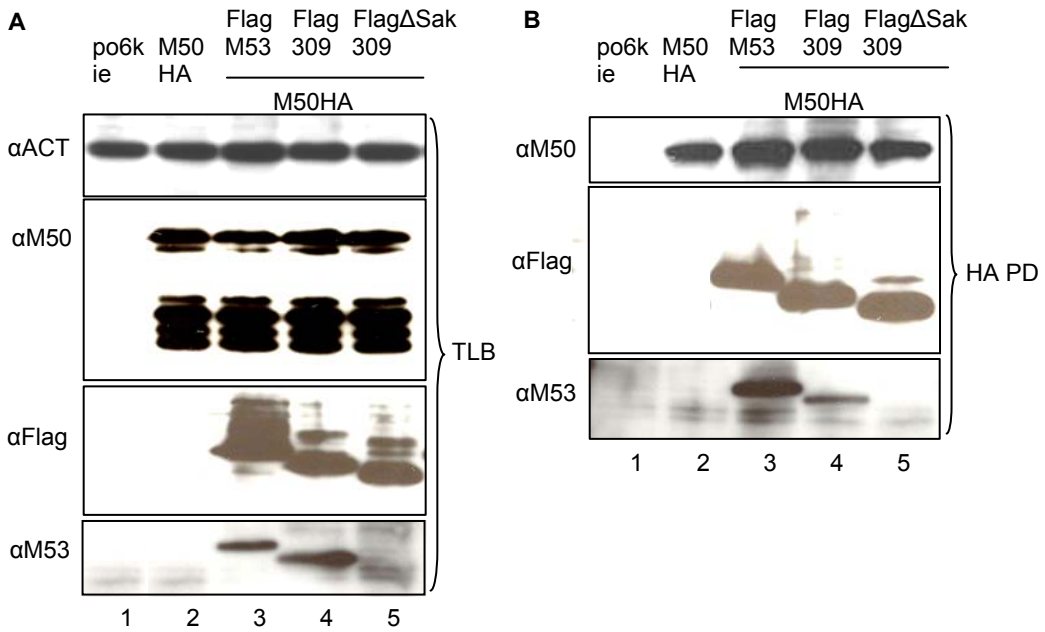

Figure 15. The FlagΔSak309 binds to M50. 293 cells were transfected with the empty vector pori6-ie (po6kie; lane 1) or the HA-tagged M50 ORF (lane 2) and co-transfected with plasmids expressing HA-tagged M50 (lanes 3 to 5) and the indicated M53 derivatives: FlagM53 (lane 3), Flag309 (lane 4), and FlagΔSak309 (lane 5). Transfected cells were lysed in total lysis buffer (TLB), and one-third of these lysates were loaded (left blots, indicated by TLB - A) and Western blotted with rabbit anti-M50 antiserum (αM50), mouse anti-Flag antiserum (αFlag), rat anti-M53 antiserum (αM53), and rabbit anti-actin antiserum (αACT), respectively. The remaining 70% of the lysates were processed by an HA pulldown (B). The eluates from the HA columns were split 1:9 and served for Western blot with rabbit anti-M50 antiserum, mouse anti-Flag antiserum (αFlag) and rat anti-M53 antiserum, respectively (rigth blots, indicated by HA PD). The expected molecular mass of the wt M50 protein is 35 kDa; the FlagM53, Flag309 and FlagΔSak30proteins migrate at about 40 kDa, 34-35 kDa and 32-33 kDa, respectively.

5.7.2 Conditional expression of Flag-tagged constructs by MCMV recombinants

The effects of Flag-tagged constructs were tested in the viral replication context. Both FlagM53 and FlagΔSak309 ORFs were subcloned by PCR into the regulation cassette and flipped-into the MCMV BAC. BACs were used to transfect MEF cells and the respective viruses were propagated on M2-10B4 cells. Then, FlagM53R and FlagΔSak309R recombinants were analyzed for the growth kinetics. Dox induction of FlagM53 protein had no effect on virus production and the infection resulted in a wt-like pattern (see Figure 16A). In contrast, in the presence of dox, the FlagΔSak309 prevented the virus replication, similar to the s309 effect (see Figure 16B).

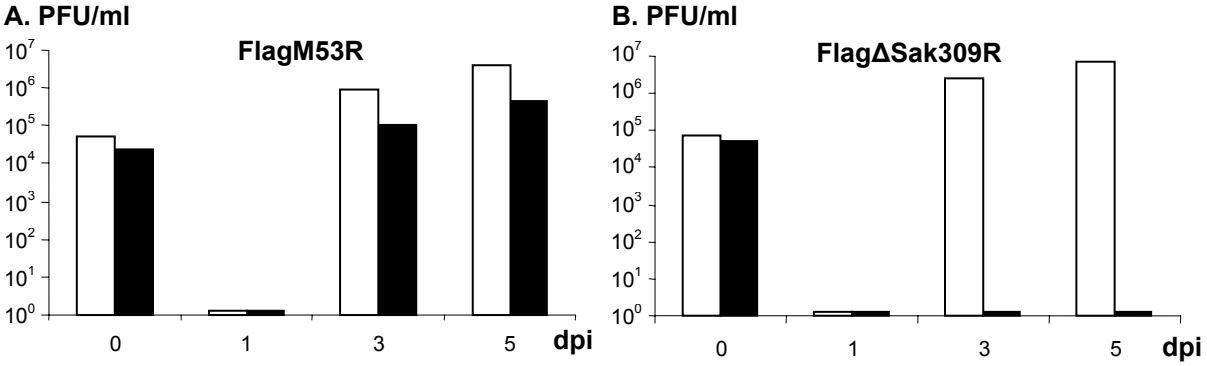

Figure 16. Flag-tag does not affect the inhibitory phenotype of s309. M2-10B4 cells were infected at an MOI of 0.1 in either the absence (white bars) or in the presence (black bars) of 1 µg/ml dox with FlagM53R and FlagΔSak309R viruses. The virus expressing the FlagM53 allele (FlagM53R) represented the positive control for second M53 gene and as negative control for a Flag-tagging effect. Cell supernatants were collected and the released progeny determined by titration on MEFs. The bar diagram shows the regulation of virus growth in response to dox administration 3 days and 5 days after infection with the FlagΔSak309 recombinant virus (FlagΔSak309R). The titer reduction is shown as a difference between the titer in the absence of dox and the titer in the presence of dox. Only titer reductions of >10-fold were considered significant. Each mutant was analyzed in duplicate in at least two independent experiments. Dpi, day post infection; PFU, particle per infectious unit.

Next, it was tested whether the Flag-tagged ORFs were expressed with late kinetics (see Figure 17), as the native protein (Loetzerich *et. al*., 2006). Therefore, NIH3T3 cells were infected at an MOI of 0.5 in the presence of dox with FlagM53R (lanes 2, 3, 6 and 7) and FlagΔSak309R recombinants (lanes 4, 5, 8 and 9) for 4 h (lanes 1 to 5) and 28 h (lanes 6 to 9). The uninfected cells represented the negative control (lane 1). The experiments were made in duplicates, one in the absence (--;

lanes 1, 2, 4, 6 and 8) and one in the presence (+; lanes 3, 5, 7 and 9) of 300 µg/ml phosphonoacetic acid (PAA), an inhibitor of DNA replication. Cells were harvested and lysed in high salt concentration (450 nm NaCl) lysis buffer. The load was tested by the cellular actin level. Because PAA is blocking late gene expression (see Figure 17), pp89/89 kDa – IE1 gene product - was used as control for viral protein expression in context of NIH3T3 cells infection.

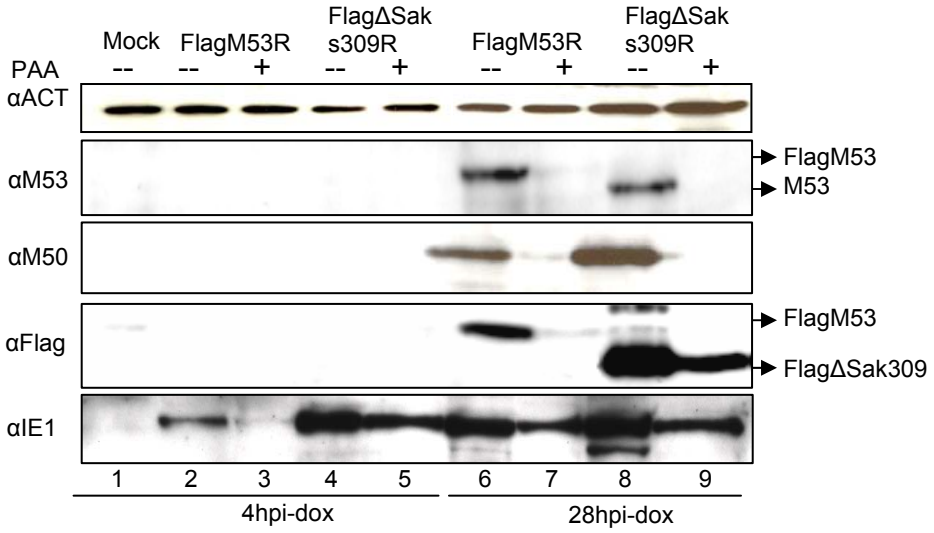

Figure 17. Flag tagging does not affect the late kinetics of DN s309. NIH 3T3 cells were infected in the presence of 1ug/ml dox at an MOI of 0.5 with the FlagM53R (lanes 2, 3, 6 and 7) and the FlagΔSak309R (lanes 4, 5, 8 and 9) viruses in the absence (--; lanes 2, 4, 6 and 8) and in the presence (+; lanes 3, 5, 7 and 9) of 300 µg/ml PAA. Uninfected cells (mock - lane 1, --) were used as negative control. Protein expression was analyzed 4 h (lanes 1-5) and 28 h (lanes 6-9) after infection by Western blotting using antibodies against pp89 (immediate-early protein, 89 kDa; αIE1), M53 (38 kDa; αM53), M50 (35 kD; αM50), Flag tag (αFlag) and actin (αACT). At 28 h post infection the native M53 (38 kDa) and M50 (35 kDa) proteins were detected in the absence of PAA, together with Flag-M53 protein (40 kDa). Accumulation of Flag-tagged M53 (Flag-M53; 40 kDa) and s309 (FlagΔSak309; 32 to 33 kDa) in the absence of PAA was associated with a late kinetics. Expression of the FlagΔSak309 is exempt from PAA control.

FlagM53 and FlagΔSak309 could not be detected after 4 hpi, neither in the absence nor in the presence of PAA. At 28 hpi, in the absence of PAA, anti-M53 antibodies identified the shift between FlagM53 (40 kDa) and native M53 (38 kDa) and anti-Flag antibodies between FlagM53 and FlagΔSak309 (32-33 kDa). At 28 hpi, in the presence of PAA, there were only weak signals for native M50 and M53. However, FlagM53R and FlagΔSak309R, in particular, expressed by the SV40

regulation cassette followed mainly the late expression kinetics but were not blocked by PAA (see Figure 17).

Next, the effect of Flag-tagged proteins on virus protein expression was tested (see Figure 18). Therefore, NIH3T3 cells were infected at an MOI of 0.5 with FlagM53R (lanes 1 and 2) and FlagΔSak309R recombinants (lanes 3 and 4) for 24 h in the absence (lanes 1 and 3) and presence of dox (lanes 2 and 4).

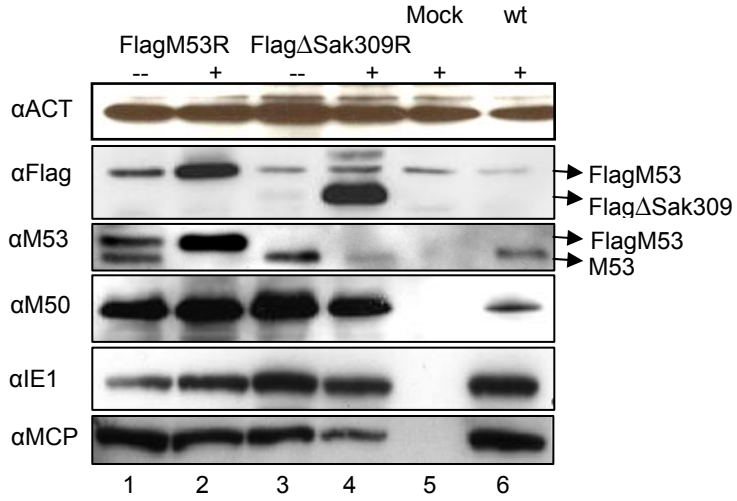

Figure 18. Flag tagging does not affect the inhibitory features of DN s309. NIH 3T3 cells were infected at an MOI of 0.5 with the FlagM53R (lanes 1 and 2) and the FlagΔSak309R (lanes 3 and 4) viruses in the absence (--; lanes 1 and 3) and in the presence (+; lanes 2 and 4) of 1 µg/ml dox. Uninfected cells (lane 5, +) and wt infection (lane 6, +) were used as negative and positive controls, respectively. Protein expression was analyzed 24 h after infection by Western blotting using antibodies against pp89 (immediate-early protein, 89 kDa; αIE1), M86 (late protein, 150 kDa; αMCP), M53 (38 kDa; αM53), M50 (35 kDa; αM50) (NEC proteins), Flag tag (αFlag) and actin (αACT). pp89 and M86 did not show significant difference after dox was added to the system. The native M53 protein (38 kDa) was detected in the absence of dox, together with Flag-M53 protein (40 kDa). Accumulation of Flag-tagged wt M53 (Flag-M53; 40 kDa) and s309 (FlagΔSak309; 32 to 33 kDa) upon dox induction was associated with a reduction in the native M53 protein levels.

FlagM53 and FlagΔSak309 expression did not affect immediately-early pp89/89kDa (IE1 gene product) or late pp86/150 kDa (major capsid protein - MCP; M86 gene product) protein expression. M53 specific rat antiserum revealed both Flag-tagged and native M53 variants after FlagM53R infection, fact indicative for leakiness of the regulation cassette. In the case of FlagΔSak309R only native M53 could be detected in the absence of dox. Dox strongly induced Flag - M53 variants associated with a loss of native M53 in FlagM53R or FlagΔSak309R infected cells.

As expected, FlagΔSak309 could not be detected by anti-M53 antiserum. M50 rabbit polyclonal antibodies revealed a strong M50 signal in the presence of FlagM53 and a certain signal reduction in the presence of FlagΔSak309. Mouse anti-Flag monoclonal antibodies revealed the leakiness of the SV40 cassette mainly for the FlagM53 mutant and confirmed the shift between FlagM53 (40 kDa) and FlagΔSak309 (32-33 kDa) proteins. Altogether, FlagΔSak309 mutant proved to be a good candidate for further analysis of the dominant negative phenotype.

5.8 Subcellular localization of NEC members

Because Flag-tagging of M53 and s309 ORFs did not affect the expression and biological properties of the proteins, the recombinants could be used to analyze the subcellular distribution of the NEC members.

The effects of conditional expression of wt M53 and DN s309 on nuclear egress of viral capsids were analyzed by confocal microscopy. NIH3T3 cells were infected with MCMV recombinants in the absence and in the presence of dox for 24 or 48 h. The cells were fixed with paraformaldehyde and stained for M53, M50 and Flag-tagged proteins.

First, the effects of dox on wt NEC members' intranuclear distribution were tested at 24 hours post infection (hpi) with wt MCMV. As shown in Figure 19 (A to H), M53 and M50 proteins colocalize at the nuclear rim independent of the absence or presence of dox.

Although the virus growth kinetics indicated no interference of FlagM53 expression with MCMV replication, the fate of the tagged copy of the wt protein could still be different at the subcellular level (see Fig 20 A to H).

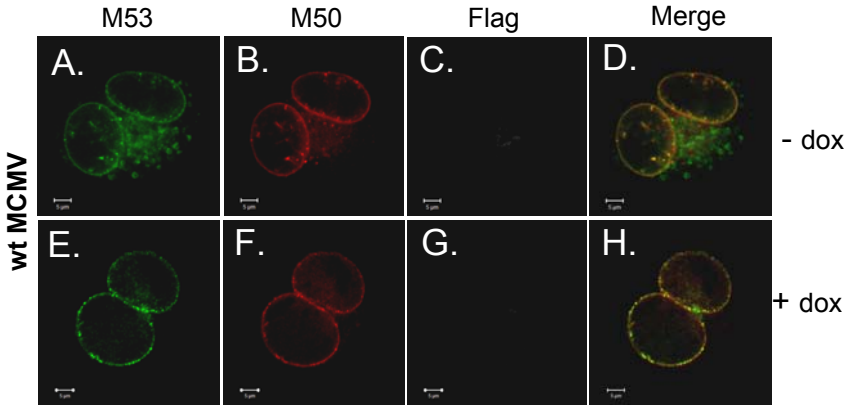

Figure 19. Subcellular distribution of the wt NEC proteins upon infection. NIH 3T3 cells were infected at an MOI of 0.2 with wt MCMV in the absence (A to D) and the presence (E to H) of 1 µg/ml dox. Analysis of wt MCMV infection in the presence of dox (E to H) was used as a control for dox effect on the wt NEC members' distribution. Twenty-four hours after infection, the localization of the M53, M50 and possible Flag cross-reactive proteins was analyzed by confocal immunofluorescence microscopy using specific primary antibodies, followed by Alexa fluorochrome-coupled secondary antibodies (Alexa 488, M53; Alexa 555, Flag tag; Alexa 633, M50). The scale bars indicate 5 µm.

Therefore, the cells were infected with the FlagM53R recombinant. In the absence of dox, a nuclear rim colocalization of the wt NEC members (see Figure 20 A to D) and a weak signal of the Flag-tagged M53 at the concave side of the nucleus could be noticed. The presence of dox induced a FlagM53 intranuclear signal with two locations: a strong nucleosolic accumulation and a nuclear rim staining. Expression of FlagM53 did not affect the interaction of the native M53 and M50 at nuclear rim (see Figure 20 E to H). These data confirm that a strong expression of M53 is not yet inhibitory for the virus replication.

FlagΔSak309R infection in the absence of dox resulted in a wt-like pattern associated with a weak nucleosolic detection of mutant protein (see Figure 20 I to L). Induction of FlagΔSak309 expression had strong effects on the wt NEC members. The native M53 protein relocalized to a nucleosolic fraction. M50 was reduced and redistributed along the nuclear rim to dotty aggregates. FlagΔSak309 mutant protein accumulated in the nucleosol but also colocalized with M50 aggregations (see Figure 20 M to P).

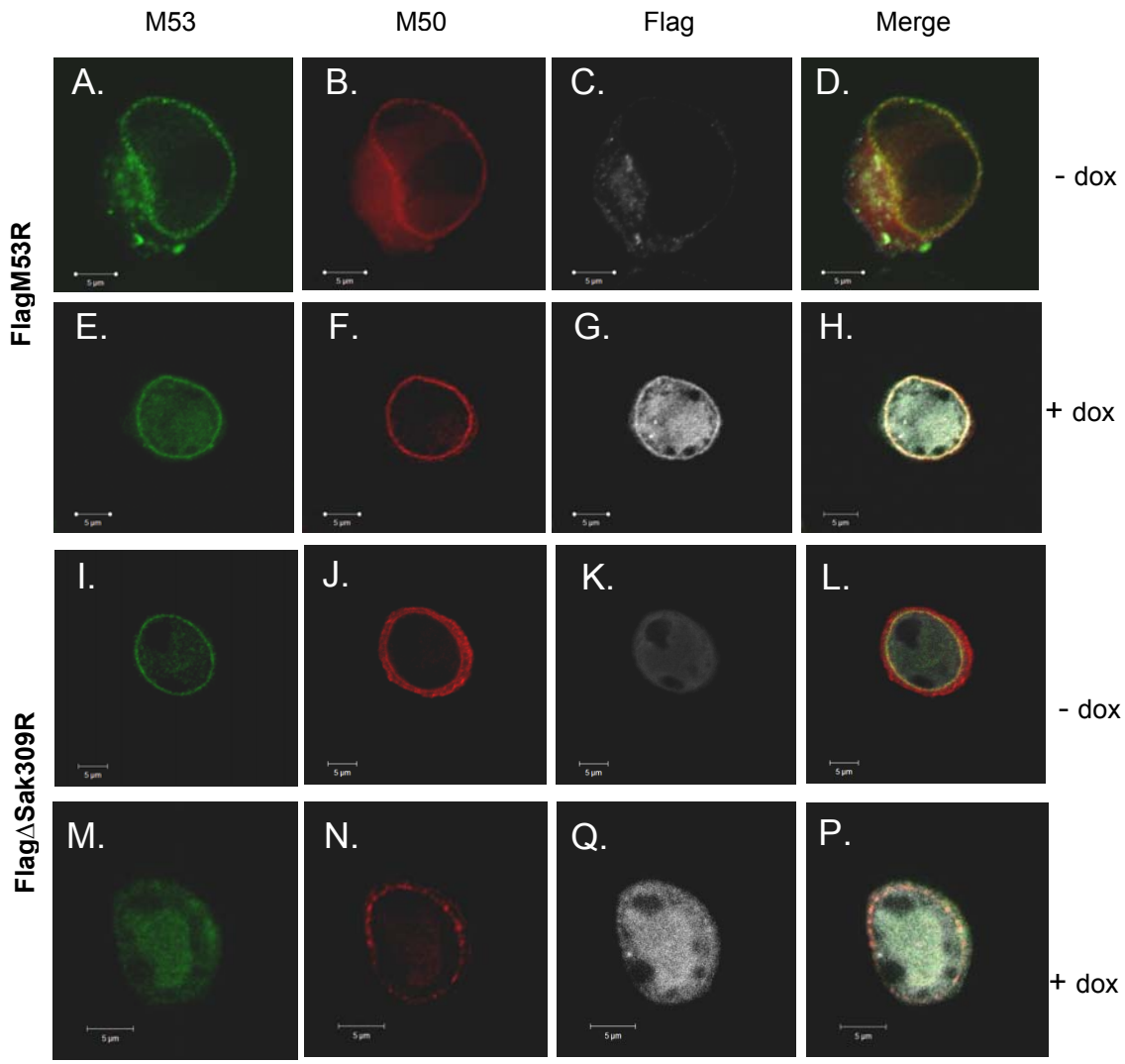

Figure 20. Subcellular distribution of the wt NEC proteins upon expression of DN s309 at 24 h after infection. NIH 3T3 cells were infected at an MOI of 0.2 with FlagM53R (A to H) and FlagΔSak309R (I to P) in the absence (A to D and I to L) and in the presence (E to H and M to P) of 1 µg/ml dox. Twenty-four hours after infection, the localization of the M53, M50, FlagM53 and FlagΔSak309 proteins was analyzed by confocal immunofluorescence microscopy using specific primary antibodies, followed by Alexa fluorochrome-coupled secondary antibodies (Alexa 488, M53; Alexa 555, Flag tag; Alexa 633, M50). The scale bars indicate 5 µm.

The analysis of the s309 nuclear egress block phenotype at 48 hpi confirmed the characteristics described above (see Figure 21 A - L). Here, even stronger effects were noticed. Dox induction of s309 resulted in a complete nucleosolic distribution of M53 and bulky aggregates of M50 colocalized with s309 along the nuclear rim (see Figure 21 M - P).

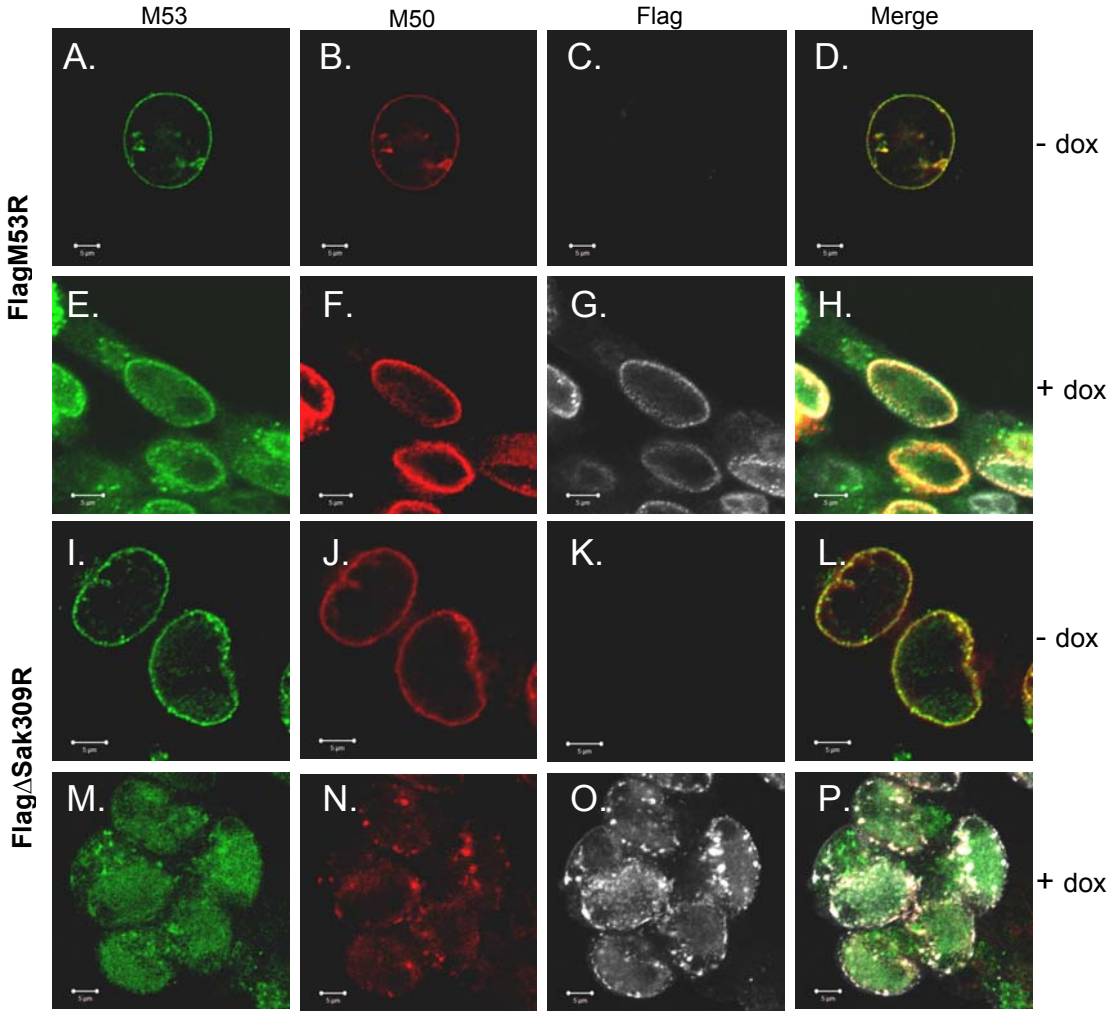

Figure 21. Subcellular distribution of the wt NEC proteins upon expression of DN s309 at 48 h after infection. NIH 3T3 cells were infected at an MOI of 0.2 with FlagM53R (A to H) and FlagΔSak309R (I to P) in the absence (A to D and I to L) and in the presence (E to H and M to P) of 1 µg/ml dox. Forty-eight hours after infection, the localization of the M53, M50, FlagM53 and FlagΔSak309 proteins was analyzed by confocal immunofluorescence microscopy using specific primary antibodies, followed by Alexa fluorochrome-coupled secondary antibodies (Alexa 488, M53; Alexa 555, Flag tag; Alexa 633, M50). The scale bars indicate 5 µm.

The s309 effect was not only a phenotype of selected cells but the general cell phenotype (see Figure 22), considering that the infection was carried out at an MOI of 0.2 and the expected values (20 infected cells out of 100) reflect the findings.

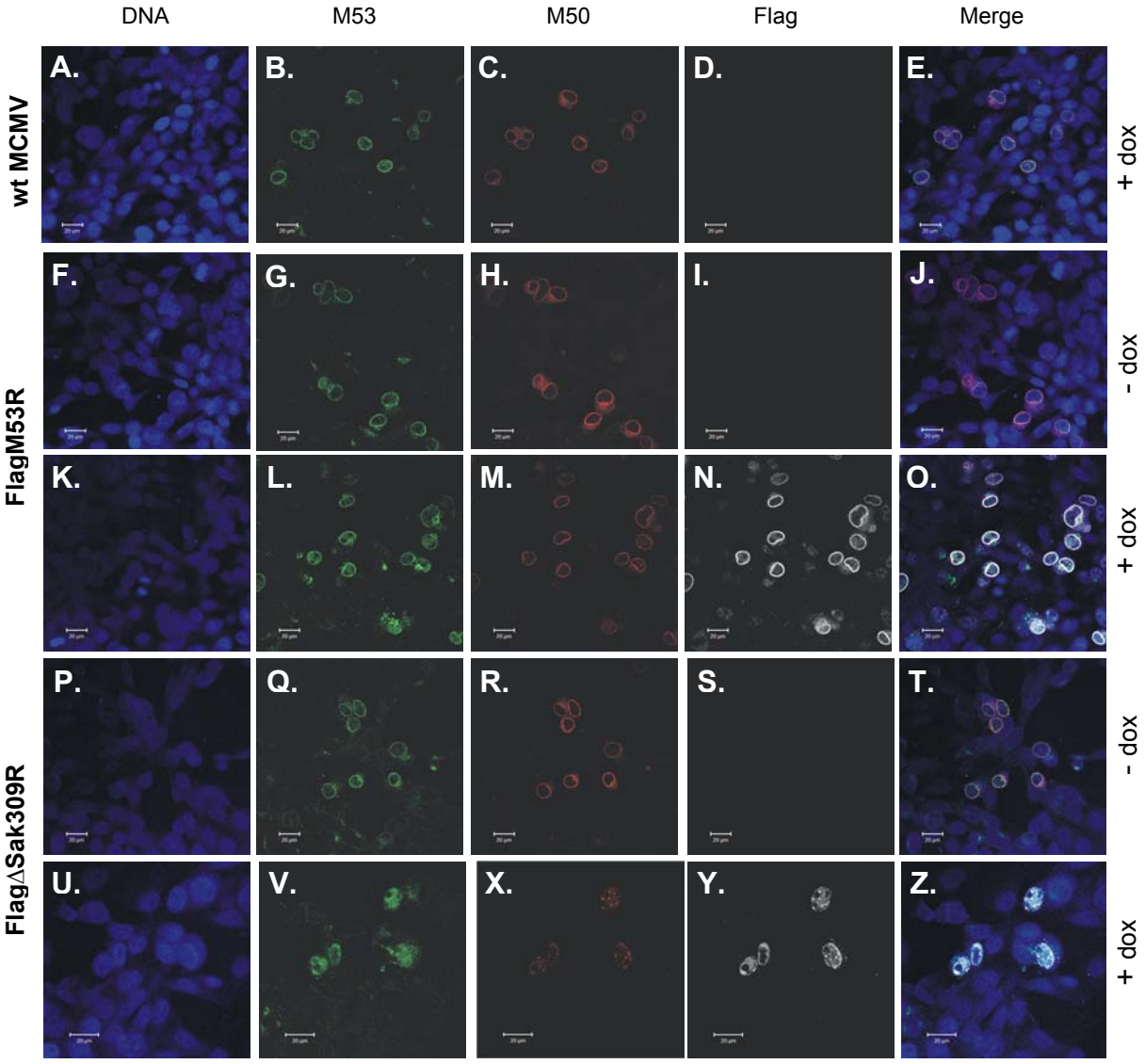

Figure 22. Generality of the DN phenotype. NIH 3T3 cells were infected at an MOI of 0.2 with FlagM53R (F to O) and FlagΔSak309R (P to Z) in the absence (F to J and P to T) and in the presence (K to O and U to Z) of 1 µg/ml dox. Analysis of wt MCMV infection (A to E, in the presence of dox) was used as a control. Twenty-four hours after infection, the localization of the M53, M50, FlagM53 and FlagΔSak309 proteins was analyzed by confocal immunofluorescence microscopy using specific primary antibodies, followed by Alexa fluorochrome-coupled secondary antibodies (Alexa 488, M53; Alexa 555, Flag tag; Alexa 633, M50). The scale bars indicate 20 µm.

5.9 Analysis of the DN s309 phenotype

Electron microscopy studies revealed that s309 induced a block of capsid export, because no capsids could be seen in the cytoplasm (see Figure 23 B) and in addition there was a defect in nuclear capsid maturation (see Figure 23 C and D). The intranuclear population was mainly represented by capsids with unusual morphology. About 50% of capsids had regular icosahedral morphology but lacked density inside

their shell and were dissimilar to the regular ring structure which is characteristic of B-capsids. In addition, there were only few mature C-capsids with the characteristic high density core. A core without density is typical for A-capsids lacking DNA. A high number of intranuclear capsids showed this phenotype.

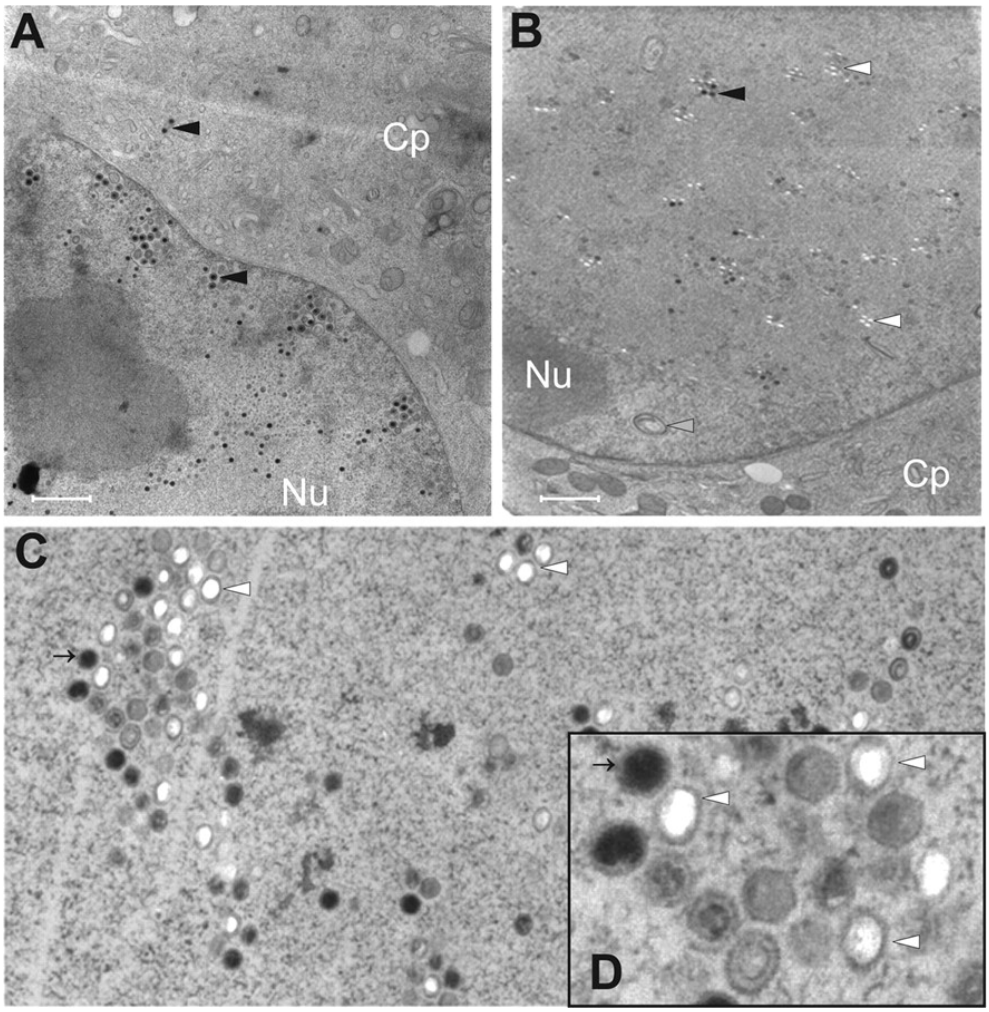

Figure 23. DN s309 prevents viral-capsid egress from the cell nucleus and induces accumulation of aberrant capsids in the nucleus. NIH 3T3 cells were infected for 72 h at an MOI of 3 with viruses that conditionally expressed s309 in the absence (A) or in the presence (B, C, and D) of 1 µg/ml doxycycline. The cells were fixed by high-pressure freezing, freeze-substituted, embedded in plastic, and analyzed by electron microscopy. The black arrowheads point at mature C-type viral capsids, and the white arrowheads point at capsids with unusual morphology. The gray arrowheads point at intranuclear membrane accumulation. (D) Electronic zoom of panel C; the upper left capsid is indicated by a thin arrow to aid orientation. The scale bars indicate 1 µm.

To test whether s309 induces a capsid maturation defect by interfering with the DNA cleavage/packaging machinery we analyzed the cleavage of viral genomes by a Southern-blot assay (see Figure 24).

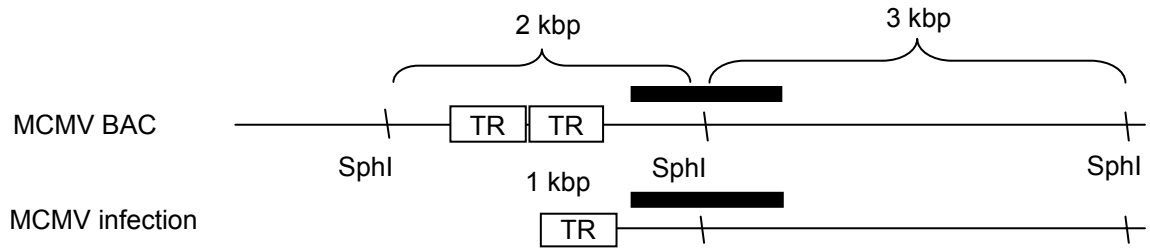

Figure 24. Schematic representation of the Southern blot assay for analysis of genome cleavage/packaging during MCMV infection. The terminal regions of MCMV genomes are depicted as they formed concatemers after replication (top) or as the genome ends were released as a consequence of a cleavage/packaging reaction (bottom). In the concatemers, the genomes are connected via the short terminal repeats (TR) of the MCMV. The hybridization probe used in this study (black bar) overlapped with both the left terminal and the next right subterminal fragments of the SphI-digested MCMV genome. The subterminal fragment (3 kbp) was detected in all genomic forms. Therefore, it served as a measure for the genome load. In the concatemeric form, the left terminal fragment is connected to the right terminal fragment. Thus, the same probe recognizes the concatemeric genome ends as a 2 kbp fragment. In the unit length genomes, the left terminal fragment is separated and recognized by the same probe as an 1 kbp fragment.

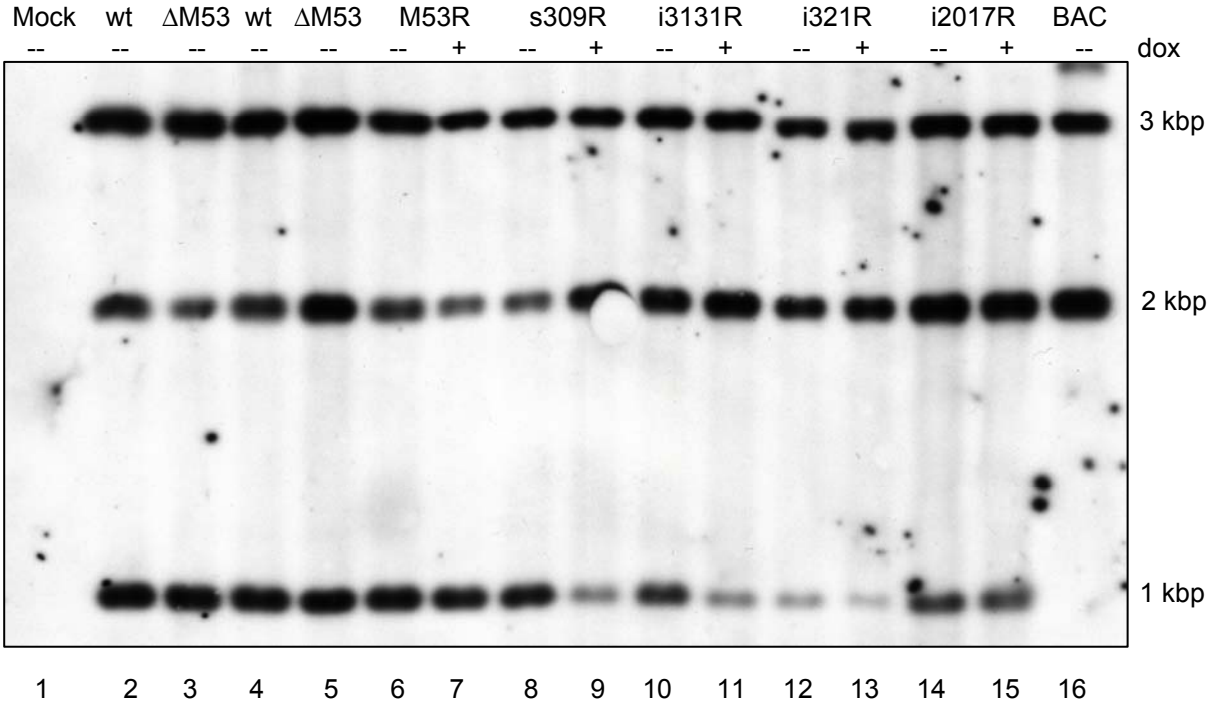

Figure 25. Southern blot analysis of a genome cleavage/packaging reaction upon induction of DN M53 mutants. NIH 3T3 cells were mock infected (lane 1) or infected with wt MCMV (lane 2, wt), ΔM53tTA-MCMV (lane 3, ΔM53), M53R (lanes 6 and 7), s309R (lanes 8 and 9), i313R (lanes 10 and 11), i321R (lanes 12 and 13) and i207R (lanes 14 and 15) at an MOI of 2 both in the absence (--; lanes 1, 2, 3, 6, 8, 10, 12 and 14) and in the presence (+; lanes 7, 9, 11, 13 and 15) of doxycycline (dox). M53 transcomplementing cells (M53tTA) were infected with the wt MCMV (lane 4, wt) and ΔM53tTA MCMV (lane 5) at an MOI of 2. Purified wt MCMV BAC DNA (lane 16, BAC) was used as a hybridization control. Forty-eight hours after infection, the nuclear DNA was extracted and digested with SphI. Then, the DNA fragments were separated by agarose gel electrophoresis and analyzed by Southern blotting using a digoxigenin-tagged probe, depicted in fig. 24. The detected fragments are indicated on the right side of the blot according to the labeling specified for fig. 24 (3 kbp – genome load; 2 kbp – concatemers; 1 kbp – unit length genome).

In herpesviruses, the unit length genomes are released from concatameric replication intermediates and transferred into pro-capsids during nucleocapsid maturation process. To test the effect of DN M53 mutants on this process, NIH3T3 cells were infected with wt MCMV and inhibitory mutants in the absence and presence of dox. After 48 h post infection the total DNA was extracted from the infected cells, cut with SphI endonuclease and probed for the MCMV genomic forms (see Figure 25). SphI cleavage of the linear genome generated an approximately 1 kbp terminal fragment representing the nucleotide positions (nt) 1-1298 according to the reference sequence (GenBank NC_004065) and a 3 kbp subterminal fragment (1299-4521 nt). A probe hybridizing with both fragments can detect the extent of DNA cleavage (see Figure 24) because in the concatemeric form of viral genomes the 1298 bp fragment is fused to another 894 bp terminal fragment (229385-230238 nt) and detected as a 2 kbp fragment. During genome packaging the intensity of the concatemers fragment (2 kbp) decreases and the intensity of the unit length genome fragment (1 kbp) increases while the 3 kbp fragment remains constant and can therefore serve as a measure for the genome load.

The circular wt MCMV BAC mimics the concatemeric viral DNA form and shows only the 2 kbp and the 3 kbp fragments (lane 16) whereas the genomic DNA derived from the wt MCMV infected cells contains also the 1kb end fragment (lanes 2 and 4). The wt pattern was noticed by using wt MCMV (lane 2) and M53R in the absence or presence of dox (lanes 6 and 7). s309R (lane 9), i313R (lane 11) and i321R (lane 13) infection revealed an accumulation of the 2 kbp fragment in the presence of dox and a reduction of the 1 kbp fragment derived from the unit length genomes compared to the absence of dox (lanes 8, 10 and 12, respectively). Notably, the infection with i207R resulted in an almost wt-like pattern (lanes 14 and

15). These data show that DNs generated by 5 aa insertion into domain CR4, but not into CR2, are associated with a DNA cleavage/packaging defect.

The ∆M53tTA MCMV mutant which lacks the M53 ORF and has to be replicated in a complementing cell line (lanes 3 and 5) showed no effect on cleavage/packaging process. The 1 kbp fragment of this mutant was detected with similar signal strength as during wt MCMV infection of the same types of cells (lanes 2 and 4). Because the null phenotype induced by M53 deletion (Loetzerich *et. al*., 2006) did not predict a cleavage/packaging defect we speculated that CR4 mutants inhibit indirectly the capsid maturation by a block or arrest of the dynamics of the cleavage/packaging process.

5.10 s309R effects on cleavage/packaging are independent of NEC formation

The s309 effect on DNA cleavage/packaging suggested that the M53 protein has multiple functions. In the next step, it was checked whether the DN effect on cleavage/packaging is associated with NEC formation.

Therefore, a mutant which might maintain the inhibitory feature of s309 but lacks the ability to bind M50 was constructed. Loetzerich *et. al*. (2006) published that 5 aa insertion at position 128 of M53 ORF destroys the capacity to bind to M50. The i128 insertion mutant was sub-cloned into the s309ORF (s309i128, see Figure 26 A) and the recombinant s309i128R BAC (MCMV BAC carrying the SV40 regulation cassette of the s309i128 mutant at the m16-m17 FRT ectopic position) was generated. Because the s309i128 mutant kept the inhibitory feature of the parental s309, the recombinant virus was analyzed under conditional expression conditions (see Figure 26 B).

Dox mediated expression of s309i128 protein down regulated the virus growth more than one thousand fold. This partial down-regulation allows at least 2 possible

explanations: i) loss of the additional effect on the NEC and ii) expression or folding problems of the s309i128 mutant.

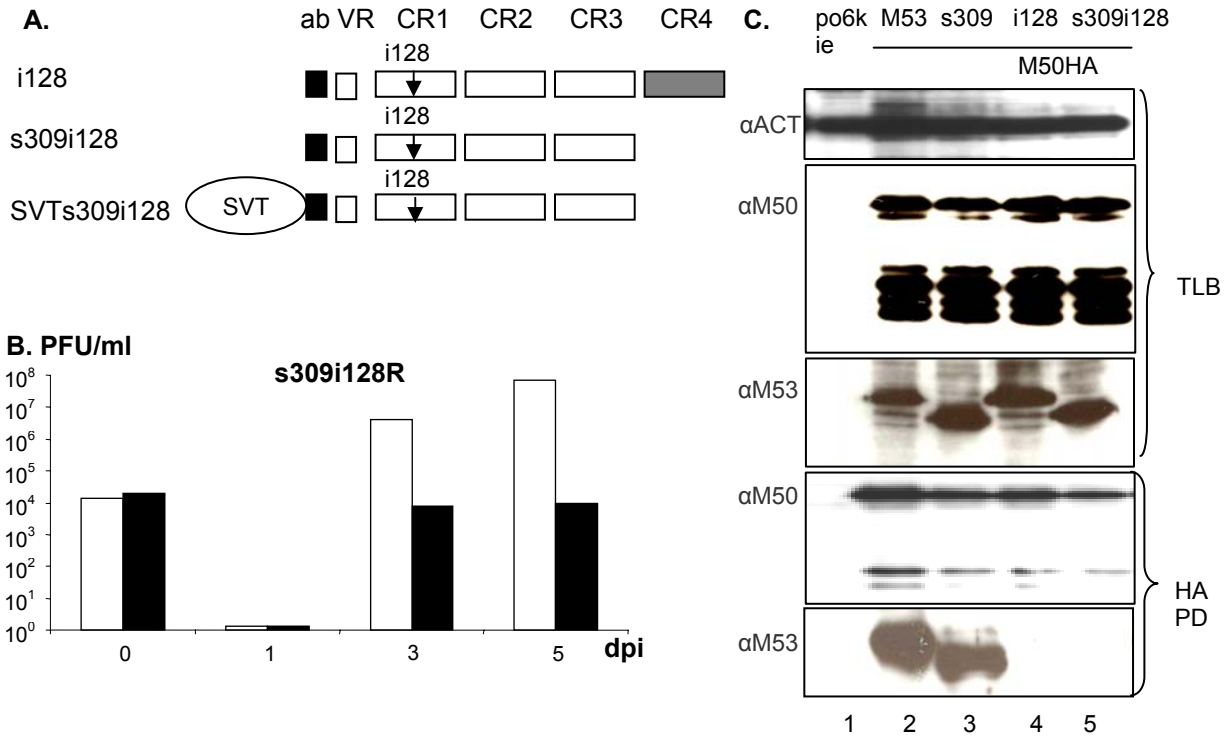

Figure 26. A. Schematic representation of the s309i128. Depicted are the domains of M53 mutants ORFs. The N-terminal site (ab) recognized by the M53-specific serum (Sak), the variable region (VR), the C-terminal four conserved regions (CR1 to CR4) and 5 aa insertion at position 128 (black arrowhead) are indicated. The s309i128 mutant expression was controlled by an SV40 enhancer driven HCMV promoter (SVT circle). B. Effects of the s309i128 inhibitory mutants on the production of viral progeny. M2-10B4 cells were infected for 5 days at an MOI of 0.1 in either the absence (white bars) or the presence (black bars) of 1 µg/ml dox. Cell supernatants were collected and the released infectious units (PFU, particle per infectious unit) were determined by titration on MEFs. The bar diagram shows the regulation of virus growth in response to dox administration 3 days and 5 days after infection with the s309i128 recombinant virus (s309i128R). The titer reduction is shown as a difference between the titer in the absence of dox and the titer in the presence of dox. Only titer reductions of >10-fold were considered significant. Each mutant was analyzed in duplicate in at least two independent experiments. Dpi, day post infection. C. The DN s309i128 lacks the M50 binding capacity. 293 cells were transfected with the empty vector pori6-ie (po6k; lane 1) and co-transfected with plasmids expressing HA-tagged M50 (lanes 2 to 5) and the indicated M53 derivatives: wild type M53 (lane 2), s309 (lane 3), i128 (lane 4) and s309i128 (lane 5). Transfected cells were lysed in total lysis buffer (TLB), and one-third of these lysates were loaded (upper three blots, indicated by TLB) and Western blotted with rabbit anti-M50 antiserum (αM50), rat anti-M53 antiserum (αM53), and rabbit anti-actin antiserum (αACT), respectively. The remaining 70% of the lysates were processed by an HA pulldown. The eluates from the HA columns were split 1:9 and served for Western blot with rabbit anti-M50 antiserum and rat anti-M53 antiserum, respectively (lower two blots, indicated by HA PD). The expected molecular mass of the M50 protein is 35 kDa; the wt M53 and the mutant i128 protein migrate at about 38 kDa and s309 together with s309i128 at about 34 to 35 kDa.

Next, protein expression and M50 binding properties of this mutant were tested (see Figure 26 C) in 293 cells co-transfected with plasmids carrying either M50HA ORF (lanes 2 - 5) or one of the next M53 ORFs: wt (lane 2), s309 (lane 3),

i128 (lane 4) and s309i128 (lane 5). The empty po6k-ie vector carrier served as negative control (lane 1). After 24 h post-transfection cells were lysed and an HA pulldown was performed using the same procedure as described in chapter 5.5. Protein expression in total lysates (see Figure 26 C) and total protein load were tested by the cellular actin level. The constructs were properly expressed and s309i128 as well as s309 shifted to 34-35 kDa. After HA pulldown, the M50 bound M53 and s309 proteins were detected whereas i128 and s309i128 were not co-precipitated. Thus, the 5 aa insertion at position 128 of the s309 ORF resulted in the expected loss of M50 binding capacity.

Further, we checked the intracellular distribution of the s309i128 protein. Therefore, NIH3T3 cells were infected at an MOI of 0.5 with s309i128R. Infection in the absence of dox resulted in a wt-like pattern distribution of M50 and M53 at the nuclear rim (see Figure 27 A to C). With respect to the leakiness of SV40 cassette, the detection levels of s309i128 protein expression were found to be lower than in case of s309 (see Figure 21). In the presence of dox, s309i128 could be detected by a strong accumulation inside of nucleus while the native M53 kept the nuclear rim localization. The fraction of relocalized native M53 – if any - could not be distinguished from the s309i128 signal due to detection by the anti-M53 antibody. The redistribution effects on M50 were weak or absent (see Figure 27 D to F). Nevertheless, s309i128 which lacks domain CR4 still blocks the virus growth, proving this effect as independent from NEC formation. The s309i128 effect was not an isolate pattern of few cells but the general phenotype as may be noticed in the Figure 27 (G to N). Therefore, the cells were counted after DNA staining with iodide (Topo-PRO-3, Invitrogen) and the dominant negative phenotype of s309i128R was evaluated for M53 and M50 redistribution. Considering that the infection was carried out at an MOI of 0.2 for 24 h and comparing the observed data with the expected

values (20 infected cells out of 100) s309i128 infection follows the wt-like pattern in the absence of dox (see Figure 28 G to J). Dox expression of s309i128 expression reliable reproduced the inhibitory phenotype (see Figure 27 K to N).

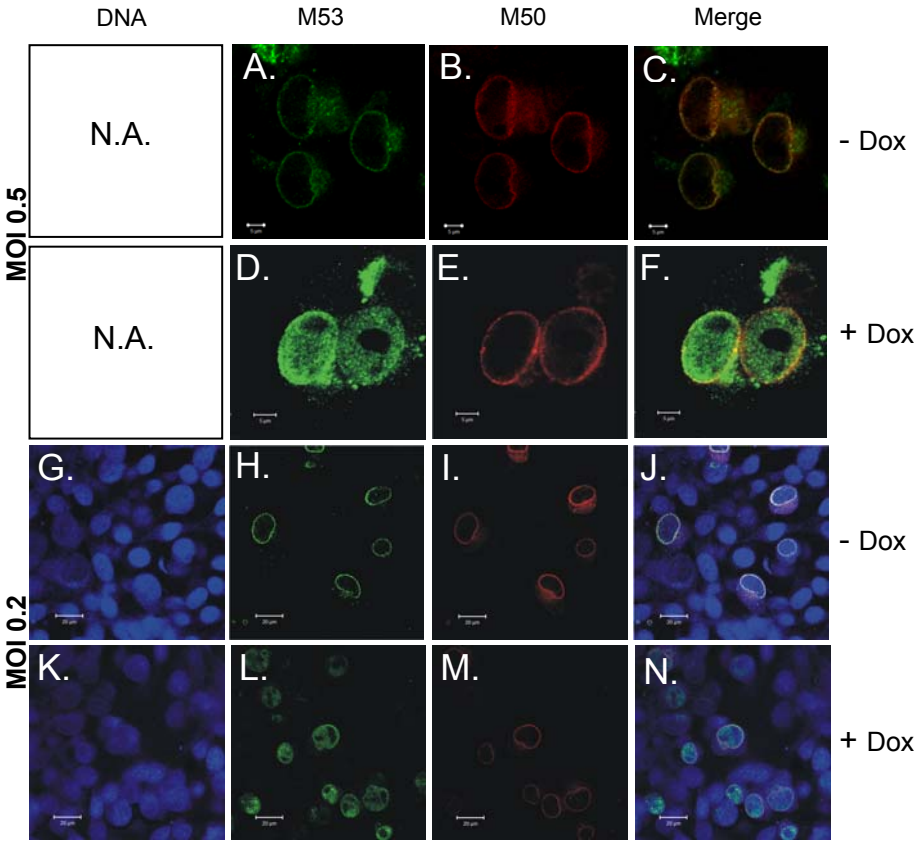

Figure 27. Subcellular distribution of the wt NEC proteins upon expression of DN s309i128. NIH 3T3 cells were infected at an MOI of 0.5 (A to F) and 0.2 (G to N) with s309i128R (A to N) in the absence (A to C and G to J) and in the presence (D to F and K to N) of 1 µg/ml dox. Twenty-four hours after infection, the localization of the M53, M50 and s309i128 proteins was analyzed by confocal immunofluorescence microscopy using specific primary antibodies, followed by Alexa fluorochrome-coupled secondary antibodies (Alexa 488, M53; Alexa 633, M50). The scale bars indicate 5 µm (A to F) or 20 µm (G to N).

Next, it was tested whether DN effect of s309i128 was also reflected at the DNA cleavage/packaging level (see Figure 28). NIH3T3 cells were infected at an MOI of 2 with recombinants overexpressing the FlagM53 (lanes 4 and 5), FlagΔSak309 (lanes 6 and 7) and s309i128 (lanes 8 and 9) in the absence (lanes 3, 4, 6 and 8) and in the presence (lanes 1, 2, 5, 7 and 9) of dox. Uninfected cells (lane 1) and the wt MCMV BAC transfection (lane 3) served as negative control for DNA cleavage/packaging. The wt infection served as positive control (lane 2). After 48 h

post infection, the total DNA was extracted from the infected cells, cut with SphI endonuclease and probed for the MCMV genomic forms (see Figure 28). Comparing the effect of Flag-tagged viruses (see Figure 28) to the initial recombinants (see Figure 25) it can be concluded that Flag-tag did not affect the s309 inhibitory capacity at the DNA cleavage/packaging level. When we compare the effect of s309i128 mutant with the DN FlagΔSak309 it was noticed that upon dox induction both mutants inhibited the cleavage reaction, indicating that this effect of M53 is independent of NEC formation.

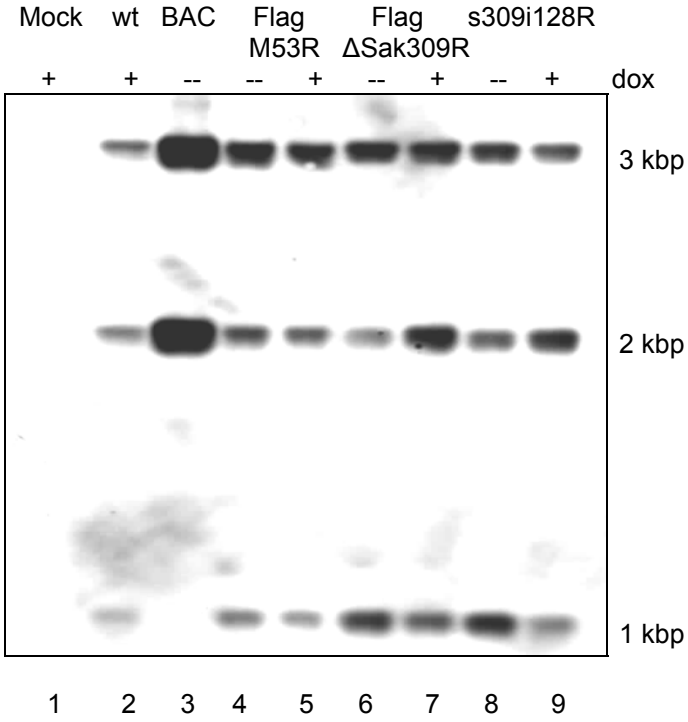

Figure 28. Southern blot analysis of a genome cleavage/packaging reaction upon induction of DN s309i128 mutant. NIH 3T3 cells were mock infected (lane 1) or infected with wt MCMV (lane 2, wt), FlagM53R (lanes 4 and 5), FlagΔSak309R (lanes 6 and 7) and s309i128R (lanes 8 and 9) at an MOI of 2 both in the absence (--; lanes 4, 6 and 8) and in the presence (+; lanes 1, 2, 5, 7 and 9) of doxycycline (dox). Purified wt MCMV BAC DNA (lane 3, BAC) was used as a hybridization control. Forty-eight hours after infection, the nuclear DNA was extracted and digested with SphI. Then, the DNA fragments were separated by agarose gel electrophoresis and analyzed by Southern blotting using a digoxigenin-tagged probe, depicted in fig. 24. The detected fragments are indicated on the right side of the blot according to the labeling specified for fig. 24 (3 kbp – genome load; 2 kbp – concatemers; 1 kbp – unit length genome).

6. Discussion

6.1 The MCMV M53 C-terminal region harbors at least two functional domains

The events leading to and regulating the nuclear egress of herpesviruses are not well understood. This process requires a complex interaction between the viral and cellular proteins. The herpesvirus nucleocapsids must acquire access to the cytoplasm in order to complete the maturation process. The nuclear envelope blocks this passage because the capsids are too big to pass intact the nuclear pores (for a review see Mettenleiter, 2002/2004/2006).

The work of many groups led to the conclusion that two viral proteins, which belong to the conserved UL31 and UL34 families, represent the major players of the nuclear egress of herpesvirus capsids. The UL34 family members (M50 in MCMV) are type II transmembrane proteins expressed with early-late kinetics. They are inserted in the endoplasmic reticulum (ER) and found distributed in both nuclear membranes (Yamauchi *et. al*., 2001). After interacting with UL31 family members (M53 in MCMV) the UL34 protein is relocated from the ER to the inner nuclear membrane (INM) (Liang and Baines, 2005; Yamauchi *et. al*., 2001) and perhaps induces a dissociation of the inner and the outer nuclear leaflets (Ye *et. al*., 2000). M53, similar to other members of UL31 family, is a nucleosolic phosphoprotein (Lötzerich, unpublished data; Chang and Roizman, 1993) translocated to the nucleus by its nuclear localization signal (NLS) (Lötzerich *et. al*., 2006; Zhu *et. al*., 1999; Chang and Roizman, 1993). There is a strict interdependence between these two gene products with regard to the localization and stability because in the absence of the UL34 protein the mRNA of the UL31 accumulates and the mature protein is degraded by the proteosomes (Ye and Roizman, 2000). A destabilization of UL34 in the absence of its partner has also been reported (Gonella *et. al*., 2005; Rupp *et. al*.,

2005; Bubeck *et. al.,* 2004; Ye and Roizman, 2000). These two proteins interact directly at the INM, as shown for viruses from all three herpesvirus subfamilies (Santarelli *et. al*., 2008; Klupp *et. al*., 2007; Loetzerich *et. al*., 2006; Schnee *et. al*., 2006; Bubeck *et. al.,* 2004, Lake and Hutt-Fletcher, 2004; Fuchs *et. al*., 2002; Muranyi *et. al*., 2002; Reynolds *et. al*., 2001). This interaction promotes the nuclear expansion and the chromatin reorganization (Simpson-Holley *et. al*., 2005/2004) as well lamina rearrangements (Park and Baines, 2006; Gonella *et. al*., 2005; Simpson-Holley *et. al*., 2005/2004; Lake and Hutt-Fletcher, 2004; Reynolds *et. al.*, 2004; Dal Monte *et. al*., 2002; Muranyi *et. al*., 2002; Scott and O'Hare, 2001), a prerequisite for nucleocapsid budding and nuclear egress.

The UL31 family members show some conserved structural features: i) they carry an NLS in their N-terminal variable region (Loetzerich *et. al*., 2006; Zhu *et. al*., 1999; Chang and Roizman, 1993) and ii) the binding site of the respective UL34 family member is located at the first conserved region (CR) (Loetzerich *et. al*., 2006; Schnee *et. al*., 2006). Data using trans-complementing cell lines, cis-complementation assays, as well in vivo binding studies (protein complementing assay - PCA) have shown that the functional cross complementation is possible between the family members but only within the same herpesvirus subfamily (Santarelli *et. al*., 2008; Schnee *et. al*., 2006).

Because the functional analysis of proteins involved in viral morphogenesis requires the context of virus replication, we decided to isolate and study the dominant negative mutants (DNs) of M53, which should provide conditional loss of function phenotypes for better understanding of nuclear egress of MCMV.

Recently, our group has published a comprehensive insertion mutagenesis library of M53 (Loetzerich *et. al*., 2006), which provided a map of susceptible sites for loss of protein functions. The respective M53 map indicated functional domains

harboring in the C terminal conserved regions (CR). Here, we tested this pool of non-viable mutants (Lötzerich *et. al.*, 2006) for their inhibitory potential. Therefore, all of them were tested for interference with virus reconstitution after tranfection in the context of a complete wt MCMV genome. Now, the majority of mutants should not affect the viral progeny production except for those with inhibitory features. This allowed us to distinguish between nonfunctional mutants with expression or folding problems and potential dominant negative mutant candidates.

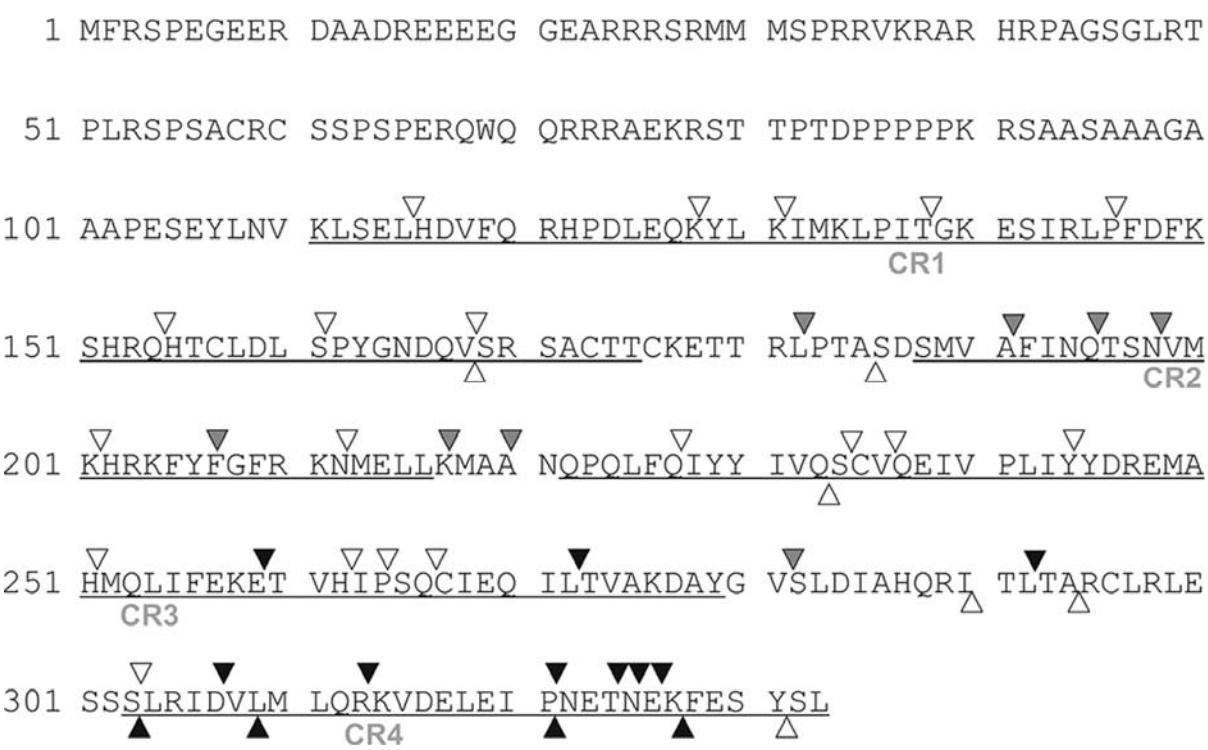

Figure 29. Screening for inhibitory mutants of the MCMV M53 gene. The amino acid sequence encoded by the M53 gene of MCMV is displayed, including the four conserved regions (CR1 to CR4; underlined), and the positions of the different mutations are indicated. Transposon insertions (arrowheads) resulting in introduction of 5 aa (depicted above the sequence) or stop codons (depicted below the sequence) are indicated along the ORF. Mutants preventing virus reconstitution after insertion into the wt MCMV BAC are labeled by black symbols, and mutants which did not interfere with virus reconstitution are depicted by white symbols. The intermediate phenotype is indicated by gray symbols.

Strikingly, the inhibitory mutants were accumulated only in the second and the fourth conserved region (CR2 and CR4; see Table 10 and Figure 29). The plaque phenotypes of the proteins mutated in these two conserved regions were different.

The CR2 mutants have shown a severely delayed reconstitution (between 4 and 6 weeks) associated with mini-plaque formation and lack of spread. This indicated that the release of infectious particles after the virus reconstitution takes place with extremely low efficiency, similar to the pattern observed after deletion of the alpha herpesvirus homologues of M53 (Klopfleisch *et. al*., 2006; Fuchs *et. al*., 2002). In contrast, if CR4 null-mutants were inserted into the wt MCMV-BAC no recombinant viruses could be reconstituted. Already these differences suggested that the M53 protein has at least 2 different C terminal functions. CR2 could is contributing to the primary envelopment of nucleocapsids at the INM, a function described for the entire UL31 family. However the second domain - CR4 - essential for other M53 function(s) might vary from that of other subfamilies. This would explain why the null phenotypes observed by deletion of UL31 members from beta herpesviruses are lethal, whereas the deletion of the homologues of alpha herpesviruses is not. The existence of a functional domain at the very C-terminus of M53 is also supported by the distribution of the inhibitory mutants: all mutants of this region including the C-terminal ones seemed to be strongly inhibitory but there is no inhibition observed by mutation of the N terminal spacer of CR4.

Taking into account that the overexpression of a viral protein might be toxic, as it is described for MCMV M50 (Muranyi *et. al*., 2002), it was necessary to study the inhibitory phenotype under the conditions which do not affect the viral gene expression in general. Therefore the specificity of the inhibitory mutants was tested by a tet-based conditional expression system, which is functional in the MCMV context (Rupp *et. al*., 2007/2005). Moreover, conditional expression of genes is superior to the use of loss-of-function mutants in order to study essential functions. The advantages are represented by the possibility to study the “off” and “on” situations with the same virus stock preparation, providing a controlled intracellular

environment, and there is no need to construct revertants to prove that the induced phenotype is strictly related to the gene of interest. Based on the expression kinetics (Loetzerich *et. al*., 2006) M53 is a true late gene, a condition when the number of viral genomes and transcripts is very high. Thus, the system requires a large mutant protein amount but also a strict regulation of the cassette because the control of DN protein expression has to be tight enough. We had to consider that a basal expression but not in a lethal amount of the DN would be selected for the mutated escape genomes with a non functional DN, as already observed (Rupp *et. al*., 2007). On the other hand abundant expression was necessary to observe inhibitory features. Altogether, the regulation of the M53 gene at the ectopic position of wt MCMV BAC should affect virus replication at a defined step, but should not affect the viral gene expression cascade in general.

Therefore, we have chosen a combination of a SV40 early-enhancer and HCMV immediately-early promoter, allowing the abundant expression of the gene of interest, inserted into the tet conditional cassette but tightly regulated in the presence of doxycycline (dox).

Confirmation of 4 mutants as dominant negatives (s309 stop mutant; i207, i313 and i321 insertion mutants) gave us tools suitable to define the functions associated with CR4 of M53 protein.

6.2 M53 CR4 functional domain and MCMV nucleocapsids egress defect

Alpha and gamma herpesviruses express the M53 homologues during the late phase of productive infection with a size range from 28 to 34 kDa (28 kDa/EBV – Lake and Hutt-Fletcher *et. al*., 2004; 29 kDa/PrV – Fuchs *et. al*., 2002; 33 kDa/KSHV size - Santarelli *et. al*., 2008; 34 kDa/HSV1 – Reynolds *et. al*., 2001). In some alpha herpesviruses the M53 homologue is expressed by early-late kinetics (ILTV,

northern-blot analysis, Helferich *et. al*., 2007). MCMV M53 (Loetzerich *et. al*., 2006) and its HCMV homologue pUL53 (Dal Monte *et. al*., 2002) are true late proteins with a size range from 38 kDa/MCMV to 42 kDa/HCMV.

At that moment, it was at interest to know if the deletion of CR4 affects MCMV M53 expression. HA pull-down experiments of DN s309 mutant after co-transfection with HA tagged M50 indicated no effect of CR4 defects on M50 binding. The non tagged protein could be detected at 34-35 kDa and at 32-33 kDa for the M53 antibody epitope deleted version of Flag309. Infection in the presence or the absence of the phosphonoacetic acid, a replication inhibitor, revealed a true late expression of the wt allele and an early-late one of the Flag-tagged mutant under the control of the SV40 early-enhancer driven expression cassette. Expression of the M53 mutants in the non-induced state was limited, offering us a good opportunity to study a tight phenotype control. One condition of dominant negative principles is to control the mutant expression without affecting the viral transcriptional program as a consequence of toxicity generally. We chose the immediately-early 1 gene product pp89 (89 kDa) and major capsid protein (150 kDa) as markers of the immediate-early and late gene expression programs, respectively. Dox induced expression of DN s309 did not affect the viral transcriptional program but selectively destabilized the M53 and lowered the degree of NEC partner M50. These data fit to the known expression interdependence of the two proteins (Gonella *et. al*., 2005; Bubeck *et. al*., 2004; Ye and Roizman, 2000). Nevertheless, the cassette was not very tightly regulated as there was already a basal s309 expression in the absence of Dox, which already affected the wt M53 distribution.

The recombinants carrying the FlagM53 and a M53 antibody epitope deleted version of the Flag309 (Flag∆Sak309), allowed differentiation between the parental and the Flag-tagged protein. Dox induced Flag∆Sak309 strongly inhibited the wt

allele. These results indicated that this mutant was suitable for further analysis of the inhibitory phenotype of CR4 mutants. Colocalization studies detected - during basal expression of the FlagΔSak309 - a wt distribution pattern with M53 and M50 colocalization at the nuclear rim, which is reflecting the results of the co-transfection experiments (Klupp *et. al*., 2007; Loetzerich *et. al*., 2006; Gonella *et. al*., 2005; Lake and Hutt-Fletcher, 2004; Reynolds *et. al*., 2004/2001; Yamauchi *et. al.*, 2001). M53 showed a tiny dotty staining along the nuclear membrane and inside of the nucleus, a pattern seen in different herpesviruses: KSHV (Santarelli *et. al*., 2008), PrV (Klupp *et. al*., 2007; MCMV (Loetzerich *et. al*., 2006), HCMV (Dal Monte *et. al*., 2002), HSV2 (Zhu *et. al*., 1999), and HSV1 (Chang and Roizman, 1993). Reynolds and the collaborators suggested that the HSV1 M53 homologue binding to the lamins A/C induces a discontinuous punctuate artificial staining due to epitope masking. This suggestion sustains the immunoelectron microscopy data of the HCMV pUL53 which induces INM pseudoinclusions (Dal Monte *et. al*., 2002). The basal expression of s309 was either not enough to affect the wt NEC members nuclear localization or we could not detect the difference due to the limitations of the system.

In contrast, dox induction of the FlagΔSak309 mutant completely changed the protein distribution by relocalization of the wt M53 molecules bulk into the nucleosol and M50 redistribution into aggregates along the nuclear rim. FlagΔSak309 protein colocalized with M50 in punctuates accumulations and filled the nuclear space with aggregates which accumulated over time. The DN M53 pattern recalls the alpha herpesviruses US3 kinase deletion phenotype with aberrant punctuate localization of wt NEC members (Park and Baines, 2006; Reynolds *et. al*., 2002/2001) and transfection of the HSV2 M53 homologue leading to nuclear aggregates which grow and finally fuse (Zhu *et. al*., 1999). These data suggest that M53 accumulates over

the time in the nucleosol if it cannot bind to M50. The excess of the CR4 deletion mutant protein, once M50 binding is saturated, has the same fate.

6.3 M53 CR4 mutants induce defects in capsid maturation

The experiments so far revealed a block in virus replication cycle at the intranuclear stage. We wished to see morphological correlates of the dominant negative phenotype by electron-microscopy (EM) studies, performed by a group of collaborators from the University of Ulm. After 72 h infection with viral recombinant overexpressing the DN s309 showed in the presence of dox a switch of majority of the nucleocapsids from the high density core type (M53R, wt-like infection) to the aberrant forms (s309R), with a weak presence of the mature capsids. The s309R nucleocapsids had accumulated in the nucleosol and were not able to initiate the primary envelopment at the nuclear rim (see Figure 24). Presence of s309 also induces nuclear membranes infoldings (see Figure 24), as noticed for the MCMV DN M50 phenotype (Rupp *et. al*., 2007), which are similar to the effect of M50 overexpression (Ye *et. al*., 2000). This similarity could be explained by s309 capacity to displace M53 from the correct interaction with wt M50. This M53 is redistributed into the nucleosol.

The DN M53 phenotype is reminiscent to the EM pattern of M53 homologues deletion which resulted in accumulation of immature capsids in the nuclear space (Granato *et. al*., 2008; Klupp *et. al*., 2001/2000; Chang *et. al*., 1997) and blocking of the nuclear egress (Fuchs *et. al*., 2002). The association between M53 deletion and nucleocapsid maturation defect was either associated with deficient DNA encapsidation (Chang *et. al*., 1997) or not (Granato *et. al*., 2008).

In order to elucidate the nucleocapsid maturation defect of MCMV M53 inhibitory mutants we designed a Southern-blot assay able to distinguish between the

viral DNA genomic forms, concatemers and unit length genomes. As control we used M53 trans-complementing cell lines which rescue the deletion mutant. The phenotype of the gene deletion mutant showed no effect on cleavage of concatemeric genomes to the unit length genomes. Reduction of the numbers of unit length genomes fit to the alpha herpesvirus HSV1 UL31 phenotype (Chang *et. al*., 1997) and the lack of effect on DNA cleavage fit to the gamma herpesvirus EBV BFLF2 pattern (Granato *et. al*., 2008). The data suggest a possible divergent functional evolution, perhaps a root for the alpha and gamma M53 homologues due to the combination of both features.

Dox induction of CR2 i207 mutants did not affect the viral DNA processing. In contrast to that, the expression of all CR4 mutants inhibited viral DNA cleavage and showed concatemers accumulation at the expense of the unit length genomes. These results are in accord with the other data (growth curves and IF analysis) and southern-blot assay suggest an association of CR4, but not of CR2, with the cleavage/packaging process.

The formation of mature capsids containing the linear double-stranded DNA genome is a complex process involving a preassembled protein shell (procapsid), concatemeric viral DNA, and at least seven other viral proteins believed to play a role in cleavage and packaging of the genome (Mettenleiter 2006/2004/2002; Homa and Brown, 1997).

The HCMV terminase responsible for cleavage of concatemeric DNA was shown to consist of two essential proteins, pUL56 and pUL89 (Thoma *et. al*., 2006; Scheffczik *et. al*., 2002). UL56 connects the viral capsids to the packaging signal of the DNA genome and has an ATP-ase activity. UL89 directly interacts with UL56 and is required for DNA cleavage (Hwang and Bogner, 2002; Scheffczik *et. al*., 2002; Salmon *et. al*., 1999/1998; Bogner *et. al*., 1998/1993). The portal (pUL104) is

involved in viral genome translocation into the capsid (Dittmer *et. al*., 2005). Successful packaging of viral DNA results in sealed DNA-filled C capsids, whereas the A capsids, containing neither DNA nor the inner layer of the capsid shell, are the results of an abortive DNA packaging.

Another four additional viral factors play a role in cleavage/packaging of the virus genome and subsequent steps of maturation, such as nuclear egress of the DNA-filled capsids. These are the HCMV proteins UL51, UL52, UL77 and UL93, which are homologous to the herpes simplex virus type 1 (HSV-1) proteins UL33, UL32, UL25 and UL17, respectively. The knowledge about these proteins is limited. HSV-1 UL33, which stabilizes the terminase and its anchorage to the capsid (Beard, Taus and Baines, 2002) is involved in viral DNA translocation into the capsid (Yang, Homa and Baines, 2007; Beard and Baines, 2004). The absence of UL17 blocks the DNA concatemers cleavage and then the DNA is not packaged (Thurlow *et. al*., 2005). Deletion of UL25 is less prohibitive (Stow, 2001). However, packaging takes place but this DNA does not seams to be stably encapsidated, having as result only few C-capsids present in the nuclei of the infected cells (McNab *et. al*., 1998). Opposite to that, the UL25-null mutant of PrV shows C-capsid formation but these capsids do not undergo nuclear export (Klupp *et. al*., 2006). UL25 forms a complex with UL17 which stabilizes the capsid during and after the DNA packaging process (Trus *et. al*., 2007; Newcomb, Homa and Brown, 2006; Thurlow *et. al*., 2006). Failure of DNA cleavage and the absence of mature C capsids after infection with an UL52 deletion HCMV recombinant suggested that also UL52 plays a role in encapsidation and/or DNA cleavage. The colocalization of UL52 with ICP8, a replication compartment marker, at the nuclear periphery suggested a role in capsid transport to replication compartments (Borst *et. al*., 2008). More, disturbing one of the complexes involved in capsid stabilization or transport result in formation of DNA-lacking capsids

composed of two concentric shells, which are believed to be default products of defective maturation, described as B-type capsids (Newcomb *et. al*., 1996).

The DN phenotype induced by CR4 mutants of the MCMV M53 protein resembles to the above described patterns. In A-type capsids, the lack of the scaffolding ring indicates a failure of capsid sealing after packaging the unit length genome. Abundant presence of B-type capsids is suggestive for a problem of encapsidation. The presence of the inner layer of the capsid shell, in absence of DNA, may indicate a failure of the packaging machinery or instability of the capsid. When we correlate the morphological features with the southern-blot results we can perhaps interpret the CR4 of MCMV M53 as a provider of a sealing/maturation signal for nucleocapsid transport from the replication compartments to the primary envelopment sites.

It should be recalled that the s309 mutant is expressed with an early-late kinetics under the control of the SV40 early-enhancer, which provides a situation where the mutant has a chance to meet other potential interaction partners even prior to the NEC formation. Certainly, one has to take in account that CMV has a long replication cycle and the definition of the early-late phase is mainly a quantitative term. Altogether, M53 protein deletion and DN s309 expression show different phenotypes because the deletion of wt allele allows formation of a functional nuclear egress up-stream complex missing a component, whereas the latter binds molecules of a subsequent maturation step but blocks the flow by lack of an essential domain.

6.4 The effect of the DN CR4 mutation on nucleocapsid maturation is independent of NEC formation

As discussed above, there is a number of proteins which are apparently involved in nucleocapsid stabilization, sealing or/and maturating. UL17 has an influence on the capsid angular shape and signals maturation upon complex formation with UL25 (HSV1; Trus *et. al*., 2007; Bowman *et. al*., 2006; Newcomb, Homa and Brown, 2006). Yet, UL25 is also essential for the nuclear egress (PRV; Klupp *et. al*., 2006). There is the possibility that the UL25 function is divergent for different viruses (Kuhn *et. al.*, 2008). pUL52 has an effect on the HCMV genome encapsidation without being a component of the cleavage/packaging machinery (Borst *et. al*., 2008), which supports the observation of HSV1 UL32 homologue being involved in capsid transport to the replication compartments (Lamberti and Weller, 1998).

The DN M53 CR4 mutant combines features of proteins involved in sealing/maturation and nuclear egress. An interesting aspect of this phenotype is that a single mutation causes a defect in two intranuclear morphogenesis steps. In order to dissect the connection between these two steps, a double knock-out recombinant was created - s309i128R. In 2006 our group published the nucleosolic accumulation of the i128 mutant after co-transfection with M50. The new M53 mutant lacks the CR4 and also the M50 binding site due to a 5 aa insertion at position 128.

Conditional expression of this mutant showed down-regulation of virus growth by more than 1000 fold but clearly less than DN s309. Given the protein stabilizing effect on the M53-M50 interaction, stability problems of the mutant protein could occur in absence of M50 binding capacity. The HA pulldown experiment performed after co-transfecting the s309i128 with a HA tagged M50 construct revealed a 34-35 kDa protein, which did not bind to M50 as expected.

Immunofluorescence studies of the s309i128R identified an intranuclear localization pattern of the NEC members different from the DN s309 with no effect on the M50 nuclear rim staining but with wt M53 allele redistribution into aggregates along the nuclear rim and/or into the nucleosol. Despite these differences between the s308i128R and FlagΔSak309R, in respect of viral genomic forms detection there were no differences in concatemers accumulation and reduction of detected unit length genomes.

Thus the DN approach assigns new functional domains which act independently in a temporal cascade.

6.5 The MCMV M53 screen and further screens

This work provided evidence that our approach based on random mutant screens to identify viral DN mutants is successful and reproducible. The number of the identified DN alleles and the quality of the two screens we performed were superior to our previous studies (Rupp *et. al*. 2007 and present study). One possibility remains that our high hit rate for DNs presented in this study was a chance finding and is due to specific properties of the tested genes. Therefore the approach needs to be tested by the analysis of several essential herpesvirus genes, to illustrate the general applicability for all herpesvirus genes and herpesviruses of the three subfamilies, and perhaps also for other virus families, too.

During this work the methodology of the inhibitory screen was optimized and the time required for the complete screen could be reduced to about half of the previous screen. This improvement makes it feasible to test a collection of MCMV genes in the future. We believe that the quality difference between the two screens is mainly dependent on the quality of the gene mutant library (Loetzerich *et. al*. 2006; Bubeck *et. al*. 2004).

Our demonstrator model, the murine cytomegalovirus is a beta herpesvirus. We could already translate some findings to the human cytomegalovirus (Rupp *et. al*., 2007), also a member of the beta herpesvirus subfamily - an important human pathogen. For the herpesvirus field in general is of limited value as the two genomes are almost collinear in the essential genes. It would be of interest to translate the approach, to test alpha and the gamma herpesvirus genes.

7. Conclusions

Completing a genetic screen for inhibitory mutants of the MCMV nuclear egress protein M53 two new functional domains were identified. One, comprising the conserved region 2 (CR2) plays a role in the nuclear egress and together with the CR1 is involved in NEC formation (Loetzerich *et. al*., 2006). Surprisingly, we discovered a second, separate functional domain, formed by CR4. Conditional expression of inhibitory CR4 mutants combined with phenotypic analysis at ultra structural and molecular level provided the first evidence that M53, besides its established role in the nuclear exports of the capsids, is also involved in the DNA cleavage/packaging and/or capsid sealing after packaging.

This data demonstrate that our random approach allows the identification of viral DN mutants of an essential herpesvirus gene without any knowledge on its protein structure. Thereby, the essential functions of a multifunctional herpesvirus protein were successfully dissected by DN mutants, providing new fundamental data on essential steps of the herpesvirus morphogenesis.

8. Outlook

Flag-tag proved to be a powerful tool in distinguishing between the wt and DN phenotypes. As consequence we were able to define the nuclear egress block mechanism by competition of the wt allele and DN gene product for NEC formation. Abolishing the M50 binding capacity of the DN s309 mutant by 5 aa insertion at the position 128 we could explain the accumulation of immature capsids in the nucleus by the DN effect on cleavage/packaging and/or sealing of the capsid. This indicates that MCMV M53 is part of a complex which connects the nucleocapsid maturation with the nuclear egress.

Then it was of interest to analyze this bridging complex and therefore we designed another TAP-tag. The tandem affinity purification (TAP) technique has been used to identify – under nondenaturing conditions – the protein complexes in yeast, but difficult to apply in mammalian cells. Replacing the two IgG binding units of Staphylococcus aureus protein A (ProtA) from the CBP-TEV-ProtA (calmodulin binding protein, CBP; TEV, tobacco etch virus cleavage site recognized by the specific TEV protease; see Rigaut *et. al*., 1999) with a minimal biotinylation-tag, the resulting Bio-TEV-ProtA allowed sufficient recovery of proteins from the mouse fibroblast monolayer in order to identify the complex members by mass spectrometry (Drakas, Prisco and Baserga, 2005).

Next, the biotinylation-tag (MSGLNDIFEAQ**K**IEWHE; Drakas, Prisco and Baserga, 2005) was combined with an HA-tag and the necessary biotin holoenzyme synthetase (*BirA*) sequence (BirA_EC-K12 strain, *BirA* - 966bp; see Schatz, 1993) remained to be inserted into the MCMV BACs. The *BirA* gene catalyzes the covalent addition of biotin to the ε amino group of the lysine side (see the **K** aa from the biotinylation-tag sequence). The minimal biotinylation substrate identified by a

peptide library screening (13 aa; see Schatz, 1993) is much smaller than the 75 amino acid required sequence of the natural substrate but it can function at either end of a fusion protein. A new Bio-HA tag (20 aa) was inserted at the *N*-terminus of our proteins, wt M53 and DN s309, in order to have the advantage of the high biotin binding affinity to the monomeric avidin resin. First the Bio-HA tap-tagged constructs were inserted at m16-m17 FRT ectopic position of the wt MCMV BAC and checked for reconstitution. Both recombinant proteins, BioHAM53 and BioHA309, kept the features of the paternal constructs (wt M53 and s309). The recombinant virus expressing the TAP-tagged wt allele (BioHAM53R) reconstituted after 5 dpi and lysed the complete MEFs monolayer after 2 weeks. BioHA309R virus was grown defective during the whole 6 weeks experiment.

The use of the new TAP-tagged recombinants described here shall provide the basis of a comparative analysis of the wt and DN protein complexes and identifies the components missing due to the M53 CR4 deletion.

The dominant negative mutant of M53 showed an unparalleled strength of inhibition. We believe that they will inhibit MCMV replication even if they are expressed in trans. This provides a basis to investigate the genetic immunization against herpesviruses, using the well established MCMV model.

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Supplementary information

S.1 List of abbreviations

α	antibodies
Δ	deletion
293	adenovirus transformed human kidney-carcinoma cells
ab	antibodies binding site
aa	amino acid/s
amp	ampicillin
APS	ammonium persulfate
β-me	β-mercapto-ethanol
BAC	bacterial artificial chromosome
BAF	barrier to auto integration factor
Bio	Biotin
Bp	base pair
BSA	bovine serum albumin
^{o}C	Celsius degree
C127	mouse mammary epithelial cells
cam	chloramphenicol
CMV	cytomegalovirus
CR	conserved region
Croma	Croatia monoclonal antibodies
d	day/s
Da	Dalton
DH10B	Escherichia coli strain for maintenance of MCMV-BAC
DM	double knock-out
DMEM	Dulbecco's modified Eagle medium
DN	dominant negative
DNA	deoxyribonucleic acid
dNTP	desoxyribonucleotide

Dox	doxycycline
DTT	1, 4 dithiotreitol
E	m16-m17 FRT ectopic position of MCMV genome
E. coli	Escherichia coli
EBV	Epstein - Barr virus
EDTA	ethylenediamine tetraacetic acid
EHV	equine herpesvirus
ER	endoplasmic reticulum
et. al.	et alii (lat., and others)
EtOH	ethanol
FCS	fetal calf serum
Fig.	Figure
Flag	Flag-tag
FLP	recombinase which recognize FRT sites
FRT	FLP recognition site
GI	gene of interest
gp	glycoprotein
h	hour/s
HA	hemagglutinin tag
HCMV	human CMV
HSV	human simplex virus
i	insertion
IF	immunofluorescence
ILTV	avian infectious laryngotracheitis virus
INM	inner nuclear membrane
kan	kanamycine
kb	kilo bases
KSHV	Kaposi sarcoma herpesvirus
l	liter
LB	Luria-Bertani medium
LBR	lamin B receptor

lg	logarithm with basis 10
M	molarity
M2-10B4	stroma-cell-line from bone marrow of BALB/c-mouse
MCMV	mouse CMV
MEF	BALB/c murine embryonal fibroblasts
MeOH	methanol
min	minute/s
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
N.A.	not applied
NCS	neonatal calf serum
NE	nuclear envelope
NEC	nuclear egress complex
NIH3T3	contact inhibited murine fibroblasts of NIH Swiss-mouse
NLS	nuclear localization signal
OD	optical density
ON	over night
ONM	outer nuclear membrane
ORF	open reading frame
pi	post infection
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
P_{CMV}	HCMV immediately-early enhancer-promoter
PCR	polymerase chain reaction
PD	pull-down experiment
PFU	particles for infectious unit
PIR1	E. coli strain for maintenance of po6k-ie vector
PrV	pseudorabies virus
P/S	penicillin/streptomycin

P_{SV40}	HCMV immediately-early promoter under control of SV40 early-enhacer
RNA	ribonucleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
s	stop codon or truncated mutant
Sak	anti-M53 antiserum
SDS	sodium dodecylsulfate
SSC	sodium chloride, sodium citrate buffer
St	Strep-tag
SV40	simian virus 40
SVEC	mouse lymphoid endothelial cells immortalized by simian virus 40
SVT	HCMV immediately-early promoter under control of SV40 early-enhacer
T	TEV cleavage site
Tab	Table
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TBST	Tris buffered saline buffer with Tween 20
TE	Tris EDTA buffer
TEMED	N, N, N', N' – tetramethylenediamine
tetO	tetracycline minimal operator
TetR	tetracycline repressor
TLB	total lysis buffer
TM	transmembrane region
TR	terminal repeats
Tris	Tris(hydroxymethyl)aminomethan
U	unit/s, enzyme activity
UL	unique long region
US	unique short region
V	volt/s
VR	variable region

VSB	virus standard buffer
v/v	volume/volume
VZV	Varicella Zoster virus
WB	western-blot
wt	wild type
w/v	weight/volume
µ	micro (10^{-6})
µg	microgram
µl	microliter
µm	micrometer
Zeo	zeocin

Amino acids

A, Ala	alanine
C, Cys	cysteine
D, Asp	aspartic acid
E, Glu	glutamic acid
F, Phe	phenylalanine
G, Gly	glycine
H, His	histidine
I, Ile	isoleucine
K, Lys	lysine
L, Leu	leucine
M, Met	methionine
N, Asn	asparagine
P, Pro	proline
Q, Gln	glutamine
R, Arg	arginine
S, Ser	serine
T, Thr	threonine
V, Val	valine

W, Trp	tryptophan
Y, Tyr	tyrosine

S.2 List of figures

S.3 List of tables

S.4 Posters and oral presentations

1. Junior Faculty Retreat, Oct. 2005, Waging am See, Germany; Mirela Popa, Zsolt Ruzsics, Mark Lötzerich, U. Koszinowski; “Dominant negative mutants of MCMV M53/p38 protein” – poster;

2. Annual meeting of the “Gessellschaft für Virologie” 2006, 15-18 March , Munich, Germany; Mirela Popa, Zsolt Ruzsics, Mark Lötzerich, Brigitte Rupp, U. Koszinowski; “Screening for dominant negative mutants of nuclear egress protein M53” – poster;

3. FORINGEN 2006 – oral presentation;

4. 32-nd International Herpesvirus Workshop 2007, 7-12 July, Asheville, NC, USA; Mirela Popa, Mark Lötzerich, Zsolt Ruzsics, U. Koszinowski; A random screen for dominant-negative mutants of the cytomegalovirus nuclear egress protein M53” –poster;

5. FORINGEN 2007 – oral presentation;

6. 18-th Annual Meeting Gesellschaft für Virologie 2008, 5-8 March, Heidelberg, Germany; Mirela Popa, Zsolt Ruzsics, Christopher Buser, Paul Walther, Ulrich H. Koszinowski; “Systemic screen for dominant negative mutants of M53 gene reveals effects on both DNA packaging and nuclear egress” – oral presentation.

S.5 Publications

Popa M, Ruzsics Z, Lötzerich M, Dölken L, Buser C, Walther P, Koszinowski UH; “Dominant negative mutants of the murine cytomegalovirus M53 gene block nuclear egress and inhibit capsid maturation”; Journal of Virology, 84(18):9035-46, 09/ 2010.

Others publications

1. Barbarii L, Constantinescu C, Popa M, Toroiman S, Stefanescu D; “Genetic variability of 6 STR loci in Romanian population”; Romanian Journal of Legal Medicine, vol. XI, 03/2003;

2. Toroiman S, Constantinescu C, Popa M, Stefanescu D; “16 versus 9 STR loci typing in paternity testing”; Romanian Journal of Legal Medicine, vol. XI, 03/2003;

3. Popa M, Constantinescu C, Barbarii L, Dermengiu D; “Mitochondrial DNA genotyping”; Romanian Journal of Legal Medicine, vol. XII, 03/2004.

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S.7 CURRICULUM VITAE

Experience:

1. Pasteur Institute, Master seed Department,

Bucharest, Romania, since July 2009

Project subject: Development of the Master seed Department;

Techniques: mammalian cells culture; chicken embryonic fibroblasts preparation; mammalian cells transient transfection; vaccine strains genotyping; virus stock preparation and quantification; virus neutralization test; gene expression (one step RT-PCR); protein expression (Western-blot); DNA packaging assay (Southern-blot); fluorescent antibodies test (Zeiss system);

2. Life and Brain, Institute of Molecular Psychiatry,

Bonn, Germany, Dec. 2008 – June 2009

Project subject: *Development and validation of adenosine receptors antagonists as new drug therapy against neurodiseases*;

Techniques: mammalian cell culture (primary and immortal); bio-luminescence report assay; ELISA; gene expression analysis; immunofluorescence; statistical analysis.

3. Max von Pettenkofer Institute, Department of Virology, Ludwig-Maximilians University,

Munich, Germany, Feb. 2005 – Dec. 2008

PhD subject: *Cytomegalovirus morphogenesis*;

Techniques: bacterial and mammalian cells culture; mouse embryonic fibroblasts preparation; plasmidial and ET cloning; BAC technology; bacterial cells chemical transformation; mammalian cells transient transfection; virus reconstitution assay; conditional expression system; virus stock preparation and quantification; protein expression and pull-down experiments (Western-blot); viral DNA packaging assay (Southern-blot); confocal microscopy (Zeiss system); cellular transcomplementation of viral proteins;

4. Institute of Biology III, Molecular Genetics laboratory; Albert-Ludwigs University,

Freiburg im Breisgau, Germany, July-Sep. 2004

Project subject: *NFI C and X transcription factors expression analysis during embryonic development*;

Techniques: mice breeding; organs preservation by paraffin embedding; manual microtomy; immunohistochemistry;

5. National Institute of Legal Medicine, Forensic Genetics Laboratory,

Bucharest, Romania, 1999 –2005

Projects: *Paternity testing; Traces DNA analysis; Romanian DNA data-base*;

Techniques: traces DNA extraction and purification; PCR; DNA fragment analysis; mitochondrial DNA sequencing; biological samples preservation; statistical analysis of the genetics data;

6. National Institute of Animal Biology, Molecular Genetics Laboratory, Bucharest, Romania, 1995-1999

Subject: *Association between the HLA/BoLA mutations and disease susceptibility*;

Techniques: cells culture DNA extraction; PCR; directed site mutagenesis; restriction fragments length polymorphism; viral genome replication assay (bovine leukaemia virus).

Education:

1. Feb. 2005 – Dec. 2008: Max von Pettenkofer Institute, Ludwig-Maximilians University, Munich, Germany, PhD – *MCMV nuclear egress complex analysis*;
2. July-Sept. 2004: Institute of Biology III, Albert-Ludwigs University, Freiburg im Breisgau, Germany, *NFIC and X transcription factors expression analysis*;
3. April-May 2004: Institute of Legal Medicine, Uniklinikum, Freiburg im Breisgau, Germany; *DNA traces analysis*;
4. May 2003: Institute of Legal Medicine, Uniklinikum Freiburg im Breisgau, Germany; *Mitochondrial DNA analysis;*
5. April 2003: Institute of Human Genetics and Anthropology, Albert-Ludwigs University, Freiburg im Breisgau, Germany; *Molecular Biology training*;
6. Sept. 2002: University of Medicine, Oradea, Romania; *Medical Genetics*;
7. Sept.-Oct. 1999: National Institute of Legal Medicine, Bucharest, Romania; *Medical genetics techniques; Genetics and the Justice*;
8. 1997: Cantacuzino Institute, Bucharest, Romania; *Molecular Biology techniques*;
9. 1994 – 1995: Babes-Bolyai University, Faculty of Biology, Cluj-Napoca, Romania; Master of Science: M*olecular Biology*;
10. 1989 – 1994: Babes- Bolyai University, Faculty of Biology, Cluj-Napoca, Romania; Bachelor of Science: *Animal Biology*.

Participations to the scientific meetings and publications:

1. Scientific session of the Faculty of Veterinary Medicine, Bucharest, 9/05/1997, Mirela (Militaru) Popa, Ioan Cureu; “Genetics control on MHC region by domestic animals and implications in their pathology”;

2. The XXVI National Conference on Immunology, Cluj Napoca, 2/10/1997, Mirela (Militaru) Popa, Ioan Cureu; “MHC implications in animal diseases susceptibility”;

3. Scientific session of Biological Sciences, Bucharest,12-16/11/1997, Mirela (Militaru)Popa, Maria Damian, Ioan Cureu; “Detecting BoLA – DR B3 2A allele presence in a bovine population by chain enzymatic amplification reaction (PCR)”;

4. Scientific symposium: “Scientific Fundamentals of development animal production”, Bucharest, 23/06/1998, Mirela (Militaru) Popa, Daniela Tudor, Stefania Tonceanu, “Investigations about MHC role on immune response adjustment”;

5. The XXVI International Conference on Animal Genetics, Auckland, New Zeeland, 9-14/08/1998, Mirela (Militaru) Popa, Maria Damian, Ioan Cureu, “PCR detection of common deletion DR B3 2A in bovine population from Romania”;

6. Scientific session of Faculty of Veterinary Medicine, Bucharest, 15-16/10/1998, Mirela (Militaru) Popa, Maria Damian, Ioan Cureu, “PCR-RFLP detection of 65 codon deletion in a bovine population from Romania”;

7. Scientific symposium of Faculty of Biology, Bucharest, 15-16/10/1998, Mirela (Militaru) Popa, Maria Damian, Ioan Cureu, “ DR B3 analysis by PCR in a bovine population – I.B.N.A. ownership”;

8. Scientific session of Cantacuzino Institute, Bucharest, 19-20/11/1998, Mirela (Militaru) Popa, Maria Damian, Ioan Cureu, “PCR detection of MHC DR B genes using microsatellites primers”;

9. The First Romanian Genetics Congress, Oradea, 19-22/09/2002, Dragos Stefanescu, Simona Toroiman, Mirela Popa, Carmen Constantinescu, “Information about STR systems in a Romanian population”;

10. Romanian Journal of Legal Medicine, vol. XI, 03/2003, Ligia Barbarii, Carmen Constantinescu, Mirela Popa, Simona Toroiman, Dragos Stefanescu, “Genetic variability of 6 STR loci in Romanian population”;

11. Romanian Journal of Legal Medicine, vol. XI, 03/2003, Simona Toroiman, Carmen Constantinescu, Mirela Popa, Dragos Stefanescu, “16 versus 9 STR loci typing in paternity testing”;

12. Romanian Journal of Legal Medicine, vol. XII, 03/2004, Mirela Popa, Carmen Constantinescu, Ligia Barbarii, Dan Dermengiu, “Mitochondrial DNA genotyping”;

13. Junior Faculty Retreat, Oct. 2005, Waging am See, Germany; Mirela Popa, Zsolt Ruzsics, Mark Lötzerich, U. Koszinowski; “Dominant negative mutants of MCMV M53/p38 protein”;

14. Annual meeting of the “Gessellschaft für Virologie” 2006, 15-18 March , Munich, Germany; Mirela Popa, Zsolt Ruzsics, Mark Lötzerich, Brigitte Rupp, U. Koszinowski; “Screening for dominant negative mutants of nuclear egress protein M53”;

15. 32-nd International Herpesvirus Workshop 2007, 7-12 July, Asheville, NC, USA; Mirela Popa, Mark Lötzerich, Zsolt Ruzsics, U. Koszinowski; A random screen for dominant-negative mutants of the cytomegalovirus nuclear egress protein M53”;

16. 18-th Annual Meeting Gesellschaft für Virologie 2008, 5-8 March, Heidelberg, Germany; Mirela Popa, Zsolt Ruzsics, Christopher Buser, Paul Walther, Ulrich H. Koszinowski; “Systemic screen for dominant negative mutants of M53 gene reveals effects on both DNA packaging and nuclear egress”.

Associations’ member:

International Society of Forensic Genetics;

Foreign languages:

English – speak, write, read, translation – advanced level;

German– speak, write, read, translation – beginner level;

French – speak, write, read, translation – beginner level;

Computer:

WINDOWS XP, MS Office – Word, Excel, Power Point – medium level;

Corel Draw - Photo paint, Adobe Reader - Photoshop 7, Graph Prism – medium level;

Vector NTI – Invitrogen, genetic design program;

LSM 510 – Zeiss, image analysis program.