

Virally mediated inhibition of Bax in leukocytes promotes dissemination of murine cytomegalovirus

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The evolutionary survival of viruses relies on their ability to disseminate infectious progeny to sites of transmission. The capacity to subvert apoptosis is thought to be crucial for ensuring efficient viral replication in permissive cells, but its role in viral dissemination *in vivo* has not been considered. We show here that the murine cytomegalovirus (MCMV) m38.5 protein specifically counters the action of Bax. As predicted from our biochemical data, the capacity of m38.5 to inhibit apoptosis is only apparent in cells unable to activate Bak. Deletion of m38.5 resulted in an attenuated growth of MCMV *in vitro*. *In vivo* replication of the Δ m38.5 virus was not significantly impaired in visceral organs. However, m38.5 played a central role in protecting leukocytes from Bax-mediated apoptosis, thereby promoting viral dissemination to the salivary glands, the principal site of transmission. These results establish that *in vivo* MCMV replication induces the activation of Bax in leukocytes, but not other permissive cells, and that MCMV interferes with this process to attain maximum dissemination.

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Apoptosis is considered to function as an innate defence mechanism against viral infection. This hypothesis has been strengthened by the fact that many viruses encode antiapoptotic proteins.¹ However, the role of many viral antiapoptotic proteins in the context of *in vivo* infection has not been determined, but rather, has been inferred from the *in vitro* analysis of viral mutants.

Bcl-2 proteins are the principal regulators of apoptosis in response to diverse stimuli.² The Bcl-2 family comprises opposing pro-apoptotic and antiapoptotic proteins that share one or more conserved regions, known as Bcl-2 homology (BH) domains. Pro-apoptotic family members can be further subdivided into the BH3-only group and the Bax/Bak subfamily; these proteins function in a coordinated manner to induce apoptosis.

The activity of the multidomain pro-apoptotic proteins, Bax and Bak, is essential for apoptosis to occur.^{3,4} The current paradigm suggests that Bax and Bak are restrained by pro-survival Bcl-2 proteins whose activity is, in turn, regulated by the BH3-only proteins.^{5–7} Although genetic studies in mice indicate that Bak and Bax are essentially redundant during development,^{3,8} some *in vitro* data suggest that apoptosis may be induced by the activation of either Bax or Bak.^{5,9} Furthermore, the inhibition of Bak is mediated by a subset of pro-survival Bcl-2 proteins, whereas all pro-survival Bcl-2 members appear to be capable of inhibiting Bax.^{5–7} These

findings suggest that apoptosis may be initiated by the selective activation of either Bax or Bak. Whether *in vivo* apoptosis is initiated by the selective activation of either Bax or Bak is a critical, yet unresolved, question. Here, we addressed this issue using a viral infection model.

Human cytomegalovirus (HCMV) is an important human pathogen that causes significant mortality in immunocompromised individuals. HCMV encodes proteins with known antiapoptotic properties,¹⁰ whose activity is likely to be required to ensure that viral replication can occur within the host. However, given the strict species specificity of the CMV family, elucidating the *in vivo* role of these proteins is not possible. As the biology of murine CMV (MCMV) is similar to that of HCMV, MCMV has proved to be a useful model for studying the *in vivo* mechanisms utilised by CMVs to subvert host immune responses. We have previously demonstrated that MCMV-infected dendritic cells (DCs) are resistant to apoptosis induced by growth factor (GF) deprivation due to an inability to fully activate Bax.¹¹ The viral mitochondria-localised inhibitor of apoptosis (vMIA) protein encoded by exon 1 of UL37 in HCMV is a potent antiapoptotic protein that functions by sequestering Bax and keeping it inactive at the mitochondrial membrane.^{12,13} Recombinant HCMV mutants lacking vMIA produced using the AD169 strain do not replicate effectively.¹⁴ Surprisingly, the inactivation of vMIA in the Towne strain of HCMV had little effect on viral replication,

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Abbreviations: BAC, bacterial artificial chromosome construct; BH, Bcl-2 homology; CMV, cytomegalovirus; DC, dendritic cell; FRT, FLP recombination target site; HCMV, human cytomegalovirus; Kan^R, kanamycin resistance; KLH, keyhole limpet haemocyanin; MCMV, murine cytomegalovirus; MEF, murine embryonic fibroblast; MOI, multiplicity of infection; ORF, open reading frame; PI, post-infection; PFU, plaque-forming unit; vMIA, viral mitochondria-localised inhibitor of apoptosis; WT, wild-type

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although cells infected with this mutant virus were more susceptible to apoptosis induced by proteasome inhibitors.¹⁵ It is unclear why the strain of HCMV used to produce vMIA mutants has such an impact on the phenotype observed. Both the AD169 and Towne strains of HCMV were developed as attenuated vaccine candidates by serial passage in fibroblasts and, consequently, do not have identical genomic structures.¹⁶ Therefore, the various Δ vMIA mutants may have different phenotypes because of compounding mutations elsewhere in the viral genome. Alternatively, the strength of the apoptotic stimulus provided by HCMV infection may vary depending on the virus strain.

Together, the current data indicate that vMIA has anti-apoptotic activity; however, the role of this protein in viral infection is unclear. The biochemical changes to Bax in MCMV-infected DC¹¹ mirror those induced by the over-expression of vMIA, suggesting that MCMV might encode a vMIA-like protein. Recently, m38.5 was identified as a potential vMIA orthologue,¹⁵ prompting us to investigate the role of m38.5 in viral infection *in vivo*. Here, we have demonstrated that m38.5 is an antiapoptotic protein that functions by specifically inhibiting Bax. Surprisingly, m38.5 activity was not required for *in vivo* viral replication in the visceral organs. By contrast, MCMV replication in salivary glands, the major site of viral transmission, was attenuated by deletion of m38.5. We determined that m38.5 activity protects virally infected leukocytes from apoptosis, thereby ensuring efficient dissemination to the salivary glands. These results demonstrate that MCMV replication *in vivo* induces the activation of Bax in only a subset of permissive cells and that MCMV inhibits this process to ensure optimal viral dissemination.

Results

m38.5 is an antiapoptotic protein that binds Bax. Initially, we sought to determine if m38.5 possesses vMIA-like activity. Cos-7 cells were transiently transfected with a Flag-tagged m38.5 construct and immunoprecipitations performed using anti-Flag antibodies. Bax was found to co-precipitate with m38.5, but not with the MCMV-encoded M36 antiapoptotic protein (Figure 1a). The ability of m38.5 to associate with either Bax or Bak was then tested. HeLa cells were transiently transfected with a Flag-tagged m38.5 construct and immunoprecipitations performed. Although a clear association between m38.5 and Bax was detected, the closely related protein Bak did not co-precipitate with m38.5 (Figure 1b). The capacity of m38.5 to interact with murine Bax and Bak was then tested using mouse embryonic fibroblasts (MEFs). MEF cell lines derived from *Bax* or *Bak* knockout mice were transfected with a construct encoding Flag-tagged m38.5 and stable cell lines generated. Co-immunoprecipitation experiments using these cell lines confirmed that m38.5 is capable of associating with Bax, but not with Bak (Figure 1c). The capacity of m38.5 to inhibit apoptosis induced by the cytotoxic drug etoposide was then tested using wild-type (WT) MEFs or MEFs lacking Bax or Bak. Treatment of fibroblasts with etoposide causes Bax- and Bak-mediated apoptosis, as either protein suffices to

induce cell killing (Supplementary Figure 1). m38.5 specifically blocked etoposide-induced killing of cells lacking Bak (Figure 1d), but not of cells from the other genotypes tested. Thus, m38.5 selectively interacts with Bax to block its killing action.

Deletion of m38.5 impairs MCMV replication *in vitro*. To evaluate the role of m38.5 in viral infection, we engineered a mutant virus, termed Δ m38.5, that lacks its expression. In constructing the Δ m38.5 virus, one must consider that the m38.5 open reading frame (ORF) overlaps with M38 (Figure 2a). The m38.5 ORF was disrupted in the previously described K181 MCMV bacterial artificial chromosome construct (BAC) pARK25¹⁷ by homologous recombination. This process involved replacing a portion of the m38.5 ORF (nucleotides 52316–52240) with the kanamycin resistance (Kan^R) gene flanked by FLP recombination target (FRT) sites. Removal of the Kan^R cassette was achieved by FLP recombination between the FRT sites. Sequencing of the resulting mutant indicated that the FRT remnant introduces several in-frame stop codons (Figure 2b) and a frameshift mutation downstream of nucleotide 52240. The result of these changes is that the m38.5 ORF is now only capable of producing a fusion protein consisting of the first 17 amino acids of m38.5 and 14 amino acids encoded by the FRT remnant. Northern blot analysis was performed on the Δ m38.5 mutant to ensure that the surrounding ORFs were not affected by the mutagenesis procedure. The ORFs in the vicinity of m38.5 use the same poly-A site; hence, probes used to detect the various RNA transcripts in this region detect multiple RNA species (Figure 2c). Transcripts for both m39 and m40 were detected in similar amounts in both the Δ m38.5 virus and WT MCMV (Figure 2d), indicating that the mutagenesis procedure had not affected the transcription of these ORFs. Owing to the small difference in their size, the m38.5 and M38 transcripts could not be distinguished in the northern blot analysis (Figure 2d). Sequencing of the Δ m38.5 virus verified that the area surrounding the transcriptional start site of M38 had not been affected. To confirm this, 5' rapid amplification of cDNA ends (RACE) products from cells infected with the Δ m38.5 virus were sequenced. We found that M38 transcripts were present in cells infected with the Δ m38.5 and that these transcripts encode the predicted sequence (data not shown). A revertant virus, m38.5 Rev, was also generated by re-inserting the full-length m38.5 ORF into the Δ m38.5 virus by homologous recombination. Confirmation that the m38.5 protein was not produced by the Δ m38.5 mutant virus was obtained by immunoblot analysis (Figure 2e). Probing with an anti-IE1-specific monoclonal antibody confirmed equivalent levels of infection in all samples (Figure 2e).

The growth characteristics of the Δ m38.5 virus were assessed in a range of permissive cell types. Cells were infected with WT, Δ m38.5 virus or m38.5 Rev virus at a multiplicity of infection (MOI) of 0.02 and viral replication at various times post-infection (PI) determined by plaque assay. The absence of m38.5 did not have an appreciable impact on viral replication during the first 2–3 days PI, but after this point, the replication of the Δ m38.5 mutant virus was consistently

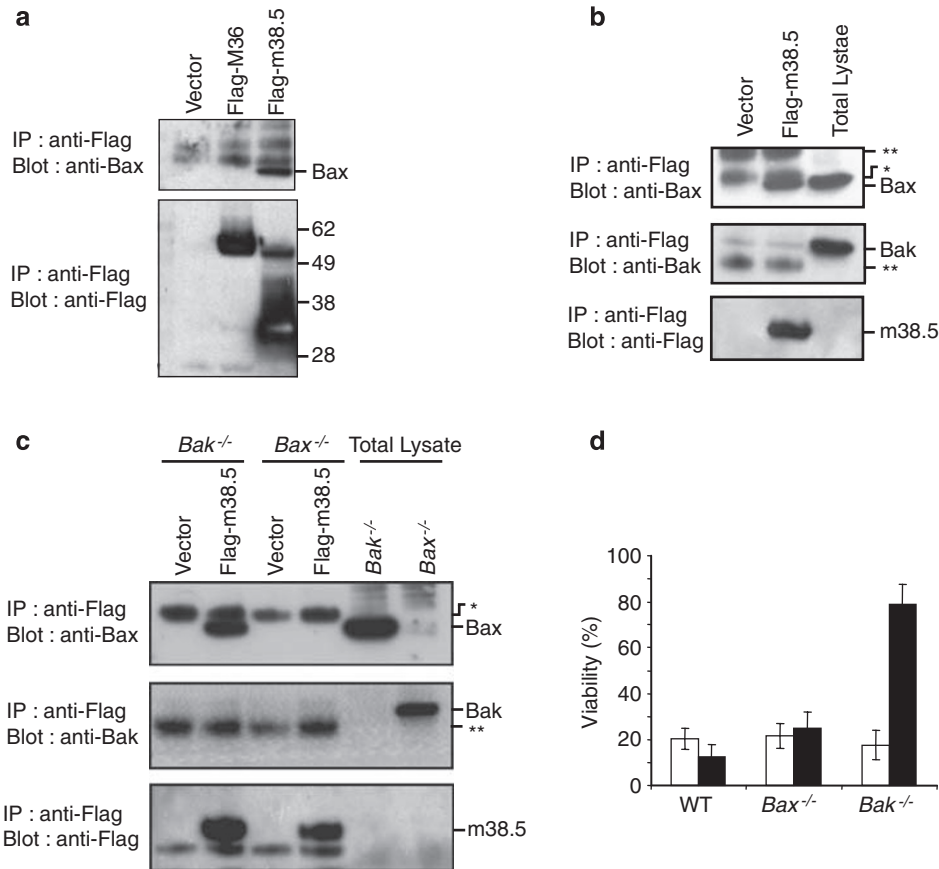


Figure 1 m38.5 inhibits apoptosis in a Bax-specific manner. **(a)** Cos cells were transiently transfected with Flag-tagged constructs encoding m38.5, M36 or empty vector. Cells were lysed in the presence of 2% CHAPS, Flag-tagged proteins immunoprecipitated and bound proteins analysed by immunoblot using the indicated antibodies. **(b)** HeLa cells were transfected with Flag-tagged m38.5 construct or empty vector. Flag-tagged proteins were immunoprecipitated and bound proteins analysed by immunoblot using the indicated antibodies. *Background band; **IgG light chain. **(c)** Lysates were prepared from the indicated MEF cell lines expressing Flag-tagged m38.5 and Flag-tagged proteins immunoprecipitated. Bound proteins were analysed by immunoblotting with the indicated antibodies. *Background band; **IgG light chain. **(d)** MEFs derived from WT, *bax*- or *bak*-deficient mice were infected with a retrovirus encoding m38.5 and GFP (filled columns) or GFP alone (open columns) and incubated for 48 h. Cells were then treated with etoposide and viability determined 16 h later ($n = 8$)

attenuated (Figure 3a). Importantly, the m38.5 Rev virus replicated as well as the WT virus in all cells tested (Figure 3a). Thus, the absence of m38.5 expression impairs MCMV replication *in vitro*. The impaired growth phenotype of the Δ m38.5 mutant could be attributed to its inability to inhibit Bax and, possibly, Bak. Importantly, when compared with the parental WT virus, growth of the Δ m38.5 virus was unaffected in cells lacking Bax, but was impaired in both WT and *bak*^{-/-} cells (Figure 3b). Hence, m38.5 promotes viral replication by preventing the activation of Bax, but not Bak.

CMVs are capable of replicating in a broad range of cell types. Next, we determined if the ability of MCMV to replicate in leukocytes was dependent on the expression of m38.5. The Δ m38.5 virus displayed a pronounced growth defect in DCs or macrophage cell lines (Figure 4a). We have previously shown that following MCMV infection, DCs become resistant to apoptosis, and this coincides with a redistribution of Bax from the cytosol to mitochondria.¹¹ Subcellular fractionation of DC infected with the Δ m38.5 virus indicated that Bax was predominately localised to the cytoplasm (Figure 4b). Furthermore, unlike DCs infected with the WT virus, DCs infected

with the Δ m38.5 mutant were susceptible to apoptosis induced by GF withdrawal (Figure 4c). Taken together, our data establish that m38.5 enhances MCMV replication by keeping Bax in an inert state on the mitochondria.

MCMV infection inhibits the activation of Bak and Bax.

Growth of the Δ m38.5 virus was equivalent to that of the WT virus in Bax-null fibroblasts, but attenuated in Bak-null fibroblasts (Figure 3b). An implication of this result is that replication of MCMV in fibroblasts must induce apoptosis exclusively through Bax. Alternatively, MCMV infection may trigger the activation of both Bax and Bak, and MCMV encodes a yet-to-be identified inhibitor of Bak. To distinguish between these possibilities, we infected WT fibroblasts or fibroblasts lacking Bax or Bak with either WT MCMV or the Δ m38.5 mutant virus, using an MOI of 3 to ensure that all cells within the culture were infected. Virally infected cells were then treated with etoposide to test their susceptibility to apoptosis. As mentioned earlier, in fibroblasts, etoposide treatment induces the activation of both Bax and Bak, with either sufficient to induce apoptosis. All fibroblast lines

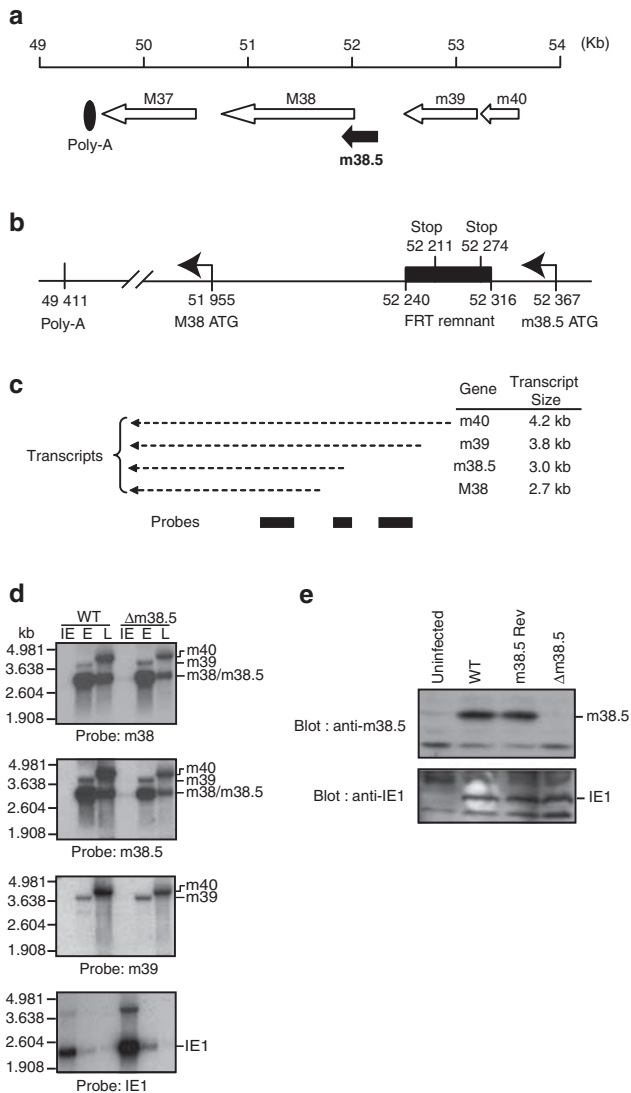


Figure 2 Genomic arrangement and analysis of Δ m38.5. (a) The genomic region of MCMV encompassing m38.5 (solid arrow) and adjoining ORFs (open arrows) is shown. Direction of gene transcription is denoted by the direction of the arrows. Location of the common polyadenylation site used by all genes in this region is denoted by the filled circle. (b) Sequencing of 5' RACE products and of viral DNA was performed and the data used to generate a map of the genomic structure of the Δ m38.5 mutant virus. The FLP remnant retained from the mutagenesis procedure causes a frameshift downstream of nucleotide 52 316 and introduces premature stop codons. (c) Size of the various gene transcripts in the region is shown. The probes used for northern analysis and their respective binding sites are shown (filled boxes). (d) RNA was isolated from fibroblasts infected with WT MCMV-K181 Perth or the Δ m38.5 mutant at immediate early (IE), early (E) and late (L) times after infection. Total RNA was separated on a denaturing agarose gel, blotted onto PVDF membrane and hybridised with DNA probes. (e) Total cell lysates were prepared from fibroblasts infected with the indicated viruses 24 h PI. Immunoblot analysis was performed using hyperimmune serum from a rat injected with an m38.5 peptide linked to KLH or an anti-IE1 monoclonal antibody

infected with WT MCMV were resistant to apoptosis induced by etoposide treatment (Figure 5), suggesting that MCMV can prevent the activation of both Bax and Bak. WT or *bak*^{-/-} fibroblasts infected with the Δ m38.5 virus were sensitive to etoposide-induced apoptosis, with only a few viable cells

remaining 18 h after the addition of the drug (Figure 5). This result is consistent with the hypothesis that cells infected with the Δ m38.5 virus lack the ability to prevent the activation of Bax. By contrast, Bax-deficient fibroblasts infected with the Δ m38.5 mutant did not undergo apoptosis after etoposide treatment (Figure 5). Therefore, in addition to encoding a Bax-specific antiapoptotic protein, MCMV must also possess a strategy to inhibit Bak activation.

Viral load within the salivary gland is dependent on m38.5 activity. As growth of the Δ m38.5 mutant virus was attenuated *in vitro*, we anticipated that viral replication would be similarly compromised *in vivo*. The capacity of the Δ m38.5 virus to replicate *in vivo* was assessed by comparing viral titres in the target organs of BALB/c mice infected with either WT or the Δ m38.5 mutant virus. Mice were infected with either WT or Δ m38.5 virus, and viral titres in target organs determined by plaque assay at various times PI. Surprisingly, viral titres in the spleen, liver and lungs of mice infected with the Δ m38.5 virus did not differ significantly from those observed in the organs of mice infected with WT MCMV (Figure 6a). By contrast, viral titres in the salivary glands were 10- to 100-fold lower for the Δ m38.5 mutant compared with the WT virus (Figure 6b). To ensure that the impaired replication observed in salivary glands was due to the loss of m38.5 activity, the growth of the m38.5 Rev virus was assessed *in vivo*. Titres of m38.5 Rev in the salivary glands were similar to those of the WT virus, whereas significantly reduced viral titres were observed in salivary glands of mice infected with the Δ m38.5 virus (Figure 6c). Therefore, although m38.5 is not required for efficient viral replication in all permissive cell types *in vivo*, the protein exerts a selective effect that promotes MCMV replication in salivary glands.

m38.5 enhances MCMV replication in the blood. The reduced salivary gland titres seen after infection with the Δ m38.5 virus may indicate a requirement for m38.5 activity for the survival of acinar cells, the predominant target of MCMV in this tissue.¹⁸ Alternatively, dissemination of the Δ m38.5 virus to salivary glands could be impaired. MCMV dissemination is mediated by myeloid progenitors that enter the circulation after being mobilised from the bone marrow.¹⁹⁻²¹ As attenuation of Δ m38.5 growth *in vitro* was most pronounced in cells of myeloid origin (Figure 4a), we investigated whether the deletion of m38.5 had any impact on viral load in the blood. Blood samples from mice infected with WT or Δ m38.5 virus were collected and the number of infected cells in the circulation determined by infectious centre assay. At day 5 after infection, the time when MCMV titres in the blood peak,²⁰ fewer cells harbouring infectious virus were detected in the blood of mice infected with the Δ m38.5 mutant (Figure 7a). This finding suggests that m38.5 may be necessary for viral replication to proceed efficiently in leukocytes, thereby ensuring maximal viral trafficking to the salivary glands.

If replication of the m38.5 mutant virus within leukocytes is impaired, this should be apparent at other sites. This possibility was investigated by examining viral replication within the spleen. MCMV replication in the spleen occurs in

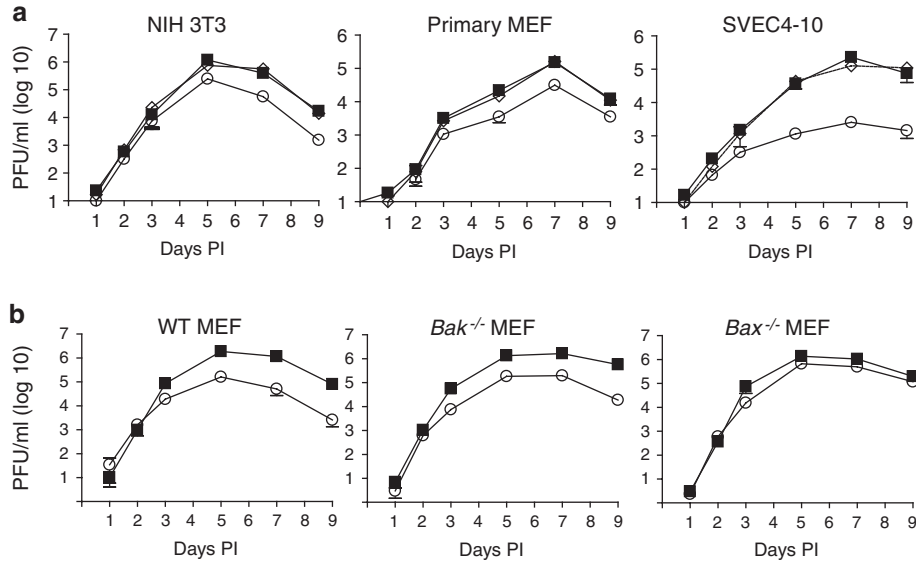


Figure 3 *In vitro* growth of the Δ m38.5 virus. (a) Primary MEFs, NIH 3T3 fibroblasts and SVEC4-10 epithelial cells were infected with either WT (filled squares), Δ m38.5 (open circles) or m38.5 Rev virus (open diamonds) at an MOI of 0.02. At the indicated times after infection, total viral yield was determined ($n = 3$). (b) Fibroblast cell lines from WT, *bax*- or *bak*-deficient mice were generated from E13–E14.5 embryos using SV40 large T antigen.⁵ The various cell lines were infected at an MOI of 0.02 with either WT MCMV (filled squares) or the Δ m38.5 mutant (open circles) and viral yield (PFU) determined at the indicated times after infection ($n = 3$)

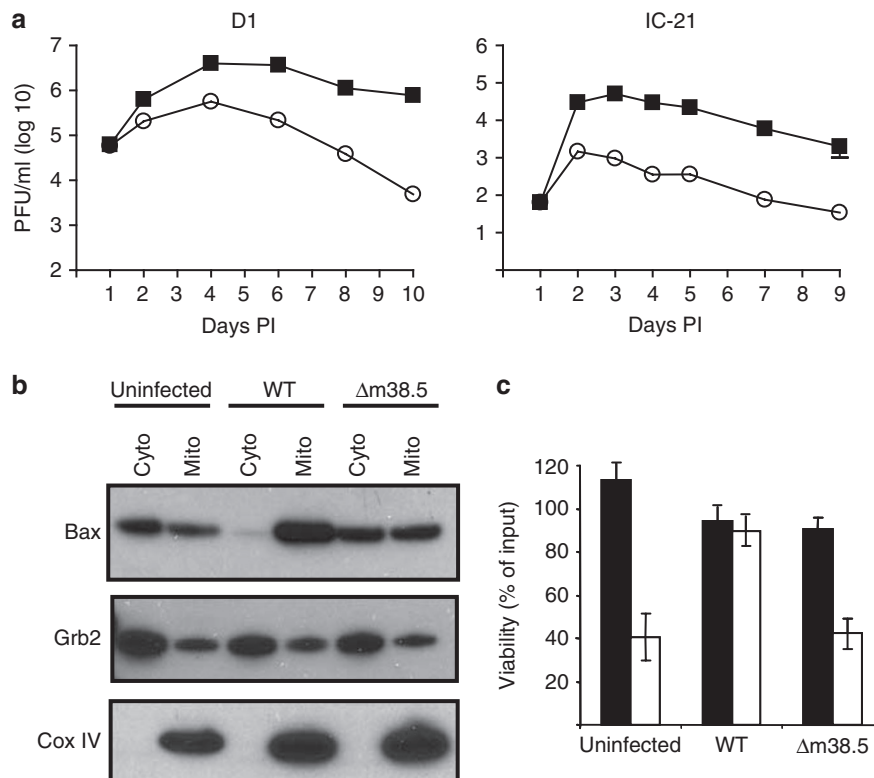


Figure 4 m38.5 promotes viral replication by binding Bax at the mitochondria. (a) D1 DCs or IC-21 macrophages were infected with WT (filled squares) or Δ m38.5 virus (open circles) at an MOI of 3 and the viral yield determined ($n = 8$ for D1 and $n = 3$ for IC-21). (b) D1 DCs were infected with WT or Δ m38.5 virus (MOI = 3) and 4 days after infection, cytoplasmic (Cyto) and mitochondrial (Mito) fractions prepared and immunoblotted with the indicated antibodies. (c) D1 DCs were infected with WT or Δ m38.5 virus (MOI = 3) and 4 days after infection the cells were plated in complete medium (filled columns) or medium lacking GF (open columns) and viability determined 18 h later ($n = 10$)

various cell types, including endothelial cells²² and leukocytes, such as macrophages¹⁹ and DCs.²³ A specific defect in viral replication within leukocytes might not have been

observed in our initial analysis as we determined viral loads in the whole organ. Therefore, spleens from mice infected with either WT or Δ m38.5 viruses were removed at day 4 PI and

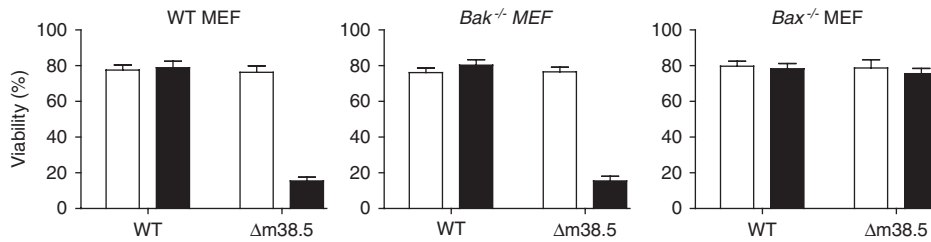


Figure 5 MCMV encodes inhibitors for both Bax and Bak. The indicated MEF cell lines were infected with either WT or Δm38.5 virus (MOI = 3) and incubated for 18 h. Etoposide (filled columns) or vehicle (open columns) was added to the various cultures and viability determined 24 h later ($n = 7$)

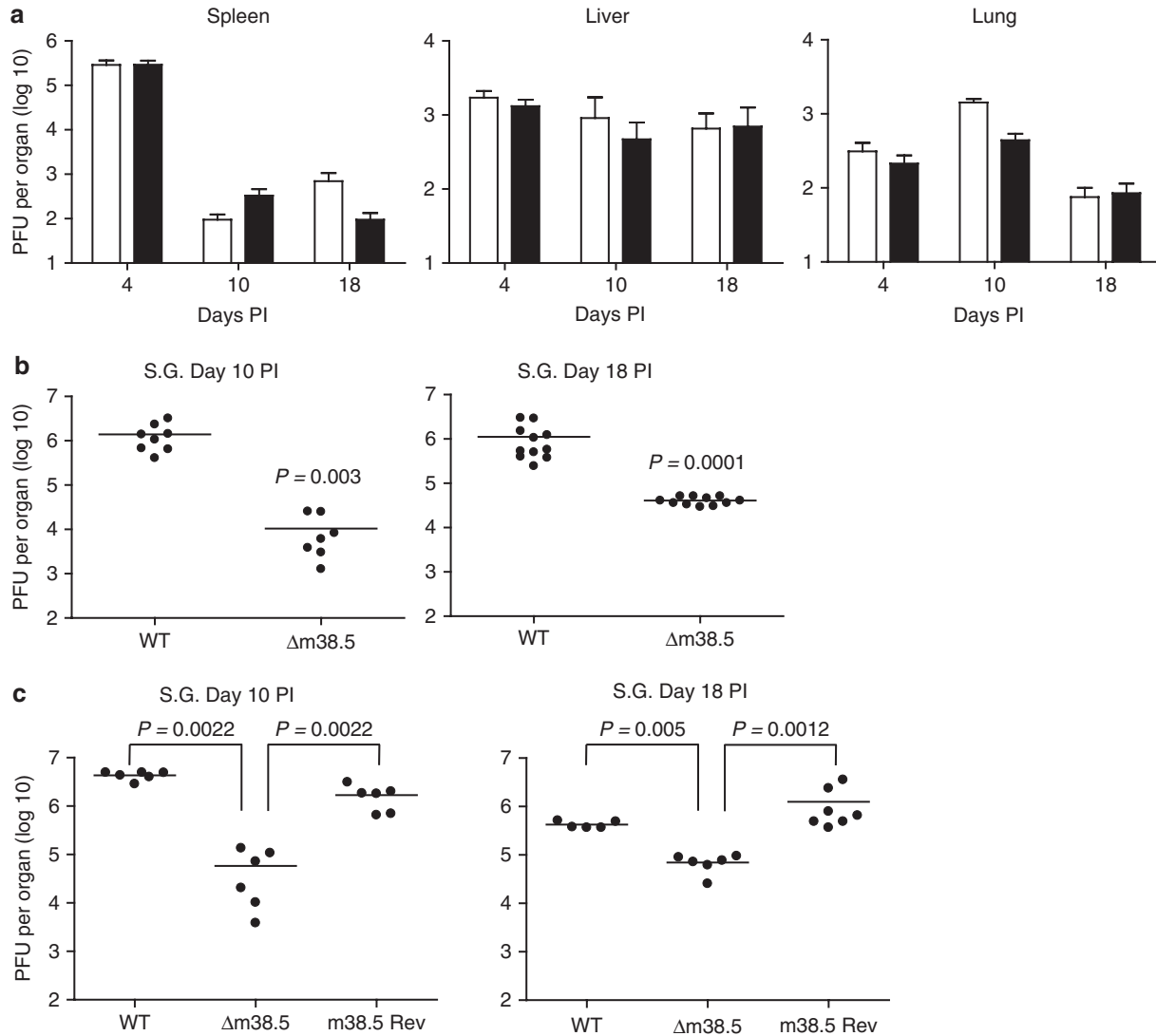


Figure 6 Loss of m38.5 reduces viral load in the salivary gland. (a) Mice were infected with WT MCMV (open columns) or Δm38.5 virus (filled columns), organs were removed at various times after infection and viral load per organ determined by plaque assay. Viral titres were quantified in three separate experiments and the data pooled, mean \pm S.D. of seven to nine mice per group is plotted. No statistically significant differences were observed at any time point. (b) Viral load in the salivary gland of mice infected with either WT or Δm38.5 virus at the indicated times after infection was determined by plaque assay. Data from four experiments have been pooled. (c) Mice were infected with the indicated viruses and viral titres in the salivary glands determined at the time points indicated. Data are pooled from two experiments

half the organ processed to purify leukocytes while excluding endothelial cells. FACS analysis indicated that greater than 95% of the cells isolated using this method were positive for the leukocyte antigen CD45 (data not shown). The leukocytes

were lysed and viral load determined by plaque assay. A significant reduction in viral replication within splenic leukocytes was observed in mice infected with the Δm38.5 virus (Figure 7b, right panel). The remaining half of the spleen was

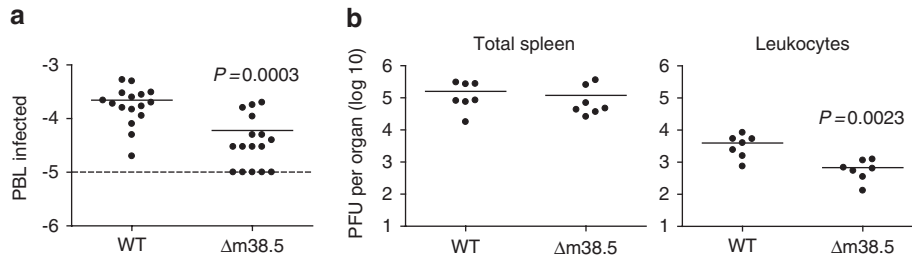


Figure 7 The role of m38.5 is specific for leukocytes. (a) Whole blood was isolated from infected mice, red blood cells removed by hypotonic lysis and the proportion of infected cells determined by infectious centre assay. (b) Spleens from mice infected with WT or $\Delta m38.5$ virus were removed and total viral burden was determined using half the spleen (left panel). The remainder of the spleen was used to determine viral load within leukocytes purified as described (right panel)

used to determine total viral load, and as with our earlier results no difference was observed in viral titres from the whole organ (Figure 6a, left panel). Together, these results establish that m38.5 activity is required to enhance viral replication within leukocytes. By contrast, deletion of m38.5 had no effect on the ability of MCMV to replicate in other permissive cell types, such as endothelial cells, *in vivo*.

Discussion

In this study, we have examined the role of m38.5 in the pathogenesis of MCMV and have established that it is a Bax-specific inhibitor of apoptosis. *In vivo*, m38.5 expression enhanced viral replication within leukocytes, thereby promoting dissemination of MCMV to the salivary glands. Our data indicate that viral interference with apoptosis not only improves viral replication, but is also required to promote efficient viral dissemination.

Given the central role played by the Bax/Bak proteins in promoting cellular destruction, it is not surprising that a number of viruses encode proteins capable of preventing their activation. For example, adenovirus, myxoma virus and Epstein–Barr virus encode antiapoptotic proteins that function by preventing the activation of both Bax and Bak.^{24–28} As the size of viral genomes is limited, encoding a single protein capable of inhibiting both Bax and Bak may provide the pathogen with a survival advantage with limited burden on the viral genome. This, however, does not seem to be the case for MCMV, as we provide evidence that Bak activation in MCMV infection is inhibited independently of m38.5-mediated activities. These findings indicate that MCMV encodes specific inhibitors of Bax (m38.5) and Bak, with the latter yet to be identified. Our *in vitro* data are consistent with recent publications demonstrating that m38.5 is a Bax-specific antiapoptotic protein.^{29–31} Interestingly, the related vMIA protein encoded by HCMV is capable of inhibiting both Bax and Bak.^{30,32} Why MCMV encodes specific and separate inhibitors of Bax and Bak, when many viruses, including the closely related HCMV, encode one antiapoptotic protein able to inactivate both pro-death mediators remains to be clarified. In this respect, it is worth noting that although Bax/Bak are essential for apoptosis, the role of these proteins in Ca^{2+} homeostasis and mitochondrial morphogenesis in healthy cells has also been established.^{32,33} Indeed, the expression of vMIA is sufficient to disrupt mitochondrial networks and induce Ca^{2+} mobilisation from the endoplasmic reticulum.^{34,35} Consistent with the inability of m38.5 to bind Bak,

expression of m38.5 does not interfere with mitochondrial morphology unless Bak is absent.³⁰ Therefore, cell lineage-specific expression of Bax or Bak viral inhibitors may allow MCMV to inhibit apoptosis, while minimising the impact on other cellular processes required for viral replication.

A central finding of this study is that rather than having a global impact on viral replication, deletion of m38.5 resulted in a reduction in viral load specifically in salivary glands. Salivary gland tropism is an important feature of CMV infection, with shedding of the virus into the saliva serving as the principal source of horizontal transmission.³⁶ Reduced salivary gland titres were the result of an impaired ability of the $\Delta m38.5$ mutant to replicate in leukocytes, cells known to be required for the dissemination of MCMV to the salivary glands. The finding that m38.5 enhanced viral replication in leukocytes, but not other permissive cell types *in vivo*, could be reconciled by the absence of Bax expression in cells or tissues where we did not observe a difference in the ability of the $\Delta m38.5$ mutant viruses to replicate. However, Bax has a broad expression pattern *in vivo* and, in particular, is expressed in both the spleen and lungs.³⁷ Hence, the capacity of the $\Delta m38.5$ virus to replicate normally in cells other than leukocytes cannot simply be attributed to a lack of Bax, but rather, suggests that MCMV infection does not lead to activation of Bax in these cells during *in vivo* infection.

In vivo replication of the $\Delta m38.5$ mutant was attenuated specifically in leukocytes, but *in vitro* replication of the $\Delta m38.5$ virus was impaired in a variety of cell types. The broader defect observed *in vitro* may be the result of cells being subjected to stress while monitoring viral growth over a period of 9 days. During this time, nutrients and GFs in the culture medium are gradually depleted, and this has the potential to act as an apoptotic stimulus. Our data indicate that cells infected with the $\Delta m38.5$ virus are less resistant to apoptotic stimuli, such as GF deprivation or etoposide treatment, than those infected with WT virus. Thus, growth of the $\Delta m38.5$ virus may be attenuated in a wide range of cell types *in vitro* due to the presence of additional apoptotic stimuli that are not at play during *in vivo* infection. Support for this hypothesis comes from the observation that the *in vitro* growth of a $\Delta m38.5$ virus is significantly enhanced by changing the culture medium daily.²⁹ The *in vitro* data clearly define m38.5 as a specific inhibitor of Bax. *In vivo*, the capacity to inhibit Bax is required to promote dissemination of MCMV.

Activation of Bax and/or Bak is a prerequisite for apoptosis to proceed in response to many stimuli.^{3,4} Bax and Bak function in a redundant fashion during development; however,

in vitro studies have suggested that Bax and Bak may be independently activated, and the ability of some BH3-only proteins to selectively antagonise the function of pro-survival Bcl-2 proteins has been noted.³⁸ Our data indicate that MCMV replication *in vivo* induces the activation of Bax in leukocytes, but not in other permissive cell types. It is unclear why MCMV infection *in vivo* results in activation of Bax in leukocytes, but this selectivity is most likely due to leukocytes expressing a different combination of pro- and antiapoptotic Bcl-2 proteins than that expressed by other MCMV-permissive cells.

In summary, we have demonstrated that m38.5 inhibits Bax activation in leukocytes. Inhibition of Bax by MCMV in leukocytes was required to achieve optimal dissemination to the salivary gland, the main site of viral transmission. We also provide evidence that MCMV inhibits the activation of Bak and that this function is independent of m38.5. How MCMV inhibits Bak is the subject of our on-going studies. A detailed understanding of how CMVs inhibit apoptosis might be relevant to the development of novel interventions against viral infection.

Materials and Methods

Cell lines and reagents. Culture conditions for cell lines and methods for the purification of viral stocks have been described previously.^{5,11,23} Cos-7 or HeLa cells were transfected with pcDNA3 encoding Flag-m38.5, Flag-M36 or vector only using Fugene-6 reagent (Roche) and total cell lysates prepared 48 h later. Similarly, MEFs were transfected with pcDNA3 encoding Flag-m38.5 or vector only using Fugene-6 reagent and stable cell lines generated by puromycin selection. Retroviral-mediated transfection was performed as described.⁵ Immunoprecipitation analysis was performed on cell lysates prepared using CHAPS detergent according to published methods.¹¹ Induction of apoptosis in DC and subcellular fractionation were performed as described.¹¹ Apoptosis in MEF was induced by the addition of 100 μ M etoposide (Sigma) and viable cells enumerated by trypan blue exclusion. Hyperimmune serum against m38.5 was produced by injecting a rat with a peptide comprising amino acids 40–53 of m38.5 linked to keyhole limpet haemocyanin. TiterMax Classic adjuvant (Sigma Chemicals) was mixed with 100 μ g of the peptide (1 : 1 ratio) and injected I.P. The rat was boosted 28 days after the primary injection. Serum was purified from whole blood 10 days after the boost and used for immunoblot analysis.

Analysis of viral growth. The Δ m38.5 mutant virus was constructed using the MCMV-K181 Perth BAC.¹⁷ Inbred BALB/c mice at 8 weeks of age were obtained from the Animal Resources Centre (Perth, Western Australia) and maintained in specific pathogen-free conditions at the Animal Services Facility of the University of Western Australia. Mice were injected intraperitoneally with 1×10^4 plaque-forming units (PFUs) of salivary gland-propagated virus stock of WT BAC-derived MCMV-K181 or Δ m38.5 virus diluted in phosphate-buffered saline containing 0.5% FBS. At various time points after infection, mice were killed and organs removed for analysis. Viral titres were quantified by plaque assay on monolayers of permissive cells.²³ Viraemia in the blood was assessed by isolating blood by heart puncture and determining the number of infected cells by culture on permissive cells.²⁰ Leukocytes in the spleen were isolated as described.²² FACS analysis indicated that > 95% of the cells isolated using this procedure were leukocytes. All animal experimentations were performed with the approval of the Animal Ethics and Experimentation Committee of the University of Western Australia and according to the guidelines of the National Health and Medical Research Council of Australia. For *in vitro* analysis of viral replication, tissue culture stocks of WT virus, Δ m38.5 or m38.5 Rev were produced by infecting NIH 3T3 fibroblasts with the respective salivary gland-propagated viruses. Viral stocks were harvested and purified as described earlier.³⁹

Generation of m38.5 constructs. The m38.5 ORF was amplified by PCR from DNA isolated from the MCMV-K181 Perth viral strain and cloned into the EcoR1 site of the pcDNA3Flag expression vector (Invitrogen) or into the pMIG

retroviral vector. DNA sequencing was employed to ensure the fidelity of the PCR product. Details of all oligonucleotides and plasmids are available from the authors.

Viral mutagenesis. A recombinant MCMV genome containing a disruption in the m38.5 ORF was constructed in *Escherichia coli*. The pSLFRTKn plasmid was used as a template⁴⁰ to generate a PCR product containing the Kan^R gene flanked by 50 bp of homology to the m38.5 target sequence and 34 bp FRT site in the same orientation. Following purification, the PCR fragment was transformed into the DH10 β strain of *E. coli* carrying the previously described K181 MCMV BAC pARK25.¹⁷ Deletion of the target sequence within the m38.5 ORF was achieved by homologous recombination, mediated by the λ red recombinase-encoding plasmid pKD46.⁴⁰ Transformants were selected at 30 °C on LB agar plates containing chloramphenicol (25 μ g/ml) to select for the ARK25 bacmid and kanamycin (25 μ g/ml). Kan^R clones were screened by PCR to confirm the insertion of the Kan^R gene. Removal of the Kan^R cassette was achieved by transforming Kan^R mutants with pCP20, which directs the excision of the Kan^R cassette through FLP recombination between the FRT sites.⁴⁰ Loss of the Kan^R gene was assessed by replica plating onto LB agar plates containing either chloramphenicol alone or chloramphenicol and kanamycin. Excision of the Kan^R gene by FLP recombination leaves an FRT remnant within the m38.5 ORF. Sequencing of Δ m38.5 indicated that the FRT remnant introduced a frameshift mutation in the m38.5 ORF (Figure 2b).

Reconstitution of virus progeny from MCMV BAC plasmids. MEFs derived from *Bax*^{-/-}*Bak*^{-/-} mice were seeded into a six-well tray at a density of 1×10^5 cells per well before transfection. pARK25 Δ m38.5 DNA was purified using the NucleoBond nucleic acid purification kit (BD Biosciences) according to the manufacturer's instructions. MEFs were transfected with viral DNA (1 μ g) using FuGENE-6 (Roche) and cultured until plaques developed. Successful transfection and reconstitution of the virus was monitored by GFP expression driven by the *EGFP* gene within the BAC backbone of ARK25. Once the monolayer exhibited maximal cytopathic effect, viral supernatants were collected. The BAC backbone was removed from the viral genome by serial passage of the virus on MEF monolayers and monitoring for the loss of GFP expression. The BAC cassette is flanked by a 249-bp MCMV sequences repeat.¹⁷ When transfected into eukaryotic cells, homologous recombination between the repeat MCMV sequences results in the excision of the BAC cassette. The reconstituted virus was denoted MCMV Δ m38.5.

Preparation of RNA and northern analysis. MEFs were infected at an MOI of 5. Infections were performed in the presence of either cyclohexamide (Sigma-Aldrich) (50 μ g/ml) for immediate early RNA or phosphonoacetic acid (Sigma-Aldrich) (20 μ g/ml) for early RNA, and isolation performed at 4 h after infection. For the collection of late RNA transcripts, MEFs were infected in the absence of metabolic inhibitors and harvested 24 h after infection. All RNA extractions were performed using TrizolTM (Invitrogen) according to the manufacturer's instructions. RNA samples were subjected to electrophoresis under denaturing conditions, blotted onto a PVDF nylon membrane (Amersham Pharmacia) and hybridised with P³²dCTP-labelled double-stranded DNA probes. Probes for M38, m38.5, M39 and IE1 were radioactively labelled using the DECAprimeTM II labelling kit (Ambion) according to the manufacturer's instructions.

3' and 5' rapid amplification of cDNA ends. 3' and 5' RACE PCR was performed using total RNA prepared from infected MEFs at immediate early, early and late times PI using the FirstChoice RLM-RACE kit (Ambion) and m38.5- or M38-specific oligonucleotides. Reactions were carried out according to the manufacturer's instructions. The cDNAs obtained were cloned into pGEMT-Easy and sequenced.

Construction of Δ m38.5 Rev. The m38.5 revertant virus (m38.5 Rev) was generated by co-transfecting MEF with Δ m38.5 viral DNA and a pBluescript construct encoding the full-length m38.5 ORF. Virus stocks isolated from the co-transfection were used to infect MEF monolayers and, 4 days later, DNA was prepared using the Puregene genomic DNA purification kit (Gentra Systems). The presence of m38.5 revertant virus was detected by PCR and the m38.5 Rev virus was cloned by limiting dilution. Briefly, MEF monolayers in 24-well plates were infected with virus stocks from the co-transfection containing the m38.5 Rev virus at an MOI of 1 PFU per well and incubated until a cytopathic effect was evident. Viral supernatants were retained and DNA prepared from the infected cells. PCR was performed to identify wells containing Δ m38.5 Rev.

Statistical analysis. All plotted data represent mean \pm 1 standard deviation. All *P*-values were determined using the non-parametric Mann–Whitney statistical test.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)