Contents lists available at SciVerse ScienceDirect

Virology

Murine gammaherpesvirus 68 ORF75c contains ubiquitin E3 ligase activity and requires PML SUMOylation but not other known cellular PML regulators, CK2 and E6AP, to mediate PML degradation

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ARTICLE INFO

ABSTRACT

All gammaherpsviruses encode at least one gene related to the cellular formylglycinamide ribonucleo-tide amidotransferase (FGARAT) enzyme but their biological roles are relatively unknown. The murine gammaherpesvirus 68 (MHV68) vFGARAT, ORF75c, mediates a proteasome-dependent degradation of the antiviral promyelocytic leukemia (PML) protein by an unknown mechanism, which is addressed in this study. We found that ORF75c interacts weakly with PML and SUMO-modified forms of PML are important for its degradation by ORF75c. ORF75c-mediated PML degradation was not dependent on two known cellular regulators of PML stability, Casein kinase II (CK2) and human papilloma virus E6-associated protein (E6AP). Finally, ORF75c had self-ubiquitination activity in vitro and its expression increased levels of ubiquitinated PML in transfected cells. Taken together, the evidence accumulated in this study provides new insights into the function of a vFGARAT and is consistent with a model in which ORF75c could mediate direct ubiquitination of PML resulting in its degradation by the proteasome.

Introduction

Promyelocytic leukemia protein (PML) or tripartite motif-containing protein 19 (TRIM19) is an interferon-inducible protein essential for the formation of nuclear organelles known as PML nuclear bodies (PML NBs), which are found in most mammalian cells (Batty et al., 2009; Lallemand-Breitenbach and de Thé, 2010; Ozato et al., 2008). PML NBs have been implicated in modulating a diverse array of functions including, tumor suppression, apop-tosis, cellular senescence, DNA repair, interferon responses and antiviral responses (Bernardi and Pandolfi, 2007; Dellaire and Bazett-Jones, 2004; Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Salomoni and Pandolfi, 2002). Eight isoforms of PML have been characterized and are generated through alter-native splicing, increasing the diversity of interacting partners and subcellular localization. PML isoform I is the most abundantly expressed isoform in human cells and is one of the two isoforms that are conserved between human and mouse (Condemine et al., 2006). Additionally, PML undergoes various post-translational modifications, including modification by a small ubiquitin-related modifier (SUMO) family at three lysine residues (Lys 65, 160 and 490), which is critical for the formation and recruitment of protein partners to PML NBs (Fu et al., 2005; Kamitani et al., 1998; Nichol et al., 2009; Zhong et al., 2000). In addition to PML itself, several other proteins are commonly found within PML NBs and have been implicated in mediating PML NB activities. These factors include, speckled protein 100 (Sp100), Daxx, SUMO-1, DNA damage response proteins (RAD50, nbs1, and mre11), p53, regulators of p53, and CREB binding protein (Negorev and Maul, 2001).

The role of PML in cellular antiviral responses is supported by the fact that PML NBs are targeted for disruption by many DNA and RNA viruses and cells lacking PML are more susceptible to viral infections (Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Reineke and Kao, 2009). Examples of herpes-virus proteins that are known to induce PML degradation or PML NB disruption include herpes simplex virus-1 (HSV-1) ICP0 (Chee et al., 2003; Everett et al., 1998, 2006), human cytomegalovirus (CMV) IE1 (Ahn and Hayward, 1997; Tavalai et al., 2006), Epstein-Barr virus (EBV) EBNA1 (Sivachandran et al., 2008), EBV Zta (Adamson and Kenney, 2001), and Kaposi's Sarcoma-associated herpesvirus (KSHV) LANA2 (Marcos-Villar et al., 2009). Altered localization and/or inactivation of PML NB components is also common. EBV EBNA-LP transcriptional coactivation functions are mediated through binding and relocalization of Sp100A from PML

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NBs (Ling et al., 2005), herpesvirus saimiri (HVS) ORF3 induces Sp100 degradation (Full et al., 2012), and EBV BNRF1 (ORF75 homolog) inactivates Daxx (Tsai et al., 2011). However, knowledge about the consequences of PML-NB disruption during human herpesvirus infection is limited largely to studies in cell culture-based systems due to the species specificity of herpesviruses. Murine gammaherpesvirus 68 (MHV68) is closely related to human gammaherpesviruses and naturally infects mice. Additionally, MHV68 can cause lymphoproliferative diseases and establish long-term chronic infections similar to human gammaherpesviruses (Nash et al., 2001; Simas and Efstathiou, 1998). Therefore, this system is ideal for investigating strategies utilized by herpesviruses for establishing long-term infections in their animal hosts.

We previously demonstrated that MHV68 infection in fibroblast cells induces a rapid proteasome-dependent degradation of PML and this was mediated by the viral tegument protein known as ORF75c (Ling et al., 2008). ORF75c has homology to the cellular formylglycinamide ribonucleotide amidotransferase (FGARAT) enzyme (Ling et al., 2008), which carries out the fourth step in de novo purine biosynthesis, although one study suggests that ORF75c no longer retains this enzymatic function (Gaspar et al., 2008). Other gammaherpesviruses also encode one or more FGARAT homologs (Full et al., 2012; Tsai et al., 2011), but their role in gammaherpesvirus pathogenesis has yet to be fully defined. To gain more insights into the function of a viral FGARAT (vFGARAT), we investigated the undetermined mechanism by which ORF75c mediates PML degradation. We found that ORF75c is present continuously throughout infection; first delivered from virion particles and then later expressed as a late gene. ORF75c induces PML poly-ubiquitination in vivo and PML SUMOylation is important for ORF75c-induced degradation. However, specific ORF75c association with PML may occur through SUMO-dependent and SUMO-independent mechanisms. Finally, ORF75c contains self-ubiquitination activity in vitro suggesting that it may be a specific PML E3 ligase, especially since other known PML E3 ligases were not required for ORF75c-mediated PML degradation. This study provides a better understanding of another interesting strategy used by gammaherpesviruses to modulate host intrinsic cellular antiviral responses through its viral FGARAT.

Results

Expression kinetics of ORF75c in mouse fibroblasts

In order to understand and interpret subsequent experiments aimed at investigating how ORF75c induces PML degradation, we first sought to determine whether ORF75c effects on PML were due entirely to incoming protein associated with virions or if there might also be newly synthesized protein expressed early on during infection. Time-course infection experiments indicated that ORF75c was
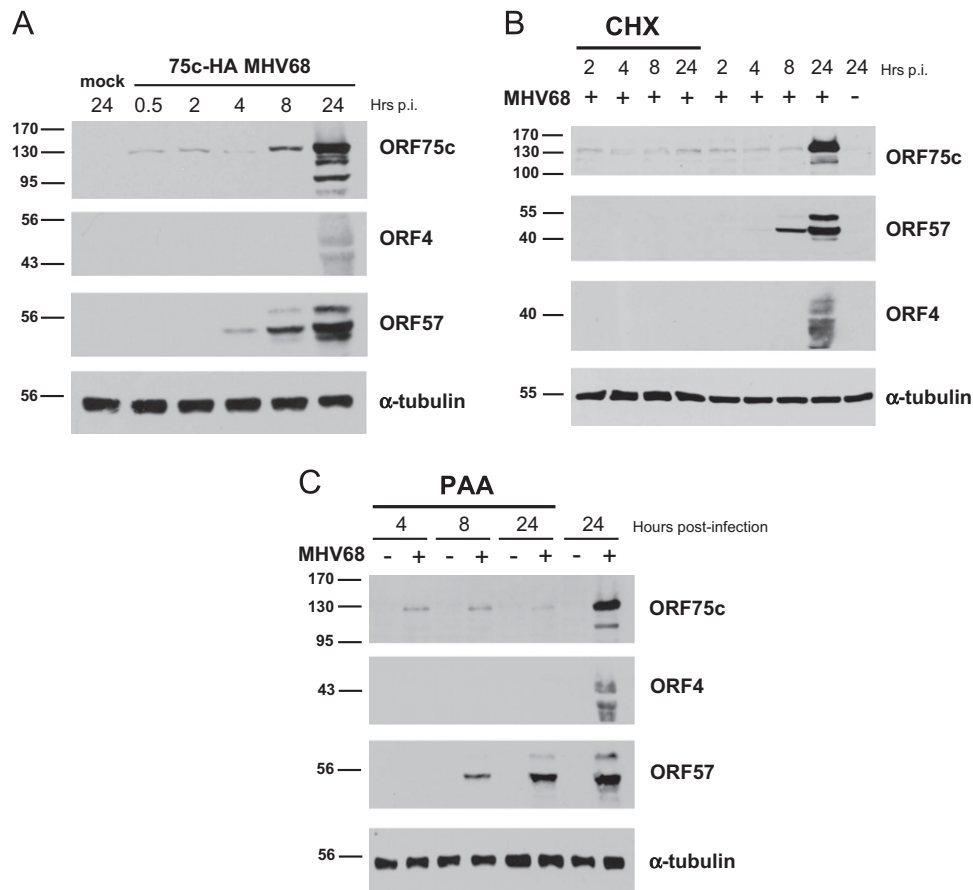

Fig. 1. Expression kinetics of ORF75c in infected mouse fibroblasts. Immunoblot analyses of selected MHV68 proteins at different time-points post-infection. (A) 3T12 cells infected with an MOI of 5 at 0.5, 2, 4, 8, and 24 h post-infection. Specific proteins detected are indicated to the right of each blot. Molecular sizes in KDa are indicated on the left. (B) Immunoblot analyses of 3T12 cells infected with an MOI of 5 at 2, 4, 8, and 24 h post-infection with and without 50 μg/ml cycloheximide treatment for 1 h before and during the infection as indicated. Specific proteins detected are indicated to the right of each blot. Molecular sizes in KDa are indicated on the left. (C) 3T12 cells infected (+) or uninfected (−) at different time points, which are indicated above the panels. Some cells were treated with 200 μg/mL phosphonoacetic acid (PAA) for 1 h before and during the infection as indicated above the panels. Specific proteins detected are indicated on the right of each blot. Molecular sizes in KDa are indicated on the left.

detectable from the start of infection. A small increase in protein was detectable at 8 h and increased significantly by 24 h post-infection (Fig. 1A). To assess whether the ORF75c detected at the beginning of infection was derived from incoming virions, we infected 3T12 cells in the presence or absence of cycloheximide (CHX). The level of ORF75c detected in both conditions was similar (Fig. 1B), suggesting that the bulk of ORF75c detected at early time-points after infection is virion derived. As a control for cycloheximide efficiency, no ORF57 (a known immediate early/early gene) was detected in the cycloheximide treated cells (Fig. 1B). Similar experiments were conducted in the presence of concentrations of phosphonoacetic acid (PAA) that are known to inhibit late gene expression (Rochford et al., 2001). The conditions were sufficient to block synthesis of a known late gene, ORF4, but not a known immediate early/early gene ORF57 as expected (Cheng et al., 2012; Martinez-Guzman et al., 2003) (Fig. 1C). Under these conditions, no additional synthesis of ORF75c appears to occur above the ORF75c delivered from virions, indicating that ORF75c displays characteristics of a late gene.

ORF75c interacts weakly with PML

To determine whether ORF75c interacts with PML, PML-Flag and ORF75c-HA epitope-tagged proteins were transiently expressed in 293T cells followed by co-immunoprecipitation analyses. PML-Flag coprecipitated ORF75c-HA but not ORF45-HA control, indicating an association between these proteins under these conditions (Fig. 2A). Attempts to coprecipitate ORF75c and PML in infected cells proved to be technically challenging due to low levels of ORF75c present at early time-points post-infection, where only virion delivered protein is present (data not shown,

Fig. 1). However, a prediction from the transient expression experiments is that both proteins would colocalize in cells. To address this question ORF75c and PML were analyzed by immunofluorescence during infection in 3T12 cells. At 60 min post-infection, ORF75c was spread throughout the cytoplasm, mostly in punctate spots (Fig. 2B). However, by 90 min post-infection most of the detectable ORF75c localized to the nucleus with a diffuse-speckled pattern, which was maintained through 3 h post-infection. Within 180 min post-infection, almost all of the PML NBs disappeared (Fig. 2B), which correlated with loss of the PML protein as we have described previously (Ling et al., 2008). High resolution deconvolution microscopy at 90 min post-infection, where both PML and ORF75c can be detected in the nucleus, indicated a small proportion of colocalization between these proteins (Fig. 2C). Infected cells treated with concentrations of MG-132 known to prevent ORF75-mediated PML degradation (Ling et al., 2008) showed similar partial localization of PML and ORF75c (data not shown).

MHV68-induced PML degradation is enhanced by PML SUMOylation but MHV68 does not ubiquitously target SUMO-modified cellular proteins

To address whether SUMO-conjugated PML is required for ORF75c-mediated PML degradation, we first looked at PML during infection in SUMO1$^{-/-}$ cells. SUMO1 did not appear to be required, as PML was degraded by MHV68 in these cells (Fig. 3A). However, the loss of SUMOylation by SUMO1 in these cells can be compensated by SUMO2 or SUMO3 (Evdokimov et al., 2008), so this finding does not exclude a role for SUMOylation in
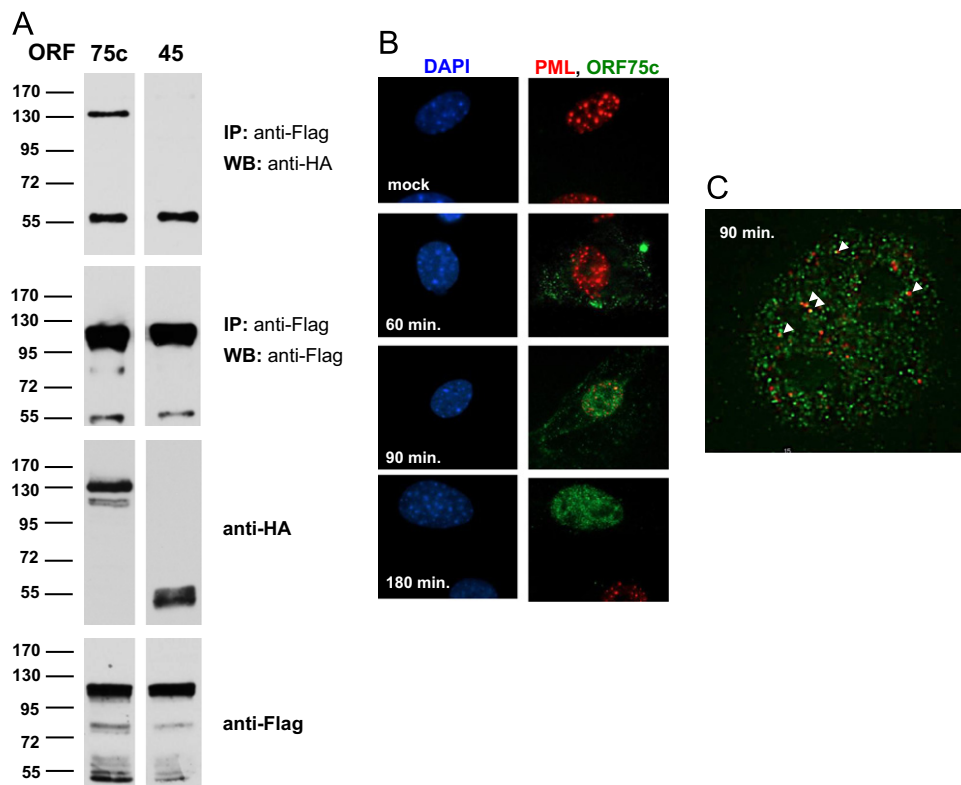

Fig. 2. ORF75c and PML interact weakly and partially colocalize with each other. (A) 293T cells cotransfected with ORF75c-HA or ORF45-HA and mPML-Flag were lysed and precipitated with mouse anti-Flag M2 antibody and then resolved by SDS-PAGE and detected by immunoblotting with anti-HA or anti-Flag M2 antibodies as indicated (top two panels). Three percent of each cell lysate was also resolved by SDS-PAGE as controls for levels of protein expression (bottom two panels). (B) MHV68-infected 3T12 cells were stained for DAPI (blue) and PML (red) or ORF75c (green) at time points indicated in panels on the left. (C) High resolution deconvolution microscopy of MHV68-infected 3T12 cells stained PML (red) and ORF75c (green) at 90 min post-infection. White arrows indicate foci staining for both PML and ORF75c.
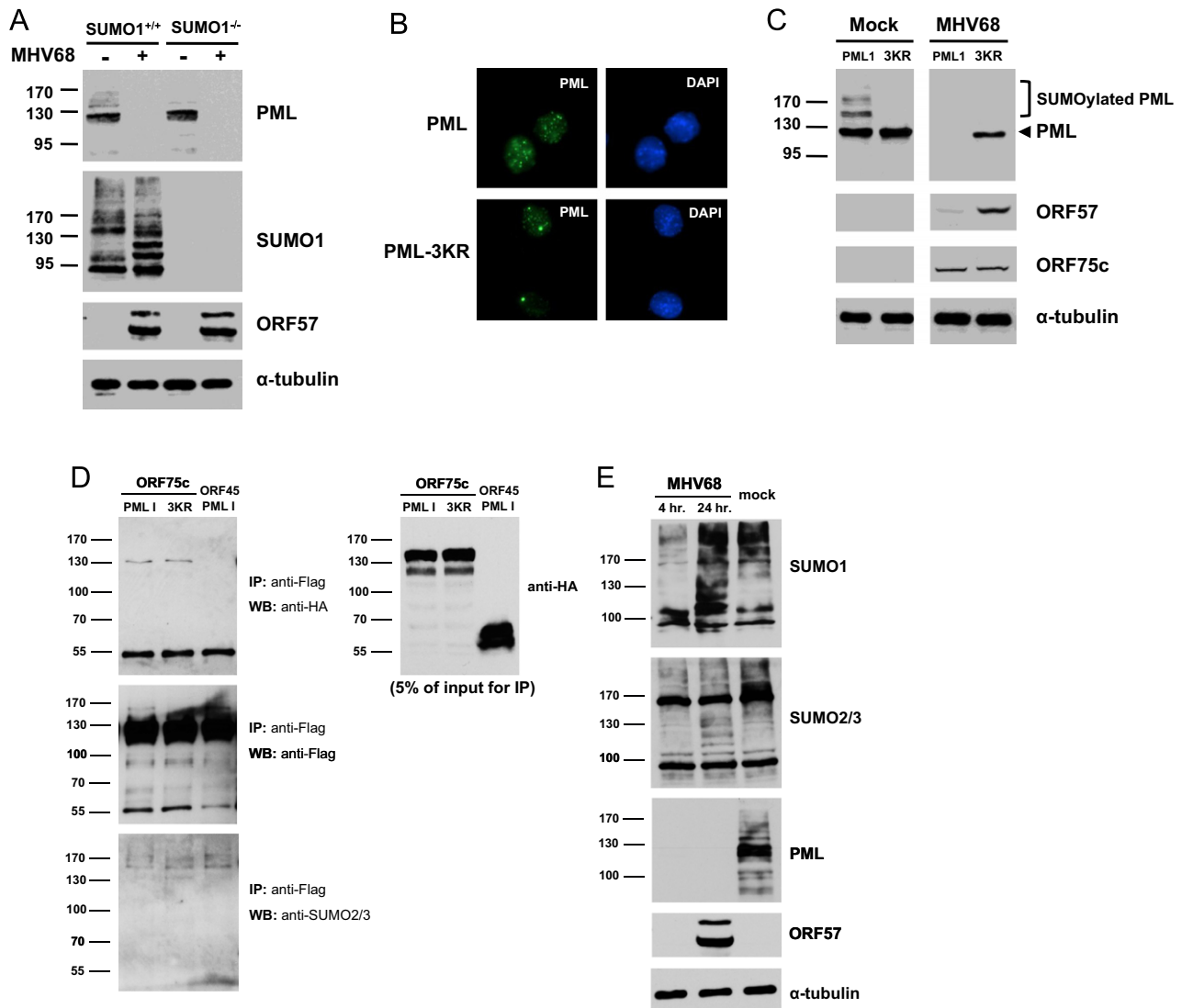
Fig. 3. SUMOylation of PML is required for ORF75c-mediated PML degradation but there is no global degradation of cellular SUMO-conjugates during MHV68 infection. (A) Immunoblot analysis of mock-infected ($-$) or MHV68-infected ($+$) wild-type (SUMO1$^{+/+}$) or SUMO1 knockout (SUMO1$^{-/-}$) fibroblast cells. Proteins were detected with antibodies indicated to the right of each panel. (B) The top two panels show PML staining (green) of mouse fibroblasts expressing wild-type PML and the corresponding DAPI stain (blue) of the same cells. The bottom two panels show PML staining (green) of mouse fibroblasts expressing PML-3KR and the corresponding DAPI stain (blue) of the same cells. (C) Immunoblot analysis of PML1 and PML-3KR cells either mock-infected or infected with MHV68 for 4 h at an MOI of 5. Proteins were detected with antibodies indicated to the right of each panel. (D) Immunoblot analysis of 293T cells cotransfected with ORF75c-HA or ORF45-HA, and PML-Flag or PML3KR-Flag and ubiquitin-Myc. Cells were lysed and precipitated with mouse anti-Flag M2 antibody and then resolved by SDS-PAGE and detected by immunoblotting with anti-HA, anti-Flag M2 or anti-SUMO2/3 antibody as indicated (left panels). Five percent of each cell lysate used for immunoprecipitation was also resolved by SDS-PAGE as controls for levels of HA-tagged protein expression (right panel). Molecular weights are indicated on the left of each blot. (E) Immunoblot analysis of 3T12 cells infected with MHV68 at MOI of 5 for 4 and 24 h. Proteins were detected with antibodies indicated to the right of each panel.

ORF75c-induced PML degradation. To address this issue further, we introduced mutations into the major SUMO-conjugated lysine residues (Lys 65, 160, and 490), which we refer to as PML-3KR (Kamitani et al., 1998; Percherancier et al., 2009). PML$^{-/-}$ cells were transduced to constitutively express wild-type PML isoform I (PML1) or PML-3KR. The PML-3KR protein formed fewer PML nuclear bodies that tended to be much brighter and larger than wild-type PML, which agreed with earlier published reports for this mutant (Lallemand-Breitenbach et al., 2001, 2008; Percherancier et al., 2009; Zhong et al., 2000) (Fig. 3B). The two cell lines were infected with MHV68 and analyzed for PML expression. Unlike wild-type PML, the PML-3KR mutant protein was not completely degraded following MHV68 infection (Fig. 3C), although a modest reduction of PML-3KR levels was consistently observed. To assess whether the differences

in degradation susceptibility are due to potential differences in association between these proteins, we conducted coimmunoprecipitation experiments, similar to those described in Fig. 2A, from cells expressing mutant or wild-type PML proteins. No differences in ORF75c association with PML or PML-3KR was observed (Fig. 3D). HSV-1 has been shown to target most of the SUMO-conjugated cellular proteins for degradation through the activity of ICP0 (Boutell et al., 2011). To determine whether a general effect on cellular SUMO-conjugated proteins also happens in the MHV68 system, we examined SUMO conjugates following MHV68 infection. Unlike HSV-1, both SUMO1 and SUMO2/3 conjugates were not globally degraded following MHV68 infection and one or more SUMO-conjugated protein species even appeared to increase in abundance following infection (Fig. 3E).

ORF75c-mediated PML degradation is not regulated by CK2 phosphorylation of PML or by E6AP

One potential mechanism by which ORF75c mediates PML degradation is through stimulating known cellular pathways that regulate PML protein stability. Previous studies have indicated that CK2-mediated phosphorylation of PML leads to its degradation through a proteasome dependent pathway (Scaglioni et al., 2006). In fact, evidence suggests that disruption of PML NBs by EBV EBNA1 is mediated by stimulating CK2 association with PML (Sivachandran et al., 2010). In addition, the cellular ubiquitin E3 ligase E6AP has also been implicated in regulating PML stability (Louria-Hayon et al., 2009). To address the role of CK2-phosphorylation, we generated a PML mutant plasmid (PML-CK2mut), which destroyed CK2 phosphorylation. PML-CK2mut exhibited a similar localization phenotype as wild-type PML when expressed in 3T12 cells (Fig. 4A). However, the PML-CK2mut was degraded as efficiently as wild-type PML when co-expressed with ORF75c (Fig. 4B). This result was supported by the finding that there was no effect on PML degradation during acute MHV68 infection in 3T12 cells in which about 70% of CK2 activity was lost after being treated with 4,5,6,7-tetrabromobenzotriazole (TBB), a selective CK2 inhibitor (Fig. 4C and D). To address the role of E6AP, PML expression was assessed during infection in E6AP$^{-/-}$ cells. MHV68 infection was sufficient to degrade PML in the absence of E6AP (Fig. 5).

ORF75c induces PML poly-ubiquitination in vivo and ubiquitinates itself in vitro

Although ORF75c induced degradation of PML is dependent on the proteasome, it has not been demonstrated that ORF75c can induce poly-ubiquitination of PML in vivo. To address this issue, ORF75c was co-expressed with PML and Myc-tagged ubiquitin in 293T cells followed by treatment with the proteasome inhibitor MG-132. Compared to co-expression with ORF75b, which does not induce PML degradation (Ling et al., 2008), ORF75c induced a significant increase in conjugation of Myc-tagged ubiquitin under these conditions (Fig. 6A). To determine whether ORF75c might contain ubiquitin E3 ligase activity itself, we expressed His-tagged ORF75c using a baculovirus expression system (Fig. 6B). The protein was purified with a metal binding resin followed by gel filtration (Fig. 6B). Highly purified ORF75c increased the overall level of ubiquitin chains and ubiquitinated proteins in the in vitro ubiquitination reactions (Fig. 6C) and demonstrated self-ubiquitination activity even without E1 and E2 (UbcH5c)
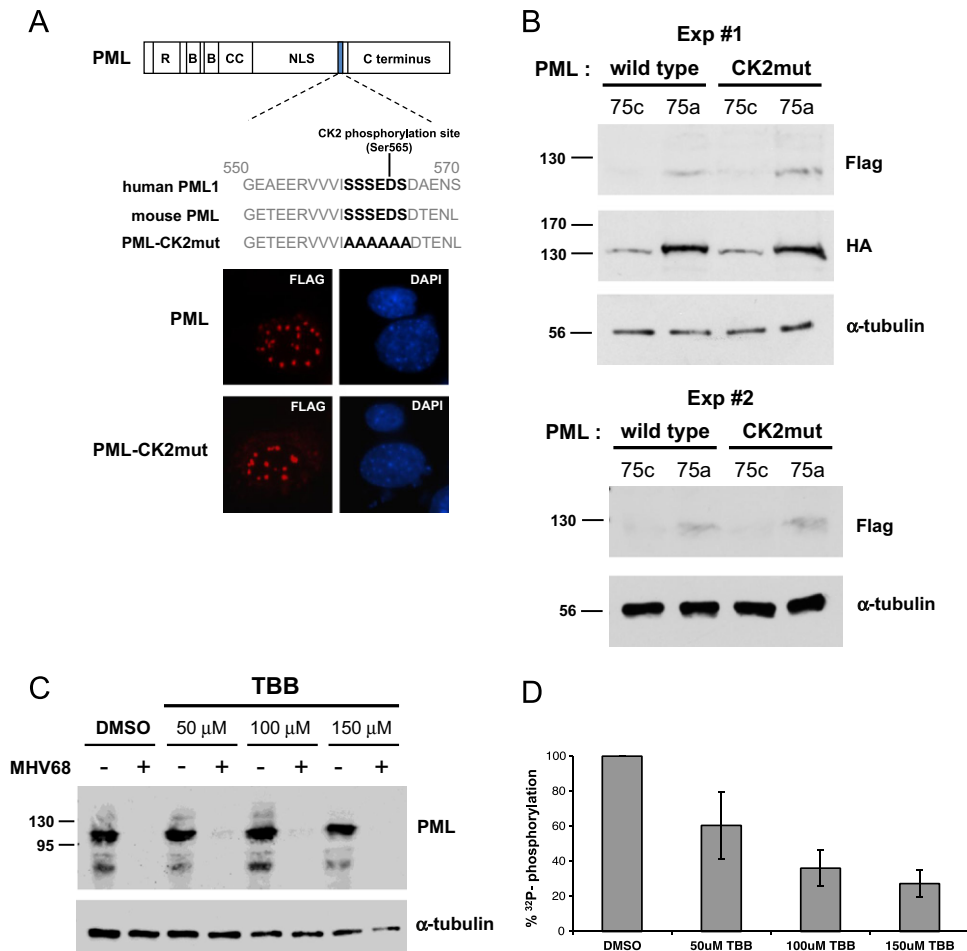

Fig. 4. ORF75c-mediated PML degradation does not require CK2 phosphorylation. (A) Schematic of PML and its conserved CK2 phosphorylation site. Residues mutated are indicated. Panels below show PML staining (red) of wild-type and PML-CK2mut proteins expressed transiently in 3T12 cells. Transiently expressed proteins were detected with anti-Flag antibody. Panels on the right show the same cells stained for DAPI (blue). (B) Immunoblot analysis of 293T cells cotransfected with plasmids encoding HA-tagged ORF75c or ORF75a together with Flag-tagged wild-type PML or the PML-CK2mut as indicated above the panels. Two independent experiments are shown and labeled as experiments 1 and 2 respectively. Proteins were detected with antibodies indicated to the right of each panel. (C) Immunoblot analysis of uninfected (−) or MHV68-infected (+) mouse 3T12 cells treated with various concentration of 4,5,6,7-tetrabromobenzotriazole (TBB) for 1 h before and during infection. Proteins were detected with antibodies indicated to the right of each panel. (D) Lysates from experiments shown in (C) were tested for remaining CK2 activity by their ability to phosphorylate a CK2-specific substrate in vitro. The percent of CK2-phosphorylation for each cell lysate treated with a different concentration of TBB compared to control cells (DMSO treated) is indicated on the y-axis. Standard deviations from three independent experiments are indicated.

enzymes in the reaction (Fig. 6D). The rabbit polyclonal anti-ORF75c antibody was used in Fig. 6D to demonstrate the equal amount of purified ORF75c in each reaction. However, this antibody was unable to detect the ubiquitin-modified forms of ORF75c. Whether ORF75c can ubiquitinate PML has not been done due to the limitation of acquiring sufficient soluble SUMO-conjugated PML protein (data not shown).
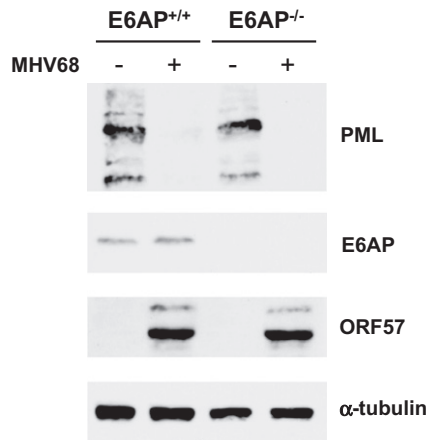
Discussion

This study shows that ORF75c is present during the entire infection cycle, first as protein most likely delivered from infectious virion particles and then later as a gene product whose expression is dependent on viral DNA replication (i.e., a late gene) (Fig. 1). The mechanism by which ORF75c targets PML for degradation appears to occur through weak or transient interactions with PML as evidenced by their association with each other in co-immunoprecipitation assays and partial colocalization in the nucleus (Fig. 2). Furthermore, interaction between these proteins appears to be facilitated by SUMO modification of PML, although ORF75c does not globally target cellular SUMO conjugated proteins for degradation (Fig. 3). While ORF75c does not stimulate the activity of other known regulators of PML protein stability, namely CK2 and E6AP, it does possess an intrinsic ability to ubiquitinate itself in vitro and is sufficient to increase the level of PML ubiquitination in transfected cells (Figs. 4–6). Taken together, the evidence accumulated in this study is consistent
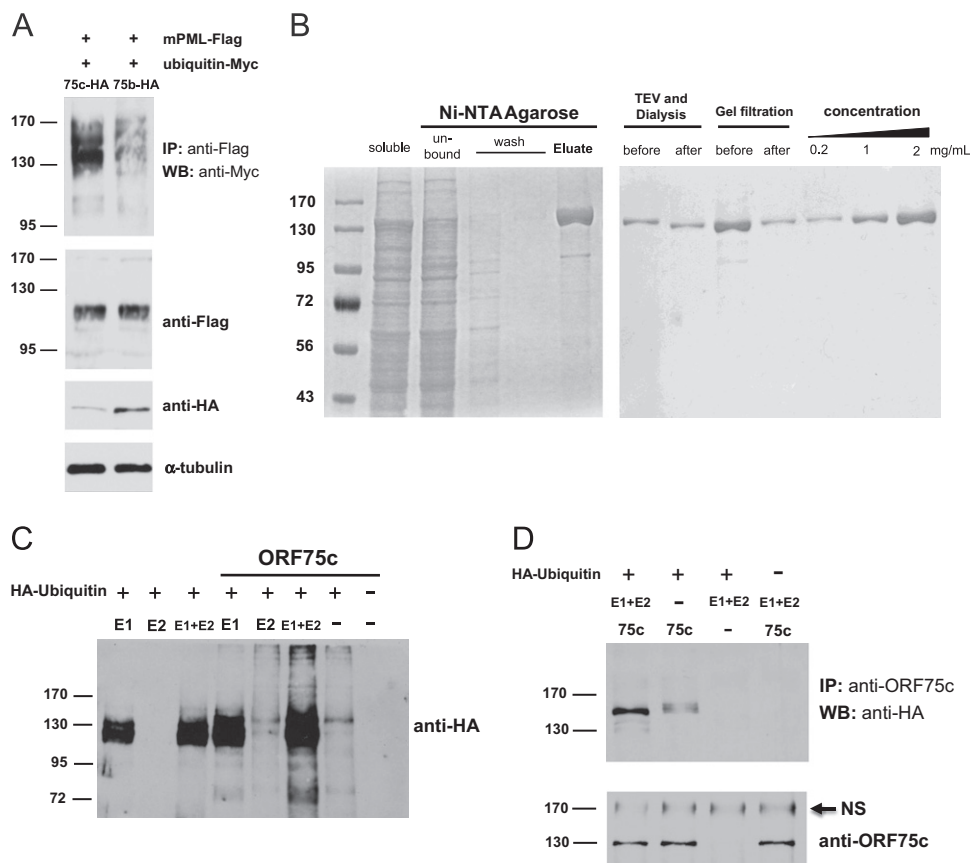

Fig. 5. ORF75c-mediated PML degradation does not require E6AP. Immunoblot analysis of mock-infected (−) or MHV68-infected (+) wild type (E6AP$^{+/+}$) or E6AP knockout (E6AP$^{-/-}$) murine fibroblast cells. Proteins were detected with antibodies indicated to the right of each panel.

Fig. 6. ORF75c mediates poly-ubiquitination of PML in vivo and self-ubiquitination in vitro. (A) Immunoblot analysis of 293T cells cotransfected with ORF75c-HA or ORF75b-HA and PML-Flag and ubiquitin-Myc. Cells were lysed and precipitated with mouse anti-Flag M2 antibody and then resolved by SDS-PAGE and detected by immunoblotting with anti-Myc antibody as indicated (top panel). Levels of protein expression in each lysate were detected by antibodies indicated to the right of each panel (bottom three panels). Molecular weights are indicated on the left of the panels. (B) Coomassie blue staining shows the purification of ORF75c from the baculovirus expression system. Molecular weights are indicated to the left of the panels. (C) Immunoblot analysis of in vitro ubiquitination reactions containing different combinations of E1, E2, HA-ubiquitin, and purified ORF75c indicated above the panel. Proteins were detected with anti-HA antibody. Molecular weights are indicated to the left of the panel. (D) Immunoblot analysis of in vitro ubiquitination reactions precipitated with anti-ORF75c antibody. Reactions containing different combinations of E1, E2, HA-ubiquitin, and purified ORF75c are indicated above the panel. Reactions were precipitated with ORF75c-specific antisera, followed by immunoblot analysis with an anti-HA antibody. The lower panel is an immunoblot probed with anti-ORF75c specific antisera of the lysates prior to immunoprecipitation. This sera often produces a nonspecific band (NS, indicated by the arrow) slightly larger than 170 kDa. Molecular weights are indicated on the left of the panels.

with a model in which ORF75c could mediate direct ubiquitination of PML resulting in its degradation by the proteasome.

Several additional characteristics of ORF75c are consistent with the hypothesis that it is a viral ubiquitin E3 ligase that can target specific proteins for ubiquitination and degradation. Although ORF75c does not contain a recognizable RING-finger domain, it does have a cys-rich region between residues 624 and 693, as noted in our earlier study (Ling et al., 2008). Such regions can be members of the HECT E3 family that typically form thioester bonds with an activated ubiquitin at the catalytic cysteine residue before transfer to target proteins, which in the case of ORF75c, would be consistent with its ability to ubiquitinate itself (Fig. 6) and that has been demonstrated for another gammaherpesvirus protein, KSHV Rta (Yu et al., 2005). Whether SUMO-conjugated PML facilitates an interaction with ORF75c remains unclear, but ORF75c does contain up to 12 consensus SUMO interaction motifs (SIMS) indicating that it has the potential to interact with SUMO-conjugated proteins. As shown elsewhere, the function of ubiquitin E3 ligases that specifically target SUMO-conjugated proteins, known as SUMO Targeted Ubiquitin Ligases (STUbLs), is mediated by their multiple SIMs (Boutell et al., 2011; Tatham et al., 2008). Which ORF75c SIMs might be important for PML degradation, presumably via facilitation of an interaction between these proteins, is unknown and is part of a separate study. However, it is likely that other specific interactions may be required since ORF75c seems to interact with PML-3KR similar to wild-type PML (Fig. 3D), although PML-3KR may still retain low levels of SUMO-conjugation on other lysine residues, which would be consistent with this observation. An alternative possibility is that ORF75c might contain a SUMO E3 ligase activity that increases the level of PML SUMOylation, which in turn fosters enhanced interaction followed by ORF75c-mediated (or possibly an unknown cellular E3 ligase) ubiquitination, leading to PML degradation. The destruction of PML is almost complete by 3 h post-infection (Ling et al., 2008) as shown in Fig. 2B, which is exceedingly rapid compared to cellular pathways that mediate PML degradation (i.e., CK2 phosphorylation or STUbL RNF4) (Lallemand-Breitenbach et al., 2008; Scaglioni et al., 2006) and so at the current time, we favor the model that ORF75c is an E3 ligase that directly modifies PML. Several herpesviruses encoded proteins known to mediate PML degradation or disruption of PML NBs are immediate early genes, suggesting that these interactions are critical early events for establishment of robust infection (Adamson and Kenney, 2001; Ahn and Hayward, 1997; Everett et al., 2006). Consistent with this, ORF75c is delivered from the viral tegument and is able to counteract the presumed antiviral activities of PML NBs at the earliest stages of infection.

Despite mounting evidence supporting the idea that ORF75c is a vial ubiquitin E3 ligase that can target PML, one limitation to this conclusion has been our inability to demonstrate direct polyubiquitination of PML in vitro. At the current time, technical challenges have prohibited production of enough soluble full length SUMO-conjugated PML protein (or the correct post-translational modified forms of PML protein) to test this hypothesis. A second limitation is that we have not fully ruled out participation by the STUbL RNF4, which is known to mediate the degradation of SUMO-modified PML following cellular stress induced by exposure to arsenic (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). Unfortunately, we have not been able to detect murine RNF4 with currently available anti-RNF4 antibodies, which has compromised our ability to evaluate knockdown experiments using either transient transfection of siRNAs or retroviral transduction of shRNAs targeting RNF4 in mouse fibroblast cells (data not shown). Finally, the failure to detect ORF75c-PML interactions via co-immunoprecipitation analysis during viral infection may simply be due to technical constraints,

since overall levels of ORF75c present at early time points post-infection (2–4 h) are low. Attempts to perform these assays at later time points, when additional ORF75c is synthesized, proved to be difficult as the toxic effects of MG-132 were apparent at 12 h post-infection when ORF75c expression begins (data not shown). Notably, despite the discovery of an association of HSV-1 ICP0 with PML almost 20 years ago, it was not until recently that evidence for a physical association between these proteins was demonstrated during HSV-1 infection and this required the use of cells expressing EYFP-PML fusion proteins of single PML isoforms (Cuchet-Lourenço et al., 2012). Similar types of reagents may be needed to examine ORF75c-PML interactions during viral infection.

Gammaherpesviruses have captured and retained cellular FGARAT proteins but their biological functions contributing to viral pathogenesis are relatively uncharacterized. This study shows that ORF75c contains intrinsic ubiquitin E3 ligase activity and preferentially targets SUMO-conjugated PML for ubiquitination and degradation by the proteasome, thereby counteracting this antiviral defense. Taken together, the evidence accumulated in this study provides new insights into the function of a vFGARAT and is consistent with a model in which ORF75c could mediate direct ubiquitination of PML resulting in its degradation by the proteasome. Because MHV68 naturally infects laboratory mice, it provides a robust model system to investigate basic mechanisms of herpesvirus pathogenesis in an animal. This system is ideal for pursuing future studies to interrogate the role of PML during herpesvirus acute infection, latency, and reactivation.

Materials and methods

Cells

Human embryonic kidney 293T cells, NIH 3T12 cells (ATCC), and other murine fibroblast cells used in this study were grown in Dulbeco's Modified Eagle Medium (DMEM)/High Glucose (Hyclone) with 10% fetal bovine serum (Gibco) and $1 \times$ antibiotic-antimycotic (Gibco), and in 5% CO_2 tissue culture incubator at 37 °C. Ube3a$^{-/-}$ (E6AP$^{-/-}$) murine fibroblast cells were kindly provided by Arthur Beaudet (Jiang et al., 1998) and SUMO1$^{-/-}$ murine fibroblast cells were kindly provided by Michael Kuehn (Evdokimov et al., 2008). PML$^{-/-}$ murine fibroblast cells were converted to express PML isoform I containing mutations at all three SUMOylation sites (PML-3KR) using previously described methods (Ling et al., 2008).

Plasmids

Plasmids encoding carboxy-terminal hemagglutinin (HA)-tagged ORF75a, ORF75b and ORF75c in the eukaryotic expression vector pCI have been described previously (Ling et al., 2008). Plasmids encoding carboxy-terminal Flag-tagged wild-type PML isoform I and a CK2-site alanine substitution mutant, SSSED-S560AAAAA (which will be referred to as PML-CK2mut) were generated by PCR using previously described methods (Ling et al., 2008). A cDNA encoding PML isoform I containing Lys-to-Arg substitution mutations at all three SUMOylation sites, K65, K160, and K490 (PML-3KR) (Kamitani et al., 1998) with no epitope tag was PCR amplified and cloned into the murine stem cell virus (MSCV) vector (Clontech) for transduction into PML$^{-/-}$ murine fibroblast cells and PML-3KR with a carboxy-terminal Flag epitope tag was cloned into pCI. A cDNA encoding a carboxy-terminal poly-histidine (6xHis)-tagged ORF75c was generated by PCR and cloned into the pFastBac HT B vector (Invitrogen). This clone was used to generate a recombinant bacmid in

DH10BAC *Escherichia coli* as described in Invitrogen's Bac-to-Bac Expression Kit handbook.

Viruses

MHV68 virus expressing HA-tagged ORF75c was generated by allelic exchange as described previously (Ling et al., 2008). Virus stocks of both wild-type MHV68 and MHV68 expressing HA-tagged ORF75c were generated by transfecting MHV68-bacterial artificial chromosome (BAC) DNA containing wild-type or HA-tagged ORF75c into 3T12 cells. Viruses were harvested as P0 stock when the cytopathic effect (CPE) of transfected cells reached approximately 50% (4–6 days). P1 stocks were derived by infecting large amounts of 3T12 cells with P0 stocks at an MOI of 0.05 and harvested at days 4–6 when the CPE of infected cells reached about 50%. Titers of P1 stocks, which were used for experiments, were determined by plaque assays on 3T12 cells as described previously (Ling et al., 2008). Baculovirus stocks and infected sf9 cell pellets expressing His-tagged ORF75c were made by the Baculovirus/Monoclonal Antibody Core Facility at Baylor College of Medicine.

Antibodies

Polyclonal antibodies against MHV68 ORF75c and ORF57 were generated by immunizing rabbits with recombinant His-tagged ORF75c and ORF57 proteins produced in baculovirus and *E. coli* respectively. To reduce the background signals on immunoblots, these two sera were pre-absorbed with boiled 3T12 cell lysates at 4 °C overnight. A mouse monoclonal antibody (9C7/A6) against MHV68 ORF4 was generously provided by Philip Stevenson (Gillet et al., 2007). Primary mouse monoclonal antibodies used in this study were against α-tubulin (Sigma), HA (Covance), Flag M2 (Sigma), mouse PML (Upstate), UBE3A (BD Biosciences) and Myc (Cell Signaling). Primary rabbit monoclonal antibodies used in this study were against HA (Upstate) and SUMO1 (abcam) and a rabbit polyclonal antibody against SUMO2/3 (abcam). Horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit IgG (Jackson ImmunoResearch laboratories) and Alexa Fluor 594 or 488-conjugated antibodies against mouse or rabbit IgG (Invitrogen) were used according to the manufacturers recommendations.

Immunoblot analysis

Transfected or infected cells were harvested and lysed in hypotonic buffer (20 mM Tris–HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, 10% (v/v) glycerol, 0.05% (v/v) NP-40, 10 mM 2-mercaptoethanol and protease inhibitor cocktail tablets) (Ruzzene et al., 2002). Then, the protein concentration of the lysates was measured by Bradford assay and equal amounts subjected to SDS-PAGE analysis. Proteins were transferred to 0.2 μm nitrocellulose membranes, and then incubated with blocking buffer (5% non-fat dried milk in PBS with 0.01% Tween 20) for 1 h at room temperature. Primary antibody was incubated in blocking buffer diluted 1:10 in PBS at 4 °C overnight. Membranes were washed with PBS for 15 min for 4 times and incubated in secondary antibody in blocking buffer diluted 1:10 in PBS for 40–50 min at room temperature. The blots were then washed with PBS again for 20 min 4 times and developed by using SuperSignal West Pico Chemiluminescent substrate (Thermo).

Immunoprecipitation

2.5×10^6 293 T cells were plated in 10 cm tissue culture dishes 1 day before cotransfection with 6 μg of plasmid expressing HA-tagged ORF75c and 4 μg of plasmid expressing Flag-tagged mouse PML isoform I using Lipofectamine (Invitrogen) according to the manufacturer's protocol. An extra dish was transfected with a pEGFP-C1 plasmid (Clontech) as a control for transfection efficiency. At 24 h post-transfection, 10 μM MG-132 (Calbiochem) was added and cells were harvested 24 h after MG-132 treatment. Cells were washed once with cold 1× PBS and lysed in cold IP buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP-40, 10 μM MG-132, 1 mM DTT and protease inhibitor cocktail tablet) for 1 h. Supernatants were incubated with 1 μL of selected antibody and rotated at 4 °C overnight. The supernatants were centrifuged to eliminate non-specific aggregates and added to 50 μL IP buffer-washed Protein G agarose (Pierce). After gentle rotation at 4 °C for 1 h, the agarose was washed with IP buffer three times and proteins were eluted by 25 μL of 2X SDS sample buffer (0.5 M Tris–HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M DTT and 0.001% (w/v) bromphenol blue). Precipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis as described above but in some cases membranes were treated with Qentix Western Blot Signal Enhancer (Thermo) to enhance protein signals before incubation with blocking buffer.

Immunofluorescence analysis

2.5×10^4 3T12 cells were plated onto coverslips overnight and then infected the next day with MHV68 expressing HA-tagged ORF75c at an MOI of 200. Infected cells were washed at 1 h after infection with 1 ml DMEM and then fixed with 4% formaldehyde at 60, 90, and 180 min after infection and stained as described previously (Ling et al., 2008). Cells treated with DMEM were used as negative control and were fixed at 180 min after treatment. In some experiments 3T12 cells were transfected with expression plasmids as described previously (Ling et al., 2008). Transfected cells were fixed at 24 h after transfection and fixed and stained as described above.

In vitro casein kinase 2 assay

To determine CK2 activity in cells treated with the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) (Calbiochem), cells were lysed in hypotonic buffer containing 1 μM okadaic acid (Calbiochem) to inhibit protein phosphatases and assayed with a Casein Kinases II Assay Kit (Sigma) according to the manufacturer's instructions. Lysates from cells treated with dimethyl sulfoxide (DMSO) (Fisher Bioreagents) were used as baseline controls.

Protein purification

Baculovirus infected cells expressing ORF75c were lysed in Ni-NTA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 350 mM Na-malonate, 10 mM imidazole, 5 mM 2-mercaptoethanol and 10% glycerol) and protein was bound to Ni-NTA agarose beads (Qiagen), eluted in buffer containing 350 mM imidazole and dialysed over night at 4 °C in gel filtration buffer (20 mM Tris pH 8.0, 150 mM NaCl, 350 mM Na-malonate, 1 mM DTT and 10% glycerol) containing TEV protease. The dialyzed protein was applied to a gel filtration column (HiLoad 16/60 Superdex 200 prep grade), and fractions containing ORF75c were collected and concentrated using a centrifugal filter (Amicon Ultracel-30K).

In vitro ubiquitination assay

Reactions were prepared in a total 30 μL, which contained 10× ubiquitin buffer (500 mM Tris–HCl pH 7.5, 1 M NaCl, 100 mM

Acknowledgments

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