Med Microbiol Immunol (2015) 204:383–394
DOI 10.1007/s00430-015-0414-1

CrossMark

ORIGINAL INVESTIGATION

An endocytic YXXΦ (YRRF) cargo sorting motif in the cytoplasmic tail of murine cytomegalovirus AP2 'adapter adapter' protein m04/gp34 antagonizes virus evasion of natural killer cells

Annette Fink[1] · Franziska Blaum[1] · Marina Babic Cac[2,3] · Stefan Ebert[1,4] · Niels A. W. Lemmermann[1] · Matthias J. Reddehase[1]

Received: 20 March 2015 / Accepted: 26 March 2015 / Published online: 8 April 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract Viruses have evolved proteins that bind immunologically relevant cargo molecules at the cell surface for their downmodulation by internalization. Via a tyrosine-based sorting motif YXXΦ in their cytoplasmic tails, they link the bound cargo to the cellular adapter protein-2 (AP2), thereby sorting it into clathrin-triskelion-coated pits for accelerated endocytosis. Downmodulation of CD4 molecules by lentiviral protein NEF represents the most prominent example. Based on connecting cargo to cellular adapter molecules, such specialized viral proteins have been referred to as 'connectors' or 'adapter adapters.' Murine cytomegalovirus glycoprotein m04/gp34 binds stably to MHC class-I (MHC-I) molecules and suspiciously carries a canonical YXXΦ endocytosis motif YRRF in its cytoplasmic tail. Disconnection from AP2 by motif mutation ARRF should retain m04-MHC-I complexes at the cell surface and result in an enhanced silencing of natural killer (NK) cells, which recognize them via inhibitory receptors. We have tested this prediction with a recombinant virus in which the AP2 motif is selectively destroyed by point mutation Y248A, and compared this with the deletion of the complete protein in a Δm04 mutant. Phenotypes were antithetical in that loss of AP2-binding enhanced NK cell silencing, whereas absence of m04-MHC-I released them from silencing. We thus conclude that AP2-binding antagonizes NK cell silencing by enhancing endocytosis of the inhibitory ligand m04-MHC-I. Based on a screen for tyrosine-based endocytic motifs in cytoplasmic tail sequences, we propose here the new hypothesis that most proteins of the *m02–m16* gene family serve as 'adapter adapters,' each selecting its specific cell surface cargo for clathrin-assisted internalization.

Keywords Adapter protein (AP2) · Cargo sorting · Clathrin-mediated endocytosis · Endocytosis motif · Immune evasion · Tyrosine-based motif YXXΦ

This article is part of the Special Issue on Cytomegalovirus.

✉ Annette Fink
finka@uni-mainz.de

[1] Institute for Virology, University Medical Center of the Johannes Gutenberg-University Mainz and Research Center for Immunotherapy (FZI), Obere Zahlbacher Strasse 67, Hochhaus am Augustusplatz, 55131 Mainz, Germany

[2] Department of Histology and Embryology, School of Medicine, University of Rijeka, Rijeka, Croatia

[3] Present Address: German Rheumatism Research Centre, Innate Immunity Group, Berlin, Germany

[4] Present Address: Section for Experimental Tumor Immunology, Department for Obstetrics and Gynaecology, University of Wuerzburg Medical School, Würzburg, Germany

Introduction

Murine cytomegalovirus (mCMV) genes *m04, m06*, and *m152* code for type-I glycoproteins that interfere with the trafficking of peptide-loaded MHC class-I (pMHC-I) molecules by disconnecting them from the constitutive vesicular flow to the cell surface for mediating immune evasion (for reviews, see [1–4]).

The MHC-I-like viral protein (MHC-Iv) m152/gp40 does not stably bind to pMHC-I but is thought to interact transiently through its luminal portion. By a poorly understood mechanism, it operates as a 'retainer' [5] in that it mediates the retention of pMHC-I complexes in a cis-Golgi ER-Golgi intermediate compartment [6–8]. This contributes to virus evasion of CD8 T cells [9, 10]. It should

be recalled that in compartments beyond the ER, MHC-I molecules are usually loaded with peptides, self or foreign, and are thus predominantly pMHC-I complexes. Notably, m152/gp40 strongly interferes with cell surface expression of recently loaded pMHC-I but is less efficient in downmodulating total cell surface pMHC-I (reviewed in [4]), a finding which is in accordance with the known retention function that blocks the transit from the ER to the cell surface rather than the recycling of cell surface-resident pMHC-I [6–8, 11]. Interestingly, m152/gp40 not only interacts with classical MHC-I allomorphs but also with MHC-I-like RAE1 family ligands of the activatory natural killer (NK) cell receptor NKG2D [12, 13]. Failure to activate NK cells due to retention of RAE1 contributes to virus evasion of NK cells [14]. As a proposed paradigm for MHC/MHC-like interactions in immune evasion, resolving the X-ray crystal structure of the quite stable m152/RAE1γ complex identified the intermolecular contact sites between m152 and the α1 and α2 helices of the luminal portion of RAE1γ, distant from the N-glycosylation sites of m152 [15]. In accordance with the crystal structure, our own work revealed that the unglycosylated m152 isoform p36 suffices for mediating evasion of both CD8 T cells and NK cells [16]. The gene products of other members of the *m145* gene family, to which *m152* belongs, namely of *m145* and *m155*, contribute to evasion of NK cells by downmodulating NKG2D ligands MULT1 and H60, respectively (reviewed in [17]).

Genes *m04* and *m06*, closely related genes of the *m02* gene family [18], code for type-I glycoproteins m04/gp34 [19] and m06/gp48 [20], respectively. Both glycoproteins share the properties to bind stably within the ER to the MHC-I α chain through their luminal portions and to act as ‘sorters’ [5] by directing the pMHC-I complexes into different cargo sorting pathways. For m06/gp48, Reusch et al. [21] have shown that a functional di-leucine motif EXXXXLL (specifically EPLARLL) in the membrane-proximal region of the cytoplasmic (C-terminal) tail links the m06-pMHC-I complexes to the tetrameric cellular adapter proteins AP1-A and AP3-A that sort the complexes from the trans-Golgi network (TGN) to late endosomes and from there to lysosomes, respectively, resulting in their degradation [20, 21]. This identified m06/gp48 as a ‘connector’ or ‘adapter adapter’ protein [22] that physically connects a cargo, pMHC-I in the specific case, with cellular APs. One can view this also in the sense that by associating with m06/gp48, pMHC-I gets equipped with an AP1-A/AP3-A binding motif for entering the corresponding cargo sorting pathway. Notably, m06/gp48 is most effective in downmodulating total cell surface pMHC-I ([23, 24], reviewed in [4]), suggesting a role also in interrupting pMHC-I recycling by routing endocytosed pMHC-I to late endosomes and lysosomes for accelerated degradation.

Less is known about the precise routing and sorting of m04-pMHC-I complexes, except that they obviously escape m152/gp40-mediated ER retention and do not end up in the lysosome but reach the cell surface [19]. There, they serve as ligands, or counter-receptors, for mostly inhibitory Ly49 family receptors on NK cells, leading to NK cell evasion by NK cell silencing [25], with a reported exception being the activatory receptor Ly49P interacting with m04/gp34-H-2D^{k} [26] (reviewed in [27]). Binding of m04/gp34 to MHC-Iα in the ER is stabilized by peptide loading and requires the luminal (N-terminal) portion and the transmembrane domain (TMD), but not the cytosolic tail [28]. We consider it plausible to propose that the interaction is by the ER-luminal domain, whereas lack of the TMD, and therefore lack of membrane anchorage, dislocates the molecule to the cytosol where it can no longer interact with ER membrane-bound (p)MHC-I. Recent work by Berry et al. [29] on the crystal structure of the m04/gp34 ectodomain and its interaction with MHC-I molecules H-2L^{d}, D^{d}, and D^{k} indeed indicated binding via an Ig-V-like structure. A role in evasion of CD8 T cells, adding to the function of m152/gp40 and m06/gp48, has been indicated by a moderately enhanced downmodulation of total cell surface pMHC-I and reduced recognition by epitope-specific T cells in the presence of m04/gp34 compared to its absence after infection with *m04* gene deletion mutants [23, 24, 30, 31].

We have been first to note that m04/gp34, like m06/gp48 and suggesting functional relatedness between these two *m02* gene family members, carries an adapter protein-binding motif in the membrane-proximal region of its cytoplasmic tail, though not a di-leucine motif but a tyrosine-based motif YXXΦ, specifically YRRF [3, 4]. This motif, most likely just incidentally, coincides with the C-terminal portion of a CD8 T cell-antigenic m04 peptide 243-YGPSLYRRF-251 that is presented by the MHC-I protein H-2D^{d} [32]. Corbett et al. [33] have sequenced the *m02–m05* region, encompassing *m04*, in a number of mCMV isolates from genetically polymorphic wild-derived mice and have noted an overall extensive sequence variation of *m04* contrasting with a high degree of conservation of *m02* family-defining motifs described previously by Rawlinson et al. [18] as well as of the region that codes for the antigenic peptide [32] (Fig. 1, based on data from [33]). Refocussed interest in the YRRF motif reveals its conservation, except for a mutation Y248C in a group of isolates. As Y is considered to be irreplaceable for the motif’s function in adapter protein binding (reviewed in [34]), this naturally occurring mutation/viral polymorphism is most likely a loss-of-function mutation, although it is suspicious that it is also kind of conserved, whereas other putative loss-of-function mutations Y248X were apparently lost in virus evolution, at least in the wild-derived mice collected for the study by Corbett et al. [33].

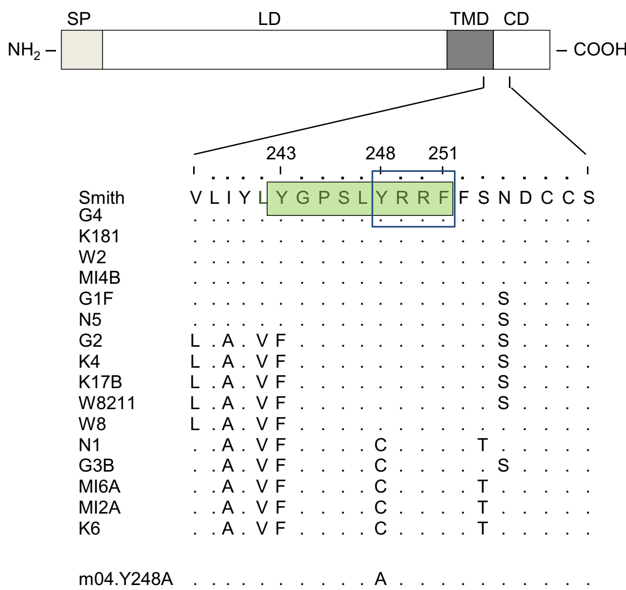

Fig. 1 Conservation of the endocytic YXXΦ motif (L)YRRF in m04/gp34. *Top* schematic representation (not drawn to scale) of the 266-amino acid protein. *SP* signal peptide, *LD* luminal domain, *TMD* transmembrane domain, *CD* cytoplasmic domain. *Bottom* Alignment of predicted amino acid sequences between positions 238–258 for mCMV laboratory strains and wild-derived isolates, as well as mutant virus mCMV-m04.Y248A. Mutations relative to the Smith strain are indicated. The antigenic peptide [32] is highlighted by *green* background *shading*, and the canonical endocytic motif is highlighted by a *blue box*. Sequences are derived from the work by Corbett et al. [33]

YXXΦ motifs are mostly associated with recruitment of adapter protein AP2 that selects cargo from the plasma membrane for clathrin-mediated endocytosis ([35]; for reviews, see [34, 36, 37]). Free pMHC-I cell surface molecules are constitutively internalized by clathrin-independent Arf6-associated endocytic carriers, and it has been shown that in uninfected fibroblasts, less than one-third of endocytosed pMHC-I molecules are recycled, whereas the remaining two-thirds enter the endolysosomal pathway for degradation (reviewed in [38]). Presence of the YXXΦ motif YRRF in the cytoplasmic tail of m04/gp34 suggests that it acts, like its relative m06/gp48, as a 'connector' or 'adapter adapter' protein. By binding to pMHC-I and physically connecting cell surface pMHC-I cargo with AP2, m04/gp34 could mediate accelerated endocytosis, followed by recycling of part of the complexes to the cell surface and degradation of the remaining complexes. With this view in mind, we previously hypothetically proposed to categorize m04/gp34 as a 'recycler' protein [3, 4].

Here we provide first evidence to conclude that the AP2 cargo sorting motif YRRF in the cytoplasmic tail of m04/gp34 is in fact used, and describe the functional phenotype of the motif mutation ARRF (Y248A) in virus evasion of NK cells.

Materials and methods

Infection of cells

Bacterial artificial chromosome (BAC)-cloned virus MW97.01, derived from BAC plasmid pSM3fr, [39] was used as 'wild-type' virus WT.BAC. Mutant virus mCMV-Δm04 was described in a previous report [23], but was replaced here with a newly generated virus mCMV-Δm04^{L} no longer containing a kanamycin cassette (author N. A. W. L., unpublished). Third-passage mouse embryo fibroblasts (MEF) were pretreated for 48 h with 20 ng of IFN-γ per ml of culture medium, if not told otherwise, or were left untreated [24]. After this period, MEF were infected with the indicated viruses at a multiplicity of infection (MOI) of 4, using the method of centrifugal infection ([40, 41] and references therein).

Generation of recombinant viruses

Virus mCMV-m04.Y248A was generated based on BAC plasmid pSM3fr by Red-mediated markerless DNA recombination described by Tischer et al. [42]. In brief, oligonucleotides pEPKan-S-m04YA_for (5′-CTTCTCGTACTGATTTACTTGTACGGACCCTCATTAGCCCGTCGTTTCTTCAGTAATGATTGTTGCTCGAGGATGACGACGATAAGTAGGG-3′) and pEPKan-S-m04YA_rev (5′-CTCTTAAGCGGTTTGAAGTTCGAGCAACAATCATTACTGAAGAAACGACGGGCTAATGAGGGTCCGTACAAGCAACCAATTAACCAATTCTGATTAG-3′) were used for PCR with plasmid pEPKan-S as a template. The resulting products were transformed into GS1783 bacteria carrying pSM3fr. After Red recombination, arabinose-induced I-*Sce*I expression, and a second round of Red recombination, BAC m04.Y248A DNA was purified and successful mutagenesis was confirmed by sequencing (GATC, Koblenz, Germany).

In viruses mCMV-m04-HA and mCMV-m04.Y248A-HA, an HA-tag (amino acid sequence YPYDVPDYA) was added to the C-terminal end of the m04/gp34 protein by established BAC mutagenesis and cloning procedures. Specifically, homologous recombination of linear PCR fragments with full-length mCMV BAC plasmid pSM3fr was performed in *Escherichia coli* strain DH10B [43]. The linear fragments for recombination were generated using the oligonucleotides HA_m04_for (5′-TTGTACGGACCCTCATTATACCGTCGTTTCTTCAGTAATGATTGTTGCTCGAACTTCAAACCGCTTAAGAGTAACTACCCATACGACGTCCCAGACTACGCTTAGAGGACGACGACGACAAGTAA-3′) and HA_rev (5′-GAAAATGGTTTACTCAAGGGGATTTTTATTTAGGGGGTTACAGGAACACTTAACGGCTGA-3′) or HA_m04.Y248A__for (5′-TTGTACGGACCCTCATTAGCCCGTCGTTTCTTCAGTAATGATTGTTGCTCGAACTTCAAACCGCTTAAGAGTAACTACCCATA

CGACGTCCCAGACTACGCTTAGAGGACGACGACG ACAAGTAA-3′) and HA_rev.

The corresponding recombinant viruses were reconstituted by transfection of the BAC plasmid DNA into MEF and were propagated for several passages until residual BAC sequences were lost as verified by PCR as described [44].

Reporter cell assay

Retrovirally transduced Ly49P$^{CD3\zeta}$ 2B4 NFAT-GFP reporter cells were described previously [45]. Primary CBA/J MEF (haplotype H-2^k), not pretreated with IFN-γ, were infected for 12 h with the indicated viruses at an MOI of ~30 (centrifugal infection with 1.5 PFU/cell) and were used as stimulator cells for the NFAT-GFP reporter cells that were added in a ratio of 1:3. After 24 h of co-cultivation, expression of GFP in the reporter cells was analyzed by flow cytometry.

CLSM analyses

The localization of HA-tagged viral protein m04/gp34 and of cellular adapter protein AP2 or AP4 was visualized by CLSM (confocal laser scanning microscope) immunofluorescence analysis, essentially as described in greater detail elsewhere [46, 47]. Primary antibodies included an HA-specific rabbit antibody (Santa Cruz Biotechnology, catalog no. sc-805), an AP2-specific mouse antibody (anti-adaptin α; BD Pharmingen, catalog no. 610502), or an AP4-specific goat antibody (anti-AP-4β; Santa Cruz Biotechnology, catalog no. sc-18460). Alexa-Fluor 546-conjugated goat anti-rabbit antibody (life technologies, catalog no. A11010), Alexa-Fluor 488-conjugated goat anti-mouse antibody (life technologies, catalog no. A11001), or Alexa-Fluor 488-conjugated donkey anti-goat antibody (life technologies, catalog no. A11055), respectively, served as secondary antibodies for fluorescence staining. Cell nuclei were stained with Hoechst dye (life technologies, catalog no. H3570). Immunofluorescence was examined with a Zeiss Laser Scanning Microscope (LSM510).

NK cell depletion

8–10-week-old female BALB/c mice were depleted of NK cells by intravenous infusion of polyclonal rabbit antibody directed against mouse asialo-GM1 (20 µl in 500 µl of PBS; WAKO Chemicals), or were left untreated. After 24 h of depletion, mice were infected intravenously with 2×10^5 PFU of the indicated viruses. Virus titers in spleen and lungs were determined on day 3 after infection from the corresponding tissue homogenates by a virus plaque (PFU) assay performed under conditions of centrifugal enhancement of infectivity as described ([40, 41] and references therein).

Determination of virus doubling times and statistical significance test

BALB/c mice were immunocompromised by hematoablative total-body γ-irradiation with a single dose of 6.5 Gy and were infected at the left hind footpad (intraplantar infection) with 1×10^5 PFU of the indicated viruses. At defined times after infection, virus multiplication was quantitated in host organs by the virus plaque (PFU) assay (see above). Virus doubling times in hours (vDT) were calculated from the slopes of log-linear regression lines as described and explained in detail elsewhere (for the method, [44]; for the principle, [48–50]; and this issue of MMI [51]).

For evaluating statistical significance of group differences, *P* values were calculated based on log-transformed virus titers by using Student's *t* test (unpaired, two-sided) with Welch's correction to account for a potentially unequal variance. Differences are considered nonsignificant for P values >0.05, significant for P values <0.05, and highly significant for P values <0.01.

Results and discussion

'Adapter adapter' protein m04/gp34 colocalizes in the cell periphery with cellular adapter AP2 but not AP4

Although YXXΦ motifs are mostly discussed in the context of sorting by cellular adapter protein AP2, there is affinity also to other adapters, in particular to AP4, depending on the specific motif sequence and its neighboring residues. While AP2 sorts cargo from the cell surface, AP4 is associated with basolateral and TGN-endosome sorting ([52, 53], reviewed in [34]). In a context unrelated to our work, Aguilar et al. [54] have tested permutations of the 7-mer motif XXXYXXΦ, i.e., YXXΦ motifs and upstream positions +1, +2, and +3, for their interaction with AP complex chains µ1–4. As extreme examples, DFYYERL was found to interact with the µ chains of AP1, AP2, AP3, and AP4, whereas DLYYDPM interacted selectively with the µ4 chain of AP4. Fortuitously, this study included the sequence ETLYRRF, which precisely matches the m04 motif plus upstream position +1, that is, the sequence LYRRF (see Fig. 1). The 7-mer motif sequence was found to interact primarily with the µ2 chain of AP2 and, to a lower degree, with the µ4 chain of AP4.

Based on this, we sought to decide whether m04/gp34 interacts with AP2 and/or AP4, with the expectation of stronger association with AP2. As antibodies recognizing native m04/gp34 for fluorescence imaging are unavailable, we constructed viruses expressing m04/gp34 with a C-terminal HA-tag and either the functional motif (L)YRRF (m04-HA) or the inactivated motif (L)ARRF (m04.Y248A-HA). Confocal laser scanning images of MEF infected with these m04-HA-tagged viruses indicated a co-localization of m04/gp34-HA and AP2 in the cell periphery, a co-localization that is lost in m04/gp34.Y248A-HA (Fig. 2a, top panel, resolved to greater detail in Fig. 2b). Such a peripheral co-localization was not seen with AP4 (Fig. 2a, bottom panel). We wish to express some caution regarding an overinterpretation of the observed overall localization, as this might be influenced by the HA-tag. In the differential analysis with viruses that both express HA-tagged m04/gp34, this parameter, however, is unlikely to account for the motif-specific differences in the co-localization with AP2.

Annulation of the AP2 sorting motif does not prevent cell surface localization of m04/gp34-pMHC-I complexes

It has been shown previously that m04/gp34 is an abundant ER glycoprotein that does not regularly reach the cell surface, except for a small fraction of it that stably binds to pMHC-I and co-travels in this complex, and exclusively in this complex, to the cell surface [19, 28, 55]. Thus, although m04/gp34 has entered the literature as a molecule that 'shuttles pMHC-I to the cell surface' [29], one can also take the view that it hijacks pMHC-I to reach the cell surface [3]. In any case, it seems to rescue a fraction of the pMHC-I molecules from retention by m152/gp40 and sorting to the lysosome by m06/gp48. As the known function of AP2 is cargo selection from the plasma membrane, we did not expect a phenotype of the AP2 motif mutation Y248A in the traveling of the m04/gp34-pMHC-I complexes to the plasma membrane in the first place, though the mutation may influence steady-state levels at the cell surface by affecting endocytosis and recycling. As antibodies specific for native m04/gp34 or for the complex are not available, we took an alternative strategy for verifying cell surface presentation of the complex in a qualitative sense. The assay is based on Ly49P reporter cells that express green fluorescent protein (GFP) upon Ly49P ligation to cell surface-located m04/gp34-pMHC-I (here H-2D^k) complexes [45].

Representing the positive and negative control groups, cells infected with WT.BAC, expressing m04/gp34 with the intact AP2 motif, stimulated the reporter cells for GFP expression, whereas cells infected with the *m04* gene deletion mutant mCMV-Δm04 failed as predicted (Fig. 3, left and center panel). Tellingly, cells infected with virus mCMV-m04.Y248A still stimulated the reporter cells (Fig. 3, right panel), which proved cell surface presentation of the Ly49P ligand complex m04/gp34-H-2D^k. It must be considered here that this reporter assay does not allow a precise quantitation of the ligand complexes, because threshold expression for stimulating the reporter cells in a yes-or-no fashion might be reached with WT and mutant virus.

Annulation of the AP2 sorting motif completes the silencing of NK cells

Whilst the contribution of m04/gp34 to evasion of CD8 T cells is moderate and only relevant under conditions when the effect of m06/gp48 and m152/gp40 on pMHC-I presentation is countered by IFN-γ [24], the main role of this protein appears to be in innate immunity by providing a cell surface ligand for mostly inhibitory NK cell receptors (see the "Introduction"). We therefore investigated the consequences of AP2 motif mutation on the silencing of NK cells in lungs and spleen (Fig. 4). To recapitulate published previous findings, we first compared virus titers in the presence or absence of NK cells after infection with WT virus, expressing the full complement of viral proteins involved in NK cell evasion by downmodulating ligands of activatory NK cell receptors or providing ligands for inhibitory NK cell receptors (for a review, see [27]), with mutant virus mCMV-Δm04, selectively lacking the NK cell silencing ligands m04/gp34-pMHC-I (Fig. 4, compare left and center panels).

Depletion of NK cells prior to infection with WT virus led to a statistically significant increase in virus titers in both organs, which indicates that NK cell evasion by WT virus is incomplete (Fig. 4, left panels). Though this information is present in published data [14], the phenomenon was not deeper thematized or experimentally pursued. In accordance with what was known before [25], absence of m04/gp34-mediated NK cell silencing led to strong virus titer reductions that were reverted by NK cell depletion (Fig. 4, center panels). Importantly, AP2 motif mutation in virus mCMV-m04.Y248A did not reproduce the phenotype of deletion of the whole protein but instead wiped out any role for NK cells, thus indicating complete NK cell silencing (Fig. 4, right panels).

Though the relative changes in virus titers were clear, a reliable comparison of absolute virus titers is only possible if the mutation does not affect the replicative fitness of the virus in organs under study by mechanisms unrelated to immune control. We therefore verified the equivalence of viral replicative capacities by log-linear growth curves for WT and AP2 motif mutant viruses in immunoablated mice (for the principle, see [51] in this issue of MMI). As shown

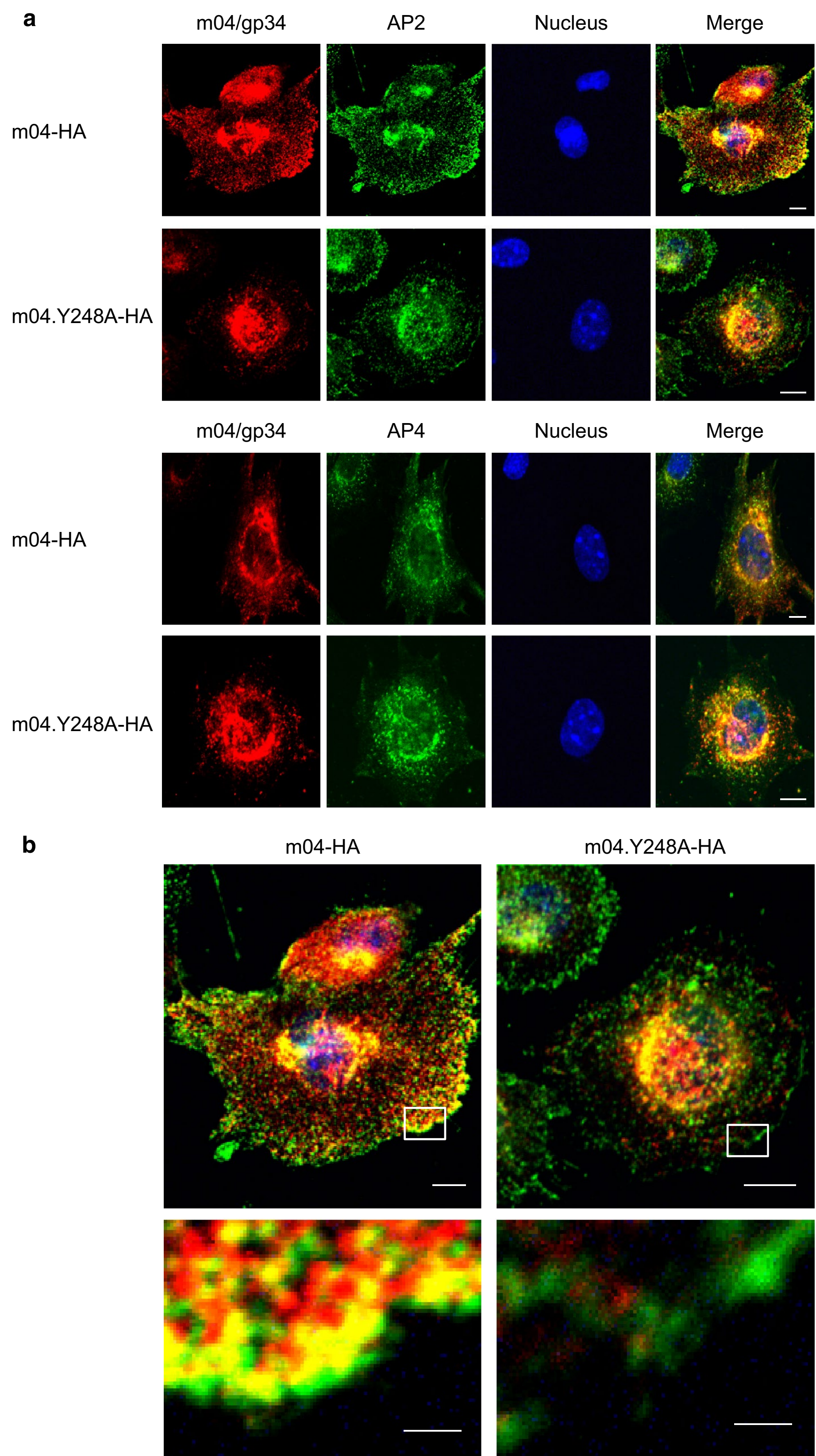

Fig. 2 CLSM analysis of m04/gp34 colocalization with cellular adapter proteins. MEF (not pretreated with IFN-γ) were infected with the indicated HA-tag mCMV recombinants at an MOI of 4 and fixed for immunofluorescence staining at 6 h postinfection. HA-tagged m04/gp34 (*red*), AP2 (*green*), or AP4 (*green*) were detected using specific antibodies directed against the HA-tag, adaptin α, and adaptin β4, respectively. Images represent optical sections **a** *Upper images*, staining of HA-tagged m04/gp34 and of AP2. *Lower images*, staining of HA-tagged m04/gp34 and of AP4. Nuclei were stained with Hoechst dye (*blue*). *Bar markers* 8 µm. **b** Enlargement of the merged images of HA-tagged m04/gp34 and AP2 (*upper panel*), and further enlargement (*lower panel*) of a *white-boxed* region at the cell periphery. *Bar markers*: 8 µm (*upper panel*), 2 µm (*lower panel*)

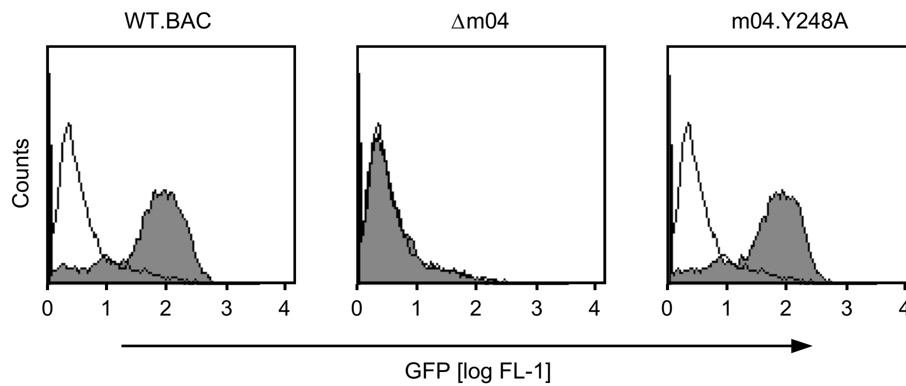

Fig. 3 Functional deletion of the endocytic AP2 motif does not prevent cell surface localization of m04/gp34-pMHC-I complexes engaging the activatory NK cell receptor Ly49P. CBA/J (H-2[k]) MEF were infected for 12 h with the indicated viruses and used to sensitize Ly49P$^{CD3\zeta}$ NFAT-GFP reporter cells. After 24 h of co-cultivation, GFP expression by the reporter cells was assessed by cytofluorometric analysis (FL-1; intensity of green fluorescence). *Shaded signals* infected stimulator cells; *open signals* uninfected stimulator cells

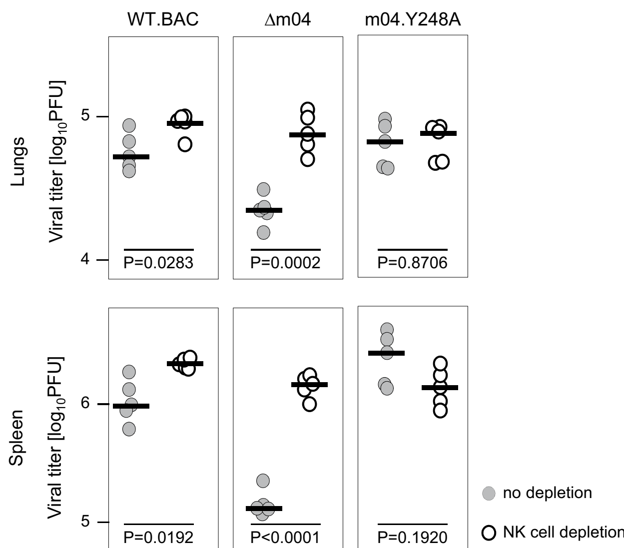

Fig. 4 Opposite effects of m04/gp34 deletion and of AP2 motif mutation on the activation of NK cells. Virus titers in lungs and spleen of BALB/c mice were determined on day 3 after intravenous infection with the indicated viruses. *Filled circles* presence of NK cells (no depletion); *open circles* absence of NK cells due to depletion with anti-asialo GM1 prior to infection. Symbols represent data from individual mice, with median values marked. *P* values <0.05 indicate a significant difference between the data sets compared

in Fig. 5a, growth regression lines were congruent within the 95 % confidence regions for both viruses regardless of the organ under study. This reflects identical growth constants, expressed as virus doubling times (vDT) [44, 51] for the two viruses within an organ, though with numerical difference between organs. Specifically, both viruses replicated faster in the lungs compared to the spleen.

After this clarification, we improved the statistical basis by compiling data for WT virus and AP2 motif mutant from two independent experiments (Fig. 5b). For the spleen, this strengthened the conclusion that WT virus is still under NK cell control and thus replicates to higher levels in the absence of NK cells ($P = 0.0065$), a difference that is wiped out by AP2 motif mutation Y248A ($P = 0.6741$). Along the same line of reasoning, WT virus replicated to lower levels than the mutant ($P = 0.0117$), a difference that is wiped out by NK cell depletion ($P = 0.7382$). In the lungs, the effect of NK cell depletion is also significant, though less pronounced ($P = 0.0429$), and is also abrogated by AP2 motif mutation ($P = 0.6412$). The more moderate effects may relate to more aggressive virus growth and a generally lesser involvement of NK cells in the control of pulmonary infection that is dominated by CD8 T cells, in particular at later times [56].

Synopsis

This study gave functional evidence for a role of the tyrosine-based YXXΦ motif (L)YRRF present in the cytoplasmic tail of mCMV protein m04/gp34. The data support the idea that this protein functions as a Janus-faced 'adapter adapter' in that it connects, like a bridge, pMHC-I molecules with the cellular adapter protein AP2 by simultaneously binding to pMHC-I and, through the motif, to AP2 (for a sketch, see Fig. 6). Consistent with AP2′s established function as an endocytic carrier that sorts cargo from the cell membrane into clathrin-triskelion-coated pits for accelerated endocytosis [22], enhanced silencing of NK cells after infection with virus mCMV-m04.Y248A, in which the motif is destroyed, suggests prolonged and/or intensified

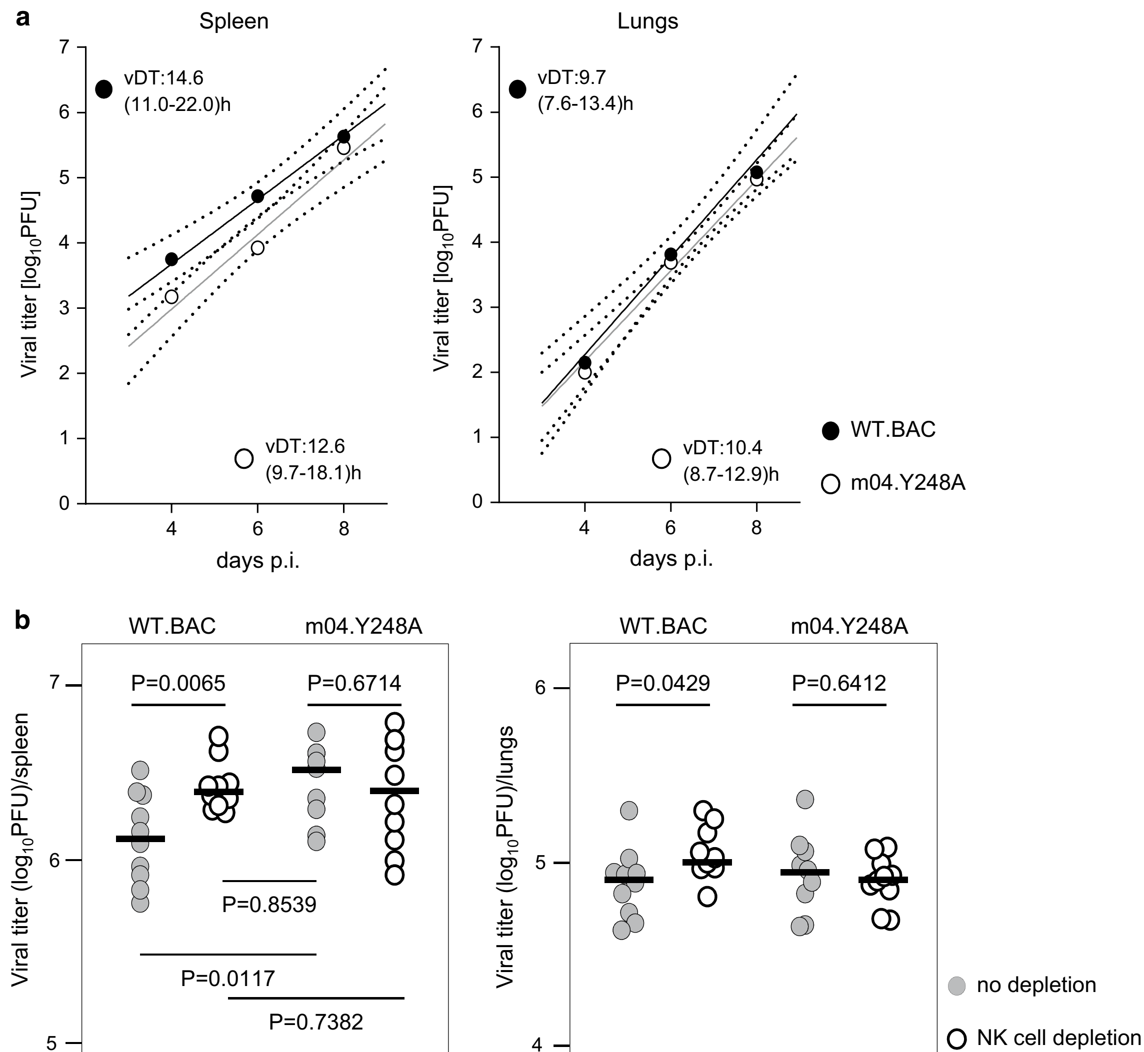

Fig. 5 Mutation Y248A of the endocytic AP2 motif results in complete silencing of NK cells. **a** Equivalent growth of WT virus (WT.BAC, *filled circles*) and AP2 motif mutant mCMV-m04.Y248A (*open circles*) in spleen and lungs of immunocompromised mice. Symbols represent median values of viral titers ($n \geq 3$), measured at the indicated times postinfection (p. i.). Shown are log-linear regression lines (WT.BAC, black; m04.Y248A, grey); the corresponding 95 % confidence areas are demarcated by *dotted curves*. vDT, virus doubling times (growth constants) in hours; the corresponding 95 % confidence intervals are given in parentheses. **b** Impact of AP2 motif mutation Y248A on NK cell activity, as revealed by virus titers in spleen and lungs. Symbols represent data from individual mice compiled from 2 independent experiments. For experimental conditions and further explanation, see the legend of Fig. 4

interaction between inhibitory Ly49 family NK cell receptors and their m04/gp34-MHC-I ligands on the cell surface of the infected cells. From this, we conclude that deletion of the motif indeed disconnects the complexes from AP2-facilitated internalization. Lucin et al. [38] have recently reviewed current knowledge of how CMV determines the residence time of cellular cargo molecules at the cell surface by perturbation of their endosomal trafficking and subsequent relocation from the cell surface into intracellular compartments and, specifically, that mCMV alters the constitutive recycling of cell surface pMHC-I by actively relocating it into an endosomal retention compartment in the early (E) phase of infection. Our data indicate that the mCMV E-phase protein m04/gp34 is one of the players.

By using an AP2-binding motif in its cytoplasmic tail for sorting pMHC-I molecules from the cell surface, m04/gp34 of mCMV shares features with lentiviral protein NEF that accelerates the endocytic internalization of CD4 molecules (reviewed in [22]). For completeness, one should not forget that many cell surface receptors bring their own YXXΦ endocytic motifs in their cytoplasmic tails, which renders them independent of ‘adapter adapters’ for internalization [57], and this applies also to viral glycoproteins such as pseudorabies virus gE, as an example [58].

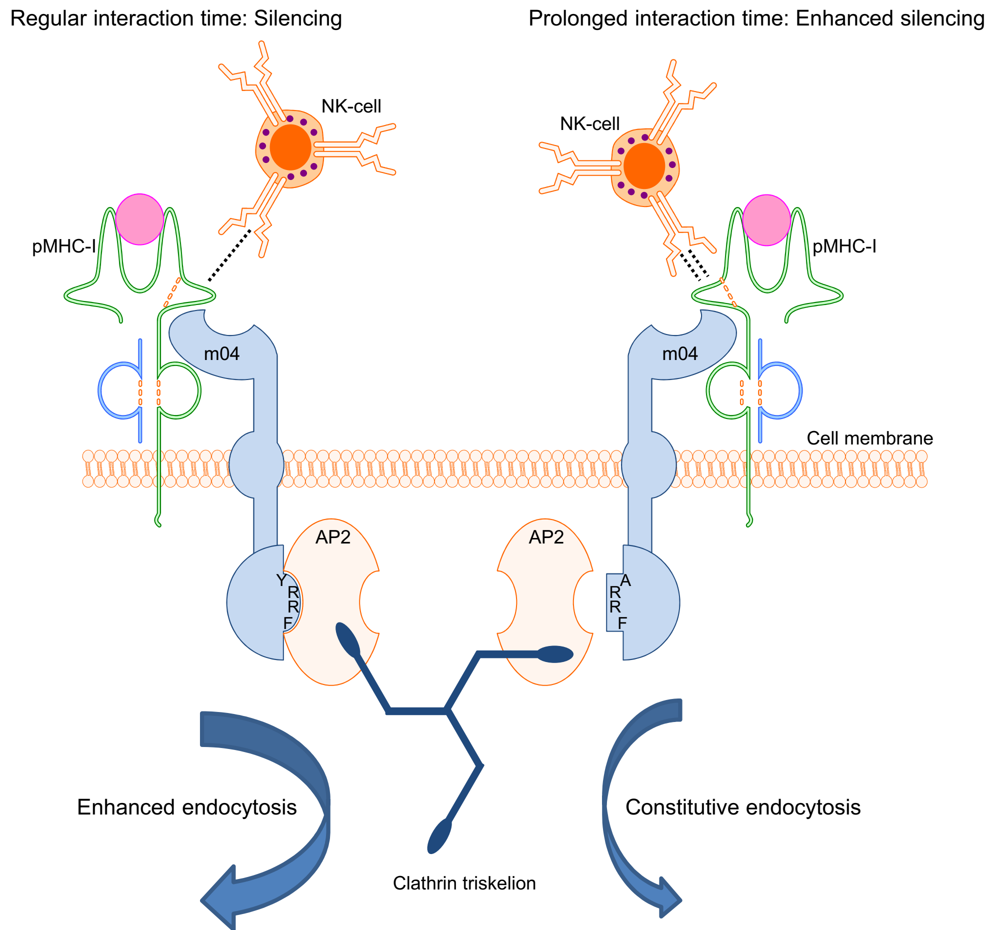

Fig. 6 Sketch of the ‘adapter adapter’ function of m04/gp34 and its disconnection from the clathrin-assisted pathway of endocytosis by mutation of the AP2-binding motif YXXΦ (YRRF) to AXXΦ (ARRF). The graphics was inspired by artwork in Oldridge and Marsh [22]

Hypothesis: Gene family *m02–m16* codes for a protein family of cargo sorting ‘adapter adapters’

Intrigued by the prototypes m04/gp34 and m06/gp48, representing ‘adapter adapters’ with a confirmed tyrosine-based sorting motif YXXΦ [this report] and a di-leucine motif EXXXXLL [21], respectively, we searched for sorting motifs in the predicted cytosolic tail amino acid sequences of the remaining members of the mCMV *m02–m16* gene family (Fig. 7). Suspiciously, canonical YXXΦ endocytic motifs are present in most family members (m02, m04, m05, m07, m08, m09, m10, and m16). In some cases (YICL of m05, YGFL of m09 in part, and YAIL of m16), the motif is located within the bioinformatically predicted TMD, which would exclude their functionality. However, as TMD predictions have a high degree of uncertainty, we would suggest that presence of a canonical endocytic motif within a predicted TMD is an argument rather to more precisely map and confirm the correct TMD experimentally. There exist also noncanonical Y-based endocytic motifs YXXGΦ [59]. Notably, m12 carries in its cytoplasmic tail a sequence YXXXGΦ, specifically YRRRGF, which might possibly represent another noncanonical endocytic motif, and we would not exclude the existence of hidden noncanonical endocytic motifs in the remaining family members. Besides m06, which contains two di-leucine motifs, of which only the TMD-proximal motif EPLARLL is a canonical motif and is actually the one that is experimentally confirmed [21], no other family member contains a clear di-leucine motif with a glutamic acid residue E in upstream position +5.

This all leads us to propose the hypothesis that most members of the m02–m16 protein family serve as AP2 ‘adapter adapters’ sorting cargo from the cell membrane by connecting it to the clathrin-triskelion route of endocytosis. Cargo specificity is likely mediated by the individual N-terminal domains/ectodomains, and we venture the prognosis that

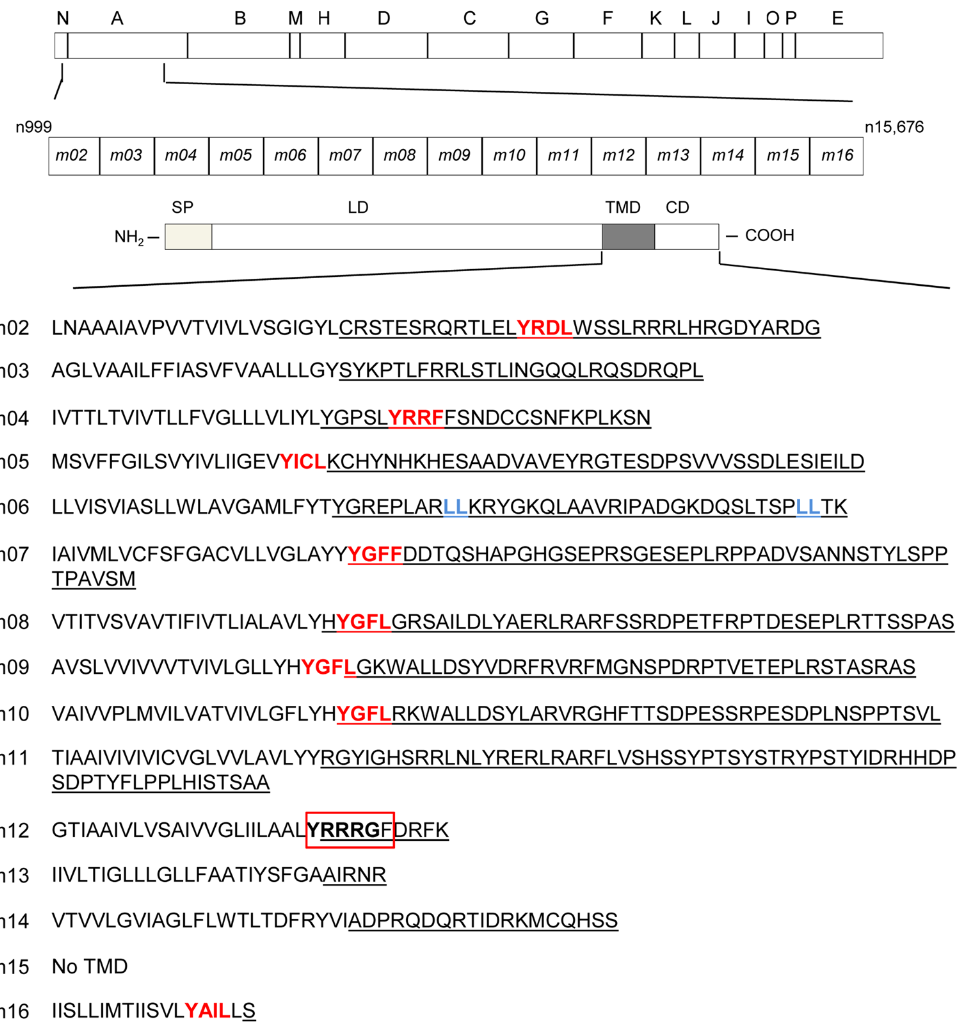

Fig. 7 Search for cargo sorting motifs in the cytosolic tails of m02-m16 family members. *Top* schematic *Hind*III map of the mCMV Smith strain genome [60] and the localization of the *m02-m16* gene family (not drawn to scale), starting at nucleotide position 999 within fragment N and reaching to 15,676 within fragment A [18]. Shown below is a schematic representation of an integral membrane type-I glycoprotein with signal peptide (SP), N-terminal luminal domain/ectodomain (LD), transmembrane domain (TMD), and cytoplasmic (C-terminal tail) domain (CD). *Bottom* Alignment of predicted amino acid sequences of m02-m16 family members, covering the putative TMD and the CD (underlined). Canonical YXXΦ endocytic motifs are displayed in *red bold letters*, di-leucine motifs are in *bold blue letters*, and a putative noncanonical endocytic motif in m12 is marked by a *red frame*. TMD predictions were made using software TMHMM on www.cbs.dtu.dk/services/TMHMM/

future research in this field will soon define the corresponding cell surface molecules downmodulated for immune evasion.

Acknowledgments This work was supported by the Deutsche Forschungsgemeinschaft, SFB490, individual project E4 'antigen presentation under the influence of murine cytomegalovirus immune evasion proteins.' NAWL received intramural funding from the young investigator program MAIFOR of the University Medical Center Mainz.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard All procedures performed in studies involving animals were approved by the ethics committee of the Landesuntersuchungsamt Rheinland-Pfalz, Permission No. 177-07/G09-1-004 in accordance with German Federal Law §8 Abs. 1 TierSchG (animal protection law). BALB/c mice were bred and housed under specified pathogen-free conditions at the Central Laboratory Animal Facility (CLAF) at the University Medical Center of the Johannes Gutenberg-University, Mainz, Germany.

References

1. Reddehase MJ (2002) Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. Nat Rev Immunol 2:831–844
2. Doom C, Hill AB (2008) MHC class I immune evasion in MCMV infection. Med Microbiol Immunol 197:191–204
3. Lemmermann NA, Böhm V, Holtappels R, Reddehase MJ (2011) In vivo impact of cytomegalovirus evasion of CD8 T-cell immunity: facts and thoughts based on murine models. Virus Res 157:161–174
4. Lemmermann NA, Fink A, Podlech J, Ebert S, Wilhelmi V, Böhm V, Holtappels R, Reddehase MJ (2012) Murine cytomegalovirus immune evasion proteins operative in the MHC class I pathway of antigen processing and presentation: state of knowledge, revisions, and questions. Med Microbiol Immunol 201:497–512
5. Hansen TH, Bouvier M (2009) MHC class I antigen presentation: learning from viral evasion strategies. Nat Rev Immunol 9:503–513
6. Del Val M, Hengel H, Hacker H, Hartlaub U, Ruppert T, Lucin P, Koszinowski UH (1992) Cytomegalovirus prevents antigen presentation blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J Exp Med 172:729–738
7. Ziegler H, Thale R, Lucin P, Muranyi W, Flohr T, Hengel H, Farrell H, Rawlinson W, Koszinowski UH (1997) A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments. Immunity 6:57–66
8. Ziegler H, Muranyi W, Burgert HG, Kremmer E, Koszinowski UH (2000) The luminal part of the murine cytomegalovirus

glycoprotein gp40 catalyzes the retention of MHC class I molecules. EMBO J 19:870–881
9. Krmpotic A, Messerle M, Crnkovic-Mertens I, Polic B, Jonjic S, Koszinowski UH (1999) The immunoevasive function encoded by the mouse cytomegalovirus gene m152 protects the virus against T cell control in vivo. J Exp Med 190:1285–1296
10. Holtappels R, Podlech J, Pahl-Seibert M-F, Jülch M, Thomas D, Simon CO, Wagner M, Reddehase MJ (2004) Cytomegalovirus misleads its host by priming of CD8 T cells specific for an epitope not presented in infected tissues. J Exp Med 199:131–136
11. Lemmermann NA, Gergely K, Böhm V, Deegen P, Däubner T, Reddehase MJ (2010) Immune evasion proteins of murine cytomegalovirus preferentially affect cell surface display of recently generated peptide presentation complexes. J Virol 84:1221–1236
12. Lodoen M, Ogasawara K, Hamerman J, Arase H, Houchins J, Mocarski E, Lanier L (2003) NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. J Exp Med 197:1245–1253
13. Zhi L, Mans J, Paskow MJ, Brown PH, Schuck P, Jonjic S, Natarajan K, Margulies DH (2010) Direct interaction of the mouse cytomegalovirus m152/gp40 immunoevasin with RAE-1 isoforms. Biochemistry 49:2443–2453
14. Krmpotic A, Busch DH, Bubic I, Gebhardt F, Hengel H, Hasan M, Scalzo AA, Koszinowski UH, Jonjic S (2002) MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. Nat Immunol 3:529–535
15. Wang R, Natarajan K, Revilleza M, Boyd L, Zhi L, Zhao H, Robinson H, Margulies D (2012) Structural basis of mouse cytomegalovirus m152/gp40 interaction with RAE1γ reveals a paradigm for MHC/MHC interaction in immune evasion. Proc Natl Acad Sci USA 109:E3578–E3587
16. Fink A, Renzaho A, Reddehase M, Lemmermann NAW (2013) The p36 isoform of murine cytomegalovirus m152 protein suffices for mediating innate and adaptive immune evasion. Viruses 5:3171–3191
17. Lenac T, Arapovic J, Traven L, Krmpotic A, Jonjic S (2008) Murine cytomegalovirus regulation of NKG2D ligands. Med Microbiol Immunol 197:159–166
18. Rawlinson W, Farrell H, Barrell B (1996) Analysis of the complete DNA sequence of murine cytomegalovirus. J Virol 70:8833–8849
19. Kleijnen MF, Huppa JB, Lucin P, Mukherjee S, Farrell H, Campbell AE, Koszinowski UH, Hill AB, Ploegh HL (1997) A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. EMBO J 16:685–694
20. Reusch U, Muranyi W, Lucin P, Burgert HG, Hengel H, Koszinowski UH (1999) A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. EMBO J 18:1081–1091
21. Reusch U, Bernhard O, Koszinowski UH, Schu P (2002) AP-1A and AP-3A lysosomal sorting functions. Traffic 3:752–761
22. Oldridge J, Marsh M (1998) Nef-an adaptor adaptor? Trends Cell Biol 8:302–305
23. Wagner M, Gutermann A, Podlech J, Reddehase MJ, Koszinowski UH (2002) Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus. J Exp Med 196:805–816
24. Fink A, Lemmermann NA, Gillert-Marien D, Thomas D, Freitag K, Böhm V, Wilhelmi V, Reifenberg K, Reddehase MJ, Holtappels R (2012) Antigen presentation under the influence of “immune evasion” proteins and its modulation by interferon-gamma: implications for immunotherapy of cytomegalovirus infection with antiviral CD8 T cells. Med Microbiol Immunol 201:513–525
25. Babic M, Pyzik M, Zafirova B, Mitrovic M, Butorac V, Lanier LL, Krmpotic A, Vidal S, Jonjic S (2010) Cytomegalovirus immunoevasin reveals the physiological role of “missing self” recognition in natural killer cell dependent virus control in vivo. J Exp Med 207:2663–2673
26. Kielczewska A, Pyzik M, Sun T, Krmpotic A, Lodoen MB, Munks MW, Babic M, Hill AB, Koszinowski UH, Jonjic S, Lanier LL, Vidal SM (2009) Ly49P recognition of cytomegalovirus-infected cells expressing H2-Dk and CMV-encoded m04 correlates with the NK cell antiviral response. J Exp Med 206:515–523
27. Vidal S, Krmpotic A, Pyzik M, Jonjic S (2013) Innate immunity to cytomegalovirus in the murine model. In: Reddehase MJ (ed) Cytomegaloviruses: from molecular pathogenesis to intervention, vol II, chap 9. Caister Academic Press, Norfolk, pp 191–213
28. Lu X, Kavanagh DG, Hill AB (2006) Cellular and molecular requirements for association of the murine cytomegalovirus protein m4/gp34 with major histocompatibility complex class I molecules. J Virol 80:6048–6055
29. Berry R, Vivian JP, Deuss FA, Balaji GR, Saunders PM, Lin J, Littler DR, Brooks AG, Rossjohn J (2014) The structure of the cytomegalovirus-encoded m04 glycoprotein, a prototypical member of the m02 family of immunoevasins. J Biol Chem 289:23753–23763
30. Holtappels R, Gillert-Marien D, Thomas D, Podlech J, Deegen P, Herter S, Oehrlein-Karpi SA, Strand D, Wagner M, Reddehase MJ (2006) Cytomegalovirus encodes a positive regulator of antigen presentation. J Virol 80:7613–7624
31. Pinto A, Munks MW, Koszinowski UH, Hill AB (2006) Coordinated function of murine cytomegalovirus genes completely inhibits CTL lysis. J Immunol 177:3225–3234
32. Holtappels R, Thomas D, Podlech J, Steffens HP, Geginat G, Reddehase MJ (2000) The putative natural killer decoy early gene m04 (gp34) of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral CD8 T cells. J Virol 74:1871–1884
33. Corbett AJ, Forbes CA, Moro D, Scalzo AA (2007) Extensive sequence variation exists among isolates of murine cytomegalovirus within members of the m02 family of genes. J Gen Virol 88:758–769
34. Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72:395–447
35. Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 269:1872–1875
36. Traub LM (2003) Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. J Cell Biol 163:203–208
37. Reider A, Wendland B (2011) Endocytic adaptors-social networking at the plasma membrane. J Cell Sci 124:1613–1622
38. Lucin P, Mahmutefendic H, Blagojevic Zagorac G, Ilic Tomas M (2015) Cytomegalovirus immune evasion by perturbation of endosomal trafficking. Cell Mol Immunol 12:154–169
39. Wagner M, Jonjic S, Koszinowski UH, Messerle M (1999) Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J Virol 73:7056–7060
40. Kurz S, Steffens HP, Mayer A, Harris JR, Reddehase MJ (1997) Latency versus persistence or intermittent recurrences: evidence for a latent state of murine cytomegalovirus in the lungs. J Virol 71:2980–2987
41. Podlech J, Holtappels R, Grzimek NA, Reddehase MJ (2002) Animal models: murine cytomegalovirus. In: Kaufmann SHE,

Kabelitz D (eds) Methods in microbiology: immunology of infection. Academic Press, London, pp 493–525
42. Tischer B, von Einem J, Kaufer B, Osterrieder N (2006) Two-step Red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 40:191–197
43. Wagner M, Ruzsics Z, Koszinowski UH (2002) Herpesvirus genetics has come of age. Trends Microbiol 10:318–324
44. Lemmermann NA, Podlech J, Seckert CK, Kropp KA, Grzimek NK, Reddehase MJ, Holtappels R (2010) CD8 T-cell immunotherapy of cytomegalovirus disease in the murine model. In: Kaufmann SHE, Kabelitz D (eds) Methods in microbiology: immunology of infection. Academic Press, London, pp 369–420
45. Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL (2002) Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science 296:11323–11326
46. Holtappels R, Simon C, Munks MW, Thomas D, Deegen P, Kühnapfel B, Däubner T, Emde S, Podlech J, Grzimek NA, Oehrlein-Karpi SA, Hill AB, Reddehase MJ (2008) Subdominant CD8 T-cell epitopes account for protection against cytomegalovirus independent of immunodomination. J Virol 82:5781–5796
47. Däubner T, Fink A, Seitz A, Tenzer S, Müller J, Strand D, Seckert CK, Janssen C, Renzaho A, Grzimek NK, Simon CO, Ebert S, Reddehase MJ, Oehrlein-Karpi SA, Lemmermann NA (2010) A novel transmembrane domain mediating retention of a highly motile herpesvirus glycoprotein in the endoplasmic reticulum. J Gen Virol 91:1524–1534
48. Wilhelmi V, Simon CO, Podlech J, Böhm V, Däubner T, Emde S, Strand D, Renzaho A, Lemmermann NA, Seckert CK, Reddehase MJ, Grzimek NK (2008) Transactivation of cellular genes involved in nucleotide metabolism by the regulatory IE1 protein of murine cytomegalovirus is not critical for viral replicative fitness in quiescent cells and host tissues. J Virol 82:9900–9916
49. Kropp KA, Simon CO, Fink A, Renzaho A, Kühnapfel B, Podlech J, Reddehase MJ, Grzimek NK (2009) Synergism between the components of the bipartite major immediate-early transcriptional enhancer of murine cytomegalovirus does not accelerate virus replication in cell culture and host tissues. J Gen Virol 90:2395–2401
50. Lemmermann NA, Krmpotic A, Podlech J, Brizic I, Prager A, Adler H, Karbach A, Wu Y, Jonjic S, Reddehase MJ, Adler B (2015) Non-redundant and redundant roles of cytomegalovirus gH/gL complexes in host organ entry and intra-tissue spread. PLoS Pathog 11:e1004640
51. Podlech J, Reddehase MJ, Adler B, Lemmermann NA (2015) Principles for studying in vivo attenuation of virus mutants: defining the role of the cytomegalovirus gH/gL/gO complex as a paradigm. Med Microbiol Immunol [Epub ahead of print]
52. Dell'Angelica EC, Mullins C, Bonifacino JS (1999) AP-4, a novel protein complex related to clathrin adaptors. J Biol Chem 274:7278–7285
53. Hirst J, Bright NA, Rous B, Robinson MS (1999) Characterization of a fourth adaptor-related protein complex. Mol Biol Cell 10:2787–2802
54. Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, Ohno H, Bonifacino JS (2001) Signal-binding specificity of the mu4 subunit of the adapter protein complex AP-4. J Biol Chem 276:13145–13152
55. Kavanagh DG, Koszinowski UH, Hill AB (2001) The murine cytomegalovirus immune evasion protein m4/gp34 forms biochemically distinct complexes with class I MHC at the cell surface and in a pre-Golgi compartment. J Immunol 167:3894–3902
56. Ebert S, Becker M, Lemmermann NA, Büttner JK, Michel A, Taube C, Podlech J, Böhm V, Freitag K, Thomas D, Holtappels R, Reddehase MJ, Stassen M (2014) Mast cells expedite control of pulmonary murine cytomegalovirus infection by enhancing the recruitment of protective CD8 T cells to the lungs. PLoS Pathog 10:e1004100
57. Owen DJ, Evans PR (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282:1327–1332
58. Tirabassi RS, Enquist LW (1999) Mutation of the YXXL endocytosis motif in the cytoplasmic tail of pseudorabies virus gE. J Virol 73:2717–2728
59. Royle SJ, Qureshi OS, Bobanović LK, Evans PR, Owen DJ, Murrell-Lagnado RD (2005) Non-canonical YXXGPhi endocytic motifs: recognition by AP2 and preferential utilization in P2X4 receptors. J Cell Sci 118:3073–3080
60. Ebeling A, Keil GM, Knust E, Koszinowski UH (1983) Molecular cloning and physical mapping of murine cytomegalovirus DNA. J Virol 47:421–433