

**Title:**

The baculovirus sulfhydryl oxidase Ac92 (P33) interacts with the *Spodoptera frugiperda* P53 protein and oxidizes it in vitro

**Running title:**

Ac92 interacts with SfP53 and oxidizes it in vitro

**Authors:**

Wenbi Wu<sup>1</sup>, Rollie J. Clem<sup>1</sup>, George F. Rohrmann<sup>2</sup>, A. Lorena Passarelli<sup>1\*</sup>

**Author affiliation:**

<sup>1</sup> Molecular, Cellular, and Developmental Biology Program, Division of Biology,  
Kansas State University, Manhattan, KS 66506-4901, USA

<sup>2</sup> Department of Microbiology, Oregon State University, Corvallis, OR 97331-3804

**Corresponding author:**

\*A. Lorena Passarelli

**Phone:** +1 785 532 3195

**Email:** [lpassar@ksu.edu](mailto:lpassar@ksu.edu)

## **Abstract**

The *Autographa californica* M nucleopolyhedrovirus (AcMNPV) sulfhydryl oxidase Ac92 is essential for production of infectious virions. Ac92 also interacts with human p53 and enhances human p53-induced apoptosis in insect cells, but it is not known whether any relationship exists between Ac92 and native p53 homologs from insect hosts of AcMNPV. We found that Ac92 interacted with SfP53 from *Spodoptera frugiperda* in infected cells and oxidized SfP53 in vitro. However, Ac92 did not interact with or oxidize a mutant of SfP53 predicted to lack DNA binding. Silencing *Sfp53* expression did not rescue the ability of an *ac92*-knockout virus to produce infectious virus. Similarly, *ac92* expression did not affect SfP53-stimulated caspase activity or the localization of SfP53. Thus, although Ac92 binds to SfP53 during AcMNPV replication and oxidizes SfP53 in vitro, we could not detect any effects of this interaction on AcMNPV replication in cultured cells.

## **Keywords**

Baculovirus; AcMNPV; Ac92; sulfhydryl oxidase; p53

## **Introduction**

Disulfide bond formation is an important step in the maturation of many proteins in eukaryotic and prokaryotic cells, since disulfide bridges are often vital for protein folding and stability. Disulfide bond formation is frequently catalyzed by sulfhydryl oxidases while their reduction can be mediated by disulfide reductases. The process of sulfhydryl oxidation in prokaryotes and eukaryotes has functional and phenotypic parallels (Sevier & Kaiser, 2002). Eukaryotes mediate redox balance using two main sulfhydryl oxidase families present in two cellular organelles. Proteins that are secreted or trafficked through the mitochondrial intermembrane space are oxidized in the endoplasmic reticulum or mitochondrial intermembrane space, respectively (Fraga & Ventura, 2012; Hakim & Fass, 2010; Herrmann *et al.*, 2009). The mitochondrial Erv (essential for respiration and viability) sulfhydryl oxidase is essential for mitochondrial biogenesis, respiratory chain function, and progression through the cell cycle. Erv-like sulfhydryl oxidases are distributed widely among eukaryotic species and many large double-stranded DNA viruses that assemble in the cytoplasm of infected cells (Fass, 2008; Hakim & Fass, 2010).

Baculoviruses are double-stranded DNA viruses that mainly infect insects and replicate in the nucleus of infected cells. During the baculovirus replication cycle, two types of progeny virions are produced, the budded virus (BV) and the occlusion-derived virus (ODV). Erv family-like sulfhydryl oxidase homologs are present in all sequenced baculovirus genomes (Rohrmann, 2011) and the homolog

encoded by the *Autographa californica* M nucleopolyhedrovirus (AcMNPV), Ac92 (P33), has been demonstrated to have sulfhydryl oxidase activity (Long *et al.*, 2009; Wu & Passarelli, 2010) and is essential for infectious budded virus production and for the formation of multiply enveloped ODV (Nie *et al.*, 2011; Wu & Passarelli, 2010). Viruses with a deletion in *ac92* or a mutation in the sequence CXXC (Wu & Passarelli, 2010), a sulfhydryl oxidase motif important for oxidation in cellular enzymes (Fass, 2008), exhibited similar phenotypes, suggesting a requirement for disulfide bond formation in the proper assembly and propagation of AcMNPV virions. The structure of Ac92 revealed that the arrangement of active-site cysteine residues and bound flavin adenine dinucleotide cofactor is similar to that observed in other Erv family sulfhydryl oxidases (Hakim *et al.*, 2011). Although Ac92 is a functional sulfhydryl oxidase and its enzymatic activity is essential for proper ODV formation and BV production, the target substrate(s) of Ac92 during baculovirus infection is unknown.

A previous study showed that Ac92 interacted with the human tumor suppressor protein p53 and enhanced p53-mediated apoptosis when these proteins were co-expressed in insect cells (Prihod'ko *et al.*, 1999). In both vertebrates and invertebrates, p53 is involved in several cellular processes, including sequence-specific transcriptional activation, cell cycle regulation, activation of DNA repair proteins, and initiation of apoptosis when DNA damage is irreparable. Recently, the p53 homolog in *Spodoptera frugiperda*, SfP53, was characterized and its role during baculovirus infection of Sf9 insect cells was studied. Baculovirus infection

stimulated a DNA damage response, as indicated by increased accumulation of SfP53 protein and other criteria. Consistent with this result, inhibition of the DNA damage response thwarted an increase in SfP53 accumulation. However, silencing *Sfp53* using RNA interference did not affect baculovirus replication or induction of apoptosis by a baculovirus lacking the anti-apoptotic gene *p35*, suggesting that these processes are independent of SfP53 in Sf9 cells (Huang *et al.*, 2011a; Huang *et al.*, 2011b).

Many factors affect the stability of p53, including DNA damage or other stress stimuli, interacting proteins (Lavin & Gueven, 2006), or post-translational modifications such as site-specific phosphorylation, acetylation, ubiquitination (Bode & Dong, 2004), or redox modification of p53 cysteine residues (Kim *et al.*, 2011). Since Ac92 interacts with human p53 when it is expressed in insect cells, it is possible that it also interacts with SfP53, affecting the stability of SfP53 or changing its redox state. However, the relationship between Ac92 and p53 homologs from *S. frugiperda* or other insect hosts of AcMNPV has not been investigated.

To characterize the relationship between Ac92 and SfP53, we performed co-immunoprecipitation to assess interaction relationships, determined protein localization during virus infection, and examined the accumulation of SfP53 and Ac92 in the presence or absence of each protein. Finally, we tested whether SfP53 was an *in vitro* substrate for Ac92 sulfhydryl oxidation, and whether expression of Ac92 affected the ability of SfP53 to induce caspase-mediated apoptosis. Our results

indicate that Ac92 can bind and oxidize SfP53 in vitro, but we did not find an obvious functional relationship between Ac92 and SfP53 in Sf9 cells.

## Results

### *Ac92 interacts with SfP53 in Sf9 cells*

A previous study demonstrated that human p53 interacted with AcMNPV Ac92 (P33) when p53 was expressed from a recombinant baculovirus in SF-21 insect cells (Prihod'ko *et al.*, 1999). To test whether SfP53, the P53 homolog in *Spodoptera frugiperda*, also interacts with Ac92, we performed co-immunoprecipitation experiments using plasmids expressing full-length HA-tagged SfP53 or specific domains or mutants of SfP53, and Flag-tagged Ac92. When HA-SfP53 and Flag-Ac92 were co-expressed in Sf9 cells, Ac92 and SfP53 co-immunoprecipitated, regardless of whether immunoprecipitation was with anti-HA and detection with anti-Flag, or vice versa (Fig. 1A). Next, we used a plasmid that expressed the putative SfP53 DNA binding domain (DBD; amino acids 71-272) to determine if Ac92 interacted with this domain. This helps delineate the interaction region and the possibility that Ac92 prevents DNA binding of SfP53. Ac92 also co-immunoprecipitated with the SfP53 DNA binding domain (Fig. 1B). Mutation of cysteines 155 and 158 of Ac92 (Ac92M1), which eliminate sulfhydryl oxidase activity (Wu & Passarelli, 2010) did not affect the interaction between Ac92 and SfP53 (Fig. 1C), indicating that the interaction spanned a larger domain or involved another Ac92 domain. We note that

although SfP53 antiserum was raised against a His-tagged SfP53, this antiserum or anti-His serum could not immunoprecipitate the Flag-Ac92 protein (from phsFHORF92, data not shown) which is also His-tagged. To test whether mutations within the SfP53 DBD affected the interaction between Ac92 and SfP53, we constructed a plasmid that expressed SfP53 with an arginine to histidine change at amino acid 252. This change abolishes DNA binding in the *Drosophila* p53 ortholog (Brodsky *et al.*, 2000). These two proteins were unable to specifically co-immunoprecipitate; similar amounts of HA-SfP53-R252H were non-specifically pulled down with anti-FLAG regardless of the presence or absence of Flag-Ac92 (Fig. 1D).

The data presented thus far indicate that Ac92 and SfP53 expressed from plasmids transfected into Sf9 cells co-immunoprecipitated, and this interaction was abolished by a mutation in the SfP53 DBD. To determine if endogenous SfP53 could interact with Ac92 expressed during virus infection, we constructed a recombinant AcMNPV bacmid, Ac92FlagRep-PG, to express a FLAG-tagged Ac92 under its natural promoter during virus infection. Since the SfP53 antiserum was raised against HA-His-tagged SfP53 recombinant protein, it cross-reacts with HA-tagged proteins, and so the virally expressed HA-tagged Ac92 was immunoprecipitated by the anti-SfP53 sera. To circumvent this problem, we constructed a recombinant virus called Ac92FlagRep-PG, where the Ac92 protein with a C-terminal Flag tag driven by the *ac92* promoter was reintroduced into an *ac92* deficient background (Fig. 2A).

Virus growth curves were performed to ensure that the Ac92Flag could rescue infectious BV production in Ac92KO. Sf9 cells were infected with the indicated recombinant viruses at a low multiplicity of infection (MOI) of 0.01 or a high MOI of 5 PFU/cell. The recombinant viruses had similar BV production (Fig. 2B), confirming that Ac92Flag expressed from its own promoter in a different genomic location could functionally rescue BV production defects in Ac92KO. When the ability of Ac92Flag to interact with endogenous SfP53 was tested, a positive interaction was detected in reciprocal co-immunoprecipitation experiments (Fig. 1E). This established that endogenous SfP53 interacts with normal levels of Ac92 expressed during virus infection in Sf9 cells.

#### *In vitro oxidation of SfP53 thiols by Ac92*

Ac92 was shown to be a sulfhydryl oxidase based on its homology to cellular and viral sulfhydryl oxidases, its ability to oxidize DTT or reduced thioredoxin, and its crystal structure (Fass, 2008; Long *et al.*, 2009; Wu & Passarelli, 2010). Since Ac92 was detected in a complex with SfP53 in infected cells, we asked whether SfP53 could serve as a substrate of Ac92. SfP53 and the SfP53 DNA binding mutant SfP53-R252H proteins were produced in *E. coli*, partially purified (Fig. 3A), and tested as substrates for Ac92 in vitro. Ac92 was able to oxidize SfP53 sulfhydryl groups in a time-dependent manner and with similar oxidation efficiencies between 5 and 120 min. However, SfP53-R252H, which does not immunoprecipitate with

Ac92, was not oxidized by Ac92 (Fig. 3B). Ac92HAM1, an Ac92 mutant unable to oxidize thiols (Wu & Passarelli, 2010), was used as the negative control to show background activity (Fig. 3B).

#### *Silencing Sfp53 did not rescue Ac92KO-PG BV production*

Since Sfp53 is oxidized by Ac92 in vitro, we speculated that oxidation of Sfp53 may be necessary for efficient virus replication. Thus, in the absence of Ac92 in Ac92KO-PG, the reduced form of Sfp53 may adversely affect BV production. To test this hypothesis, we silenced *Sfp53* expression prior to transfection of Sf9 cells with AcWT-PG or Ac92KO-PG bacmid DNA and monitored BV production. Silencing of Sfp53 was confirmed by immunoblotting cell lysates after dsRNA addition (Fig. 4A). BV produced after transfecting cells with AcWT-PG (Wu & Passarelli, 2010) or Ac92KO-PG DNA were collected to generate growth curves. Cells transfected with AcWT-PG bacmid DNA revealed a steady increase in virus production, as expected (Fig. 4B). However, no infectious BV was produced in cells transfected with Ac92KO-PG bacmid DNA after silencing *Sfp53* or when the non-specific *cat* dsRNA was used (Fig. 4B). Thus, reducing the levels of *Sfp53* did not compensate for the inactivation of Ac92.

#### *Ac92 does not enhance Sfp53-mediated apoptosis in Sf9 cells*

Overexpression of Sfp53 induces caspase activity and apoptosis in Sf9 cells (Huang *et al.*, 2011a). Since Ac92 interacts with and oxidizes thiols in Sfp53, we asked whether

Ac92 affected SfP53-mediated apoptosis. Expression of full-length SfP53 or the SfP53 DBD, pHA-SfP53-DBD, were able to stimulate similar levels of caspase activity (Fig. 5, columns 2 and 3). Although the DNA binding mutant, pHA-SfP53-R252H, was not significantly different from the full-length SfP53, it consistently stimulated reduced levels of caspase activity (Fig. 5, compare columns 2 and 3 to 4). Inhibition by the caspase inhibitor zVAD-FMK demonstrated assay fidelity (columns 5 to 7). Ac92 expression did not stimulate significant caspase activity on its own (columns 8 and 9), nor did it stimulate HA-SfP53-mediated caspase activation above levels induced by SfP53 alone or with an empty vector (Fig. 5, compare column 11 with 10 or 2).

*Ac92 does not affect the cellular localization of SfP53 and partially co-localizes with SfP53 in Sf9 cells*

The interaction between Ac92 and SfP53 after plasmid transfections and during virus infections suggests that at least some of Ac92 and SfP53 co-localize in cells. To confirm this and determine whether Ac92 expression could affect the localization of SfP53 in insect cells, immunofluorescence experiments were performed with anti-SfP53 in plasmid-transfected or virus-infected cells. TO-PRO-3 (red color) was used to visualize DNA in the nucleus, where viral DNA replicates (Fig. 6). In mock-treated cells, SfP53 was localized throughout the nucleus (Fig. 6A). However, in infected cells, SfP53 mainly localized to a central body within the nucleus, which may have been the virogenic stroma. Localization of SfP53 was not affected by the

presence (AcWT-PG-infected) or absence (Ac92KO-PG DNA-transfected) of *ac92* (blue color, Fig. 6A), suggesting that Ac92 did not affect the localization of SfP53 in virus-infected cells. AcWT-PG-infected or Ac92KO-PG DNA-transfected cells were visualized by expression of GFP expressed from the polyhedrin locus (green color, Fig. 6A).

Ac92 localization, visualized by expression of a fusion of Ac92-GFP, was mainly observed in a punctate staining pattern in the cytoplasm around the outside of the nucleus of pAc92GFP-transfected cells. However, in cells infected with Ac92GFP-PH, Ac92-GFP was localized within the nucleus around the viral DNA replication center (ring zone) (Fig. 6B), although more diffuse Ac92-GFP fluorescence was observed throughout the nucleus. Note the presence of occlusion bodies within the nucleus of the cells in the lower bright field image and coinciding with Ac92-GFP localization (Fig 6B, bright field merge). Since Ac92-GFP was mainly cytoplasmic in transfected cells and mainly nuclear in infected cells, it is possible that another viral gene is necessary to localize Ac92-GFP within the nucleus. These results indicate that Ac92 and SfP53 only partially co-localized during infection; the majority of Ac92 was around the inner periphery of the nucleus and did not co-localize with SfP53.

*Overexpression or silencing of Ac92 during virus infection does not affect SfP53 accumulation*

To determine whether expression of Ac92 affected SfP53 accumulation, we overexpressed Ac92 or silenced *ac92* during virus infection. Sf9 cells were transfected with p<sub>h</sub>sFHORF92 (Flag-Ac92) or p<sub>FH</sub>-Ac92M1 (Flag-Ac92M1), expressing a functional or non-functional Ac92, respectively, and the expression of endogenous SfP53 was monitored by immunoblotting using SfP53 antisera. No difference was observed in SfP53 levels between cells transfected with control (pHSGFP) or Ac92-expressing plasmids (p<sub>h</sub>sFHORF92 and p<sub>FH</sub>-Ac92M1) (Fig. 7A). Next, to study the effect of Ac92 overexpression or native expression during virus infection on the accumulation of endogenous SfP53, Sf9 cells were transfected with pHSGFP as a control or Flag-Ac92-expressing plasmid, and at 24 h p.t., cells were infected with Ac92HARep-PG. Thus, cells either expressed Ac92 from virus infection or overexpressed it from virus-infected and plasmid-transfected cells. Cells were collected at different time points and the expression level of SfP53 was determined by immunoblotting. Again, no obvious differences were detected in SfP53 levels when Ac92 was overexpressed during virus infection (Fig. 7B). The HA-tagged Ac92 expressed from the Ac92HARep-PG recombinant virus was also detected with SfP53 antisera because anti-SfP53 was raised against an HA-tagged SfP53 recombinant protein (Huang *et al.*, 2011a) (Fig. 6B).

The effect of silencing *ac92* during virus infection on the accumulation of endogenous SfP53 was also investigated. Sf9 cells were transfected with *ac92* dsRNA 24 h prior to infection with Ac92HARep-PG. The expression of SfP53 in Sf9 cells was not

affected when *ac92* was silenced during virus infection compared to control *cat* dsRNA (Fig. 7C). Note that *ac92* knockdown was also confirmed in the same immunoblot since the SfP53 antisera also recognized HA-tagged Ac92 (Fig. 7C).

#### *Overexpression or silencing of SfP53 during virus infection does not affect Ac92 accumulation*

The effect of SfP53 expression on Ac92 accumulation was also studied. SfP53 was overexpressed by transfection of pHA-SfP53, as confirmed by immunoblotting, but no obvious effects were observed on the accumulation of Ac92 expressed from Ac92HA-Rep-infected cells compared to cells transfected with pHSGFP as a negative control (Fig. 8A). Similarly, when SfP53 was silenced with dsRNA during virus infection, Ac92 accumulation was not affected (Fig. 8B). These results indicate that SfP53 and Ac92 did not affect the expression of each other during virus infection.

## **Discussion**

The Ac92 protein is a functional sulfhydryl oxidase that is required for infectious BV production and for the formation of multiply-enveloped ODV ( Nie *et al.*, 2011; Wu & Passarelli, 2010). Homologs of *ac92* are found in all sequenced baculovirus genomes, further emphasizing the importance of this gene in baculovirus replication (Rohrman, 2011). Previous studies revealed an association between Ac92 and the human p53 protein (Prihod'ko *et al.*, 1999). In this report, we examined the relationship between Ac92 and SfP53, the *S. frugiperda* p53 homolog.

Similar to other well characterized p53 homologs, SfP53 is predicted to encode an activation domain, a central core sequence-specific DNA binding domain, and a multifunctional C-terminal domain (Huang *et al.*, 2011a). A number of proteins interact with the activation domain or the C-terminal domain of p53, including general transcription factors, replication and repair proteins, transcriptional activators, or kinases, but not many proteins appear to bind to the p53 DNA binding domain (Ko & Prives, 1996; Lavin & Gueven, 2006). In addition to cellular proteins, a few virally encoded proteins associate with p53 (Ko & Prives, 1996), *e.g.*, the SV40 large T-antigen (Linzer & Levine, 1979), adenovirus E1b-55-kDa (Sarnow *et al.*, 1982), and human papilloma virus E6 (Werness *et al.*, 1990).

Ac92 interacted with full-length SfP53 and the SfP53 DNA binding domain (SfP53-DBD) in co-immunoprecipitations (Fig. 1A, B and E). We do not know if this complex includes other cellular or viral proteins. The p53 DNA binding domain binds few reported proteins (*e.g.*, Tumor protein p53 binding proteins, TP53BP1 and TP53BP2 (Iwabuchi *et al.*, 1994)), so it is possible that the virus uses Ac92 to block binding of SfP53 to cellular proteins or to prevent its binding to cellular DNA, inhibiting cellular transcriptional activation. In contrast, Ac92 did not interact with SfP53-R252H, which is predicted to be a DNA binding domain mutant based on homology to human and *Drosophila* p53 (Fig. 1D). Interestingly, the Ac92 cysteine mutant, Ac92M1, though unable to catalyze disulfide bond formation, was able to

interact with SfP53 (Fig. 1C), suggesting that the conformation of the protein may not have been significantly altered and/or that the SfP53 cysteines are not involved in the interaction. In contrast, SfP53-R252H, where only one amino acid in the DNA binding domain was changed, was unable to interact with or be oxidized by Ac92. Structural properties may have affected the interaction of SfP53 DNA binding domain and abolished the interaction of SfP53 with Ac92. Curiously, the expression level of SfP53 DNA binding mutant SfP53-R252H was consistently higher than that of the wild-type SfP53 (data not shown). Some of the measured caspase activity (Fig. 5, column 4) may be in part attributed to higher levels of protein in the lysates. It is well known that the expression of human p53 is maintained at a low level by ubiquitin-mediated degradation via the E3 ubiquitin ligase MDM2. Although there are no detectable orthologs of MDM2 in insects, the expression of SfP53 was also low in uninfected Sf9 cells, but its accumulation increased following baculovirus infection or DNA damage (Huang *et al.*, 2011b). Higher accumulation of SfP53-R252H in uninfected Sf9 cells may suggest a more stable and conformationally altered form or poor degradation leading to increased accumulation. Thus, the finding that Ac92 could not interact with or oxidize SfP53-R252H was consistent with this hypothesis.

Oxidation of p53 thiol groups in cysteines is a post-translational modification (Hainaut & Milner, 1993; Rainwater *et al.*, 1995). The DNA binding activity of p53 can be modulated by metal chelators and oxidizing agents. Thiol oxidation disrupts p53 conformation and abolishes DNA binding; conversely, the reduced state of

cysteine residues favors p53 folding into its wild-type conformation and leads to specific DNA binding (Hainaut & Milner, 1993). The roles of individual cysteines in the regulation of p53 function have been investigated by substitution of serine for each cysteine in murine p53. Mutation of cysteines located in the zinc-binding region markedly reduced in vitro DNA binding and blocked transcriptional activation; while a mutation of non-zinc binding cysteines in the DNA-binding domain, partially impaired p53 function (Rainwater *et al.*, 1995). In this study, Ac92 interacted with the SfP53 DNA binding domain and catalyzed SfP53 cysteine oxidation, suggesting Ac92 might inhibit SfP53 function by cysteine oxidation. However, silencing SfP53 did not rescue BV production in Ac92 knockout virus. Thus, the specific function of SfP53 during baculovirus replication and the advantage of its oxidation for the virus remain elusive.

We tested if accumulation of either SfP53 or Ac92 were affected in the presence or absence of the other gene. It has been shown that overexpression of SfP53 in Sf9 cells induces caspase activity (Huang *et al.*, 2011a), but caspase activity was not enhanced by Ac92 (Fig. 5). In contrast, Ac92 stimulated human p53-mediated apoptosis in Sf9 cells (Prihod'ko *et al.*, 1999). SfP53 is a nuclear protein like other p53 homologs. The localization of SfP53 at viral replication centers was not affected by Ac92 (Fig. 6A). When Ac92 was expressed alone, it mainly localized in condensed centers within the cytoplasm around the outside of the nucleus, while expression during baculovirus infection included nuclear foci at the ring zone of infected cells (Fig. 6B). Although

p53 is a nuclear protein, it might function outside the nucleus through protein-protein interactions, *e.g.*, p53 can translocate to the mitochondria and interact with anti-apoptotic proteins such as BCL2 and BCL-X<sub>L</sub> to induce apoptosis (Mihara *et al.*, 2003). We do not know in which cellular compartment Ac92 and SfP53 interact; they might interact in the cytoplasm where there is a high concentration of proteins or in the nucleus where multiple-ODV formation, a process influenced by Ac92, takes place. Both the nucleus and cytoplasm are reducing compartments and the oxidizing function of Ac92 could serve as a functional switch.

Overexpression of Ac92 or silencing *ac92* expression during baculovirus infection did not affect the steady-state expression levels of SfP53 (Fig. 7). We also investigated whether SfP53 affected Ac92 by overexpressing SfP53 or silencing *Sfp53* during virus infection, and found that the expression level of Ac92 was not affected (Fig. 8). Overall, using the assays described, we could not detect any direct effects between the two proteins during virus infection. This is not necessarily surprising since it was confirmed that SfP53 was not responsible for the DNA damage response triggered during baculovirus infection and silencing of *Sfp53* did not affect baculovirus replication in Sf9 cells (Huang *et al.*, 2011b). Although the role of SfP53 during baculovirus replication is not clear, identifying the targets of Ac92 may shed light into the functional requirement of Ac92 during baculovirus replication. In this report, assays that defined a relationship between Ac92 and SfP53 were carried out in vitro or using cell free assays. The correlation between Ac92 and SfP53 in vivo was not

clearly established in this cell line, but the results may be different in other cell types. During a natural infection of an insect, it is possible that the interaction of Sfp53 with Ac92 is important in certain cell types that is not reflected in cultured Sf9 cells. Additional in vitro and in vivo experimentation are required to define more specific functions and establish any dependency or correlation between these genes.

## **Materials and methods**

### *Viruses, cell lines and bacterial strains*

The Sf9 insect cell line, clonal isolate 9 from IPLB-Sf21-AE cells, derived from the fall armyworm *Spodoptera frugiperda*, was purchased from ATCC and cultured at 27° C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), penicillin G (60 µg/ml), streptomycin sulfate (200 µg/ml), and amphotericin B (0.5 µg/ml).

### *Construction of plasmids and recombinant AcMNPV bacmids*

Plasmid pHA-Sfp53 was constructed as previously described (Huang *et al.*, 2011a) in which Sfp53 was HA-tagged at the N terminus and expressed under control of the AcMNPV *ie-1* promoter. To construct the plasmid pHA-Sfp53-R252H, arginine 252 was changed to histidine as described (Chiu *et al.*, 2008) using primers Sfp5352FT (5'-GGTGCCACGTGTGTGCCTGTCCTCGCCGGGACATGGTGA-3'), Sfp5353FS (5'-TGTCCTCGCCGGGACATGGTGA-3'), Sfp5332RT (5'-GGCACACACGTGGGCACCCACTTTCTGACGTCCGTAGACT-3'), and

Sfp5333RS (5'-CACTTTCTGACGTCCGTAGACT-3') and pHA-Sfp53 as the template. PCR products were digested with DpnI (New England Biolabs) for 2 h and denatured at 99 °C for 3 min, followed by annealing during two cycles of 65 °C for 5 min and 30 °C for 15 min. *Escherichia coli* DH5a cells were transformed using the PCR products. The mutation in the final product pHA-Sfp53-R252H was confirmed by DNA sequencing. To construct the plasmid pHA-Sfp53-DBD, containing the predicted Sfp53 DNA binding domain with an N terminus HA tag under control of the AcMNPV *ie-1* promoter, primers Nco.Nde.HA.sfp53-aa71 (5'-CCATGGCACATATGTACCCATACGACGTCCCAGATTACGCCGGCCGACC GCCGCACTTTCCTTC-3') and Sfp53.aa272.Nco (5'-TTATGCCATGGACTTG TAGACACCCTCGGCCGTCTC-3') were used to amplify the Sfp53 DNA binding domain (aa 71-272). The PCR product was cloned into pCR II vector (Invitrogen) and the nucleotide sequence was verified. The resulting plasmid was cut by NcoI to release HA-Sfp53-DBD fragment and the fragment was ligated to pGR09-5.5 (Huang *et al.*, 2011a) to generate the final construct pHA-Sfp53-DBD. The plasmid phsFHORF92 (Prikhod'ko *et al.*, 1999), obtained from the plasmid collection of Dr. Lois K. Miller, contains Ac92 with an N-terminal Flag tag expressed under the control of the *Drosophila* heat shock protein (hsp) 70 promoter. pFH-Ac92M1 was constructed as described (Chiu *et al.*, 2008). Briefly, four primers Ac9255FT (5'-GCAGCCATGGCACGCGATCATTATATGAACGTCA-3'), Ac9256FS (5'-CGCGATCATTATATGAACGTC-3'), and Ac9237RT

(5'-TGCCATGGCTGCCTGTAGTATGAAAAATACGTTG-3'), and Ac9238RS (5'-CTGTAGTATGAAAAATACGTTG-3') were used in PCR amplifications with p<sub>h</sub>sFHORF92 as a template. PCR products were treated with DpnI. The mutation in p<sub>h</sub>FH-Ac92M1 was confirmed by DNA sequencing. To express an Ac92-C-terminal EGFP (enhanced green fluorescent protein) fusion protein under the AcMNPV *ie-1* promoter, pAc92GFP, a plasmid with an N-terminal Flag-tagged Ac92 was used as a template for PCR with primers 5'-GCTAGCGCCATGGACTACAAGGACGACGACGATGACAAAATACCGCTGACGCCGCTTTTTTCTC-3' (with NcoI and NheI sites and a Flag tag) and 5'-TTAGGCCATGGATTGCAAATTTAACAATTTTTTGTATTCTC-3' (with an NcoI site). PCR products were cloned into pCR II and the insert sequenced. The FLAG-Ac92 fragment was released by digesting with NcoI and purified. Plasmid pEGFP (Pearson *et al.*, 2000) was digested with XhoI and Sall to remove a 1-kbp fragment from the 3' regulatory region and self-ligated. This construct was then digested with NcoI and ligated with an NcoI-digested FLAG-Ac92 fragment yielding the final construct pAc92GFP.

The plasmid pHis6-SfP53 (Huang *et al.*, 2011a) contains the SfP53 with a 6X his tag at the N terminus cloned into the pET28a expression vector to express and purify SfP53. To express the SfP53 DNA binding mutant SfP53-R252H in *E. coli*, pET28a-SfP53-R252H was constructed. The vector pET28a was digested by NdeI/HindIII and the fragment gel-purified. pHA-Sfp53-R252H was cut with

NdeI/HindIII and the HA-SfP53-R252H fragment was gel-purified and ligated into pET28a digested with the same enzymes to construct the expression plasmid pET28a-SfP53-R252H. pHSGFP (Crouch & Passarelli, 2002) carries *egfp* under control of the *Drosophila* heat shock protein 70 promoter.

Recombinant bacmids AcWT-PG, Ac92KO-PG, and Ac92HARep-PG were previously described (Wu & Passarelli, 2010). Briefly, AcWT-PG is an AcMNPV-based bacmid containing the *polyhedrin* gene and its promoter and *egfp* under control of the AcMNPV *ie-1* promoter. Ac92KO-PG is similar to AcWT-PG but *ac92* has been deleted. Ac92HARep-PG is based on Ac92KO-PG and contains a C-terminally tagged *ac92* with the *ac92* promoter in the *polyhedrin* locus. Ac92FlagRep-PG and Ac92GFP-PH recombinant bacmids were constructed by introducing *ac92*, a C terminus Flag-tagged or as an EGFP C-terminal fusion, respectively, into the *ac92* deletion bacmid background. Two primers, Ac92510 (5'-GAAGGCCTAATGCACATATGTCTTATAC-3') and Ac92310 (5'-CGCTCTAGATTACTTATCGTCGTCATCCTTGTAATCTTGCAAATTTAACAATTTTTTGTA-3') were designed to amplify a PCR product containing *ac92* with a C-terminal Flag tag and its native promoter. The PCR product was digested with StuI/XbaI and ligated into pFB-PG-pA (Wu & Passarelli, 2010) to generate pFBPG-Ac92Flag. Two primers, GFP5 (5'-TCTAGAGGCAAAGGAGAAGAACTT-3') and GFP3 (5'-CTGCAGTTATTTGTATAGTTCATC-3') were designed to amplify *egfp* without

the start codon from the template pFB1-PH-GFP (Wu *et al.*, 2006). The PCR fragment was cut with PstI/XbaI and ligated into the pFB-PHR (Hu *et al.*, 2010) to generate the plasmid pFB-PHR-GFP. Primers Ac9252 (5'-GAGCTCAATGCACATATGTCTTATAC-3') and Ac9231 (5'-GGCTCTAGATTGCAAATTTAACAATT-3') were designed to amplify a fragment that contained the *ac92* promoter and open reading frame without the stop codon and then the fragment was cut with SacI/XbaI and ligated to pFB-PHR-GFP to generate pFB-PHR-Ac92GFP. Electrocompetent *E. coli* DH10B cells containing helper plasmid pMON7124 and the Ac92KO bacmid (Wu & Passarelli, 2010) were transformed with the donor plasmid pFBPG-Ac92Flag or pFB-PHR-Ac92GFP to generate the Ac92FlagRep-PG or Ac92GFP-PH recombinant bacmids, respectively. Bacmid DNA was isolated and successful transposition was confirmed by PCR. The correct recombinant bacmids were electroporated into DH10B cells and screened for tetracycline sensitivity to ensure that the isolated bacmids were free of helper plasmids. Bacmid DNA was extracted and purified with QIAGEN Large-Construct Kit and quantified by optical density.

#### *Plasmid or bacmid DNA transfections and viral growth curve analysis*

To transfect plasmid or bacmid DNA, DNAs were mixed with a non-commercial liposome reagent and added to Sf9 cells as previously described (Crouch & Passarelli, 2002). Cells were incubated with the DNA/liposome mixture at 27° C for 5 h and then washed twice with TC-100 media, followed by addition of TC-100 media containing

10% fetal bovine serum (designated as the zero time point) and incubation at 27° C.

To generate viral growth curves, Sf9 cells ( $1.0 \times 10^6$  cells/35 mm-diameter dish) were transfected with bacmid DNA as described above. The supernatants of the transfected cells containing budded virions were collected at various time points to determine titers by TCID<sub>50</sub> end-point dilution assays (O'Reilly *et al.*, 1992) on Sf9 cells.

#### *Co-immunoprecipitation assays*

Co-immunoprecipitations were done as previously described (Lehiy *et al.*, 2013). Sf9 cells in 60-mm-diameter culture dishes were transfected as described above with 5 µg of the indicated plasmids expressing Flag- or HA-tagged genes. At 20 h post transfection (p.t.), cells were heat-shocked for 30 min at 42° C and harvested after 4 h. Alternatively, Sf9 cells in 60-mm-diameter culture dishes were infected with Ac92FlagRep-PG virus at a MOI of 5 PFU/cell and harvested at 48 h p.i. Transfected or infected cells were pelleted at 2,000 ×g for 3 min and lysed with NP-40 IP/lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1% NP-40) and two cycles of freeze-thawing. The lysate was clarified by centrifugation at 10,000 ×g for 5 min and precleared by adding 50 µl of a 50% slurry of protein G beads (Sigma), followed by incubation at 4° C for 1 h with rolling. The supernatant was then transferred to a new microcentrifuge tube and mixed with the indicated antibody. After incubation at 4° C for 3 h with rolling, 50 µl of a 50% slurry of protein G beads was added to the mixture and incubated for another 1 h at 4° C with rolling.

The beads were collected by centrifugation and washed five times with IP/lysis buffer for 15 min each. Following addition of 25  $\mu$ l of 2 $\times$  Protein Loading Buffer (PLB, 0.25 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.02% bromophenol blue), the samples were heated at 100 $^{\circ}$  C for 5 min. Samples were immediately analyzed by immunoblotting or stored at -20 $^{\circ}$  C until use.

### *Immunoblotting*

Cells were collected and washed with phosphate-buffered saline (PBS), pH 6.2 (Potter & Miller, 1980), resuspended in PBS with an equal volume of 2 $\times$  PLB, and incubated at 100 $^{\circ}$  C for 5 min. Protein samples were resolved by SDS-12% PAGE, transferred onto a PVDF membrane (MILLIPORE), and probed with one of the following primary antibodies: (i) mouse monoclonal anti-HA-HRP antibody (Cell Signaling); (ii) mouse monoclonal anti-FLAG antibody (Sigma); or (iii) rabbit polyclonal antisera raised against Sfp53 (Huang *et al.*, 2011a), followed by incubation with horse radish peroxidase-conjugated secondary antibodies (Sigma), as needed. Blots were developed using SuperSignal West Pico Chemiluminescent substrate (Pierce) and exposed to X-ray film.

### *Protein purification and sulfhydryl oxidase activity assays*

His-tagged Sfp53 or Sfp53-R252H protein was produced from plasmid pHis6-Sfp53 or pET28a-Sfp53-R252H, respectively, in *E. coli* strain BL21, using protein expression and purification methods as previously described (Wu & Passarelli, 2010).

Purified proteins were stored at -80° C and used as substrates for sulfhydryl oxidase activity. Sulfhydryl oxidase activity assays were carried out as previously described (Long *et al.*, 2009; Wu & Passarelli, 2010). Briefly, purified Sfp53 or Sfp53-R252H protein was incubated with Ac92HA or Ac92HAM1 protein at 37° C, and at designated time points, aliquots were mixed with Ellman's reagent (5',5'-dithio-bis(2-nitrobenzoic acid, DTNB, Sigma) and thiol content was measured by absorbance at 412 nm, calculating an extinction coefficient of 13.6 mM<sup>-1</sup>cm<sup>-1</sup>.

#### *Gene silencing by RNA interference (RNAi)*

RNAi was performed as previously described (Huang *et al.*, 2011b). Full-length open reading frame sequences were used as templates for PCR, with the T7 promoter sequence TAATACGACTCACTATAGGG added at each 5' end. Primers T7-CAT-dsRNA-F

(5'-TAATACGACTCACTATAGGGTATCCCAATGGCATCGTAAAGAACA-3') and T7-CAT-dsRNA-R

(5'-TAATACGACTCACTATAGGGACAAACGGCATGATGAACCTGAAT-3') were used to amplify chloramphenicol acetyl transferase (*cat*) template; primers

T7-Ac92-dsRNA-F

(5'-TAATACGACTCACTATAGGGACTTATAACATTTTGC GTGTTTGAT-3') and T7-Ac92-dsRNA-R

(5'-TAATACGACTCACTATAGGGATCGTTAATGTGGTTGTGAAAAGTC-3')

were used to amplify *ac92* template; primers T7-Sfp53-dsRNA-F

(5'-TAATACGACTCACTATAGGGATCGTTCACCAGGCGGGCTACC-3') and T7-Sfp53-dsRNA-R

(5'-TAATACGACTCACTATAGGGCCATGTCCACGTTCCCGCAGTAGT-3') were used to amplify *Sfp53* template. These templates were used to produce double stranded RNAs (dsRNAs) by in vitro transcription using the AmpliScribe T7 High Yield Transcription Kit (Epicentre), according to the protocol of the manufacturer. dsRNA (150 µg) was delivered into Sf9 cells by transfection to silence expression of *ac92* or *Sfp53*, with *cat* dsRNA used as a negative control.

#### *Caspase activity*

Sf9 cells were seeded in 35-mm-diameter culture dishes and transfected with 3 µg of the indicated plasmids. Cells were heat-shocked at 20 h p.t. to increase hsp70-Ac92 expression and were collected 4 h after heat shock. Caspase activity was measured using the mammalian caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethylcoumarin) (DEVD-AFC; MP Biomedicals) as described previously (Wang *et al.*, 2008). To inhibit caspase activity, the caspase inhibitor zVAD-FMK (MP Biomedicals) was added at a final concentration of 20 µM.

#### *Immunofluorescence*

Sf9 cells ( $1 \times 10^6$ ) were seeded on coverslips in 35-mm-diameter culture dishes and transfected with 3 µg of pAc92GFP plasmid, with 1 µg of Ac92KO-PG bacmid DNA,

or infected with AcWT-PG or Ac92GFP-PH recombinant virus at an MOI of 1 PFU/cell. At 24 h p.t. or p.i., the supernatant was removed and the cells were washed twice with PBS, pH 6.2 (Potter & Miller, 1980), and fixed in 2.5% formaldehyde in PBS for 10 min at room temperature (RT). The fixed cells were washed three times in PBS for 5 min per wash, followed by permeabilization in 0.1% NP-40 (Sigma) in PBS for 10 min at RT. Cells were washed three times in PBS for 5 min per wash before incubation with blocking buffer (5% BSA, 0.3% Triton-100 in PBS) for 1 h at RT, followed by incubation with Sfp53 anti-sera (1:500) in PBS with 1% BSA, 0.3% Triton X-100 overnight at 4° C. Cells were washed three times in blocking buffer for 5 min each, followed by 1 h incubation with an Alexa Fluor 555-conjugated goat anti-rabbit antibody (1:1000) in the dark at RT. Cells were washed three times for 5 min each in PBS, followed by incubation with 1  $\mu$ M TO-PRO-3 (Invitrogen) in PBS for 15 min at RT. The cells were subsequently washed three times for 10 min each in PBS. Coverslips were mounted on a glass slide with Fluoromount-G (SouthernBiotech) and stored at 4° C in the dark until examined with a Carl Zeiss LSM 5 PASCAL Laser Scanning Confocal Microscope.

### **Acknowledgments**

We thank Ning Huang for helpful experimental advice and providing reagents. This work was supported by the NIH award 5R01AI091972-02 to R.J.C. and A.L.P. This is contribution number 13-394-J from the Kansas Agricultural Experiment Station.

## References

- Bode, A. M. & Dong, Z. (2004).** Post-translational modification of p53 in tumorigenesis. *Nat. Rev. Cancer* **4**, 793-805.
- Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M. & Abrams, J. M. (2000).** Drosophila p53 binds a damage response element at the reaper locus. *Cell* **101**, 103-113.
- Chiu, J., Tillett, D., Dawes, I. W. & March, P. E. (2008).** Site-directed, ligase-independent mutagenesis (SLIM) for highly efficient mutagenesis of plasmids greater than 8kb. *J. Microbiol. Methods* **73**, 195-198.
- Crouch, E. A. & Passarelli, A. L. (2002).** Genetic requirements for homologous recombination in *Autographa californica* nucleopolyhedrovirus. *J. Virol* **76**, 9323-9334.
- Fass, D. (2008).** The Erv family of sulfhydryl oxidases. *Biochimica et Biophysica Acta-Molecular Cell Research* **1783**, 557-566.
- Fraga, H. & Ventura, S. (2012).** Protein oxidative folding in the intermembrane mitochondrial space: more than protein trafficking. *Current Protein & Peptide Science* **13**, 224-231.
- Hainaut, P. & Milner, J. (1993).** Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer Res.* **53**, 4469-4473.
- Hakim, M. & Fass, D. (2010).** Cytosolic disulfide bond formation in cells infected with large nucleocytoplasmic DNA viruses. *Antioxid. Redox Signal.* **13**, 1261-1271.
- Hakim, M., Mandelbaum, A. & Fass, D. (2011).** Structure of a baculovirus sulfhydryl oxidase, a highly divergent member of the erv flavoenzyme family. *J. Virol* **85**, 9406-9413.
- Herrmann, J. M., Kauff, F. & Neuhaus, H. E. (2009).** Thiol oxidation in bacteria, mitochondria and chloroplasts: Common principles but three unrelated machineries? *Biochimica et Biophysica Acta-Molecular Cell Research* **1793**, 71-77.
- Hu, Z., Yuan, M., Wu, W., Liu, C., Yang, K. & Pang, Y. (2010).** *Autographa californica* multiple nucleopolyhedrovirus ac76 is involved in intranuclear microvesicle formation. *J. Virol.* **84**, 7437-7447.
- Huang, N., Clem, R. J. & Rohrmann, G. F. (2011a).** Characterization of cDNAs encoding p53 of *Bombyx mori* and *Spodoptera frugiperda*. *Insect Biochem. Mol. Biol.* **41**, 613-619.
- Huang, N., Wu, W., Yang, K., Passarelli, A. L., Rohrmann, G. F. & Clem, R. J. (2011b).** Baculovirus infection induces a DNA damage response that is required for efficient viral replication. *J. Virol.* **85**, 12547-12556.
- Iwabuchi, K., Bartel, P. L., Li, B., Marraccino, R. & Fields, S. (1994).** Two cellular proteins that

- bind to wild-type but not mutant p53. *Proc. Natl. Acad. Sci. U S A* **91**, 6098-6102.
- Kim, D. H., Kundu, J. K. & Surh, Y. J. (2011).** Redox modulation of p53: mechanisms and functional significance. *Mol. Carcinog.* **50**, 222-234.
- Ko, L. J. & Prives, C. (1996).** p53: Puzzle and paradigm. *Genes & Development* **10**, 1054-1072.
- Lavin, M. F. & Gueven, N. (2006).** The complexity of p53 stabilization and activation. *Cell Death and Differentiation* **13**, 941-950.
- Lehiy, C. J., Wu, W., Berretta, M. F. & Passarelli, A. L. (2013).** *Autographa californica* M nucleopolyhedrovirus open reading frame *109* affects infectious budded virus production and nucleocapsid envelopment in the nucleus of cells. *Virology* **435**, 442-452.
- Linzer, D. I. & Levine, A. J. (1979).** Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17**, 43-52.
- Long, C. M., Rohrmann, G. F. & Merrill, G. F. (2009).** The conserved baculovirus protein p33 (Ac92) is a flavin adenine dinucleotide-linked sulfhydryl oxidase. *Virology* **388**, 231-235.
- Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P. & Moll, U. M. (2003).** p53 has a direct apoptogenic role at the mitochondria. *Mol. Cell* **11**, 577-590.
- Nie, Y., Fang, M. & Theilmann, D. A. (2011).** *Autographa californica* multiple nucleopolyhedrovirus core gene *ac92* (*p33*) is required for efficient budded virus production. *Virology* **409**, 38-45.
- O'Reilly, D. R., Miller, L. K. & Luckow, V. A. (1992).** *Baculovirus expression vector: a laboratory manual*. New York, USA: Freeman, W.H. and Co.
- Pearson, M. N., Groten, C. & Rohrmann, G. F. (2000).** Identification of the *Lymantria dispar* nucleopolyhedrovirus envelope fusion protein provides evidence for a phylogenetic division of the Baculoviridae. *J. Virol.* **74**, 6126-6131.
- Potter, K. N. & Miller, L. K. (1980).** Transfection of two invertebrate cell lines with DNA of *Autographa californica* nuclear polyhedrosis virus. *J. Invertebr. Pathol.* **36**, 431-432.
- Prikhod'ko, G. G., Wang, Y., Freulich, E., Prives, C. & Miller, L. K. (1999).** Baculovirus p33 binds human p53 and enhances p53-mediated apoptosis. *J. Virol.* **73**, 1227-1234.
- Rainwater, R., Parks, D., Anderson, M. E., Tegtmeyer, P. & Mann, K. (1995).** Role of cysteine residues in regulation of p53 function. *Mol. Cell Biol.* **15**, 3892-3903.
- Rohrmann, G. F. (2011).** *Baculovirus Molecular Biology*. Bethesda, MD: National Library of Medicine (US), National Center for Biotechnology Information.
- Sarnow, P., Ho, Y. S., Williams, J. & Levine, A. J. (1982).** Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. *Cell* **28**, 387-394.

- Sevier, C. S. & Kaiser, C. A. (2002).** Formation and transfer of disulphide bonds in living cells. *Nat. Rev. Mol. Cell Biol.* **3**, 836-847.
- Wang, H., Blair, C. D., Olson, K. E. & Clem, R. J. (2008).** Effects of inducing or inhibiting apoptosis on Sindbis virus replication in mosquito cells. *J. Gen. Virol.* **89**, 2651-2661.
- Werness, B. A., Levine, A. J. & Howley, P. M. (1990).** Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**, 76-79.
- Wu, W., Lin, T., Pan, L., Yu, M., Li, Z., Pang, Y. & Yang, K. (2006).** *Autographa californica* multiple nucleopolyhedrovirus nucleocapsid assembly is interrupted upon deletion of the 38K gene. *J. Virol.* **80**, 11475-11485.
- Wu, W. & Passarelli, A. L. (2010).** *Autographa californica* multiple nucleopolyhedrovirus Ac92 (ORF92, P33) is required for budded virus production and multiply enveloped occlusion-derived virus formation. *J. Virol.* **84**, 12351-12361.

## Figure legends

### Fig. 1. Coimmunoprecipitation of Ac92 and SfP53.

Sf9 cells were cotransfected with plasmids (A-D) or infected (E) with virus as indicated to the left. At 24 h p.t. or 48 h p.i., cells were collected and lysed for immunoprecipitation using the antibodies indicated above each lane. Transferred proteins were probed with the indicated antibodies at the top. Input, input cell lysates; IP/Ctrl. IgG, control immunoprecipitation with mouse IgG; IP/anti-FLAG, immunoprecipitation with anti-FLAG antibody; IP/anti-HA, immunoprecipitation with anti-HA antibody; IP/PI-serum, immunoprecipitation with SfP53 preimmune sera; IP/anti-SfP53, immunoprecipitation with SfP53 antisera. Plasmid names: Flag-Ac92, p<sub>h</sub>sFHORF92; HA-SfP53, p<sub>h</sub>HA-SfP53; HA-SfP53-DBD, p<sub>h</sub>HA-SfP53-DBD; Flag-Ac92M1, p<sub>h</sub>FH-Ac92M1; HA-SfP53-R252H, p<sub>h</sub>HA-SfP53-R252H

### Fig. 2. Construction of recombinant bacmids and analysis of BV production in Sf9 cells.

(A) Schematic diagram of Ac92FlagRep-PG and Ac92GFP-PH showing *polyhedrin* (*polh*) and *enhanced green fluorescence protein* (*egfp*) inserted into the *polh* locus by

*Tn7*-mediated transposition. (B) BV production measured by TCID<sub>50</sub> endpoint dilution assays. Titers were determined by TCID<sub>50</sub> endpoint dilution from supernatants of cells infected with AcWT-PG, Ac92FlagRep-PG or Ac92GFP-PH at MOI of 0.01 or 5 PFU/cell and collected at the designated time points.

**Fig. 3.** Protein purification and sulfhydryl oxidase assay.

(A) His-tagged SfP53 (left panel) or SfP53-R252H protein (right panel) was expressed in *E. coli*, partially purified, and analyzed by SDS-PAGE followed by Coomassie Blue staining. Sup, supernatant; M, markers (B) Sulfhydryl oxidase assays were carried out with purified SfP53 or SfP53-R252H as substrate and purified Ac92HA. Representative results of three independent repeats are shown. Ac92 cysteine mutant protein Ac92HAM1 was used as a negative control. The measurement and calculation of free thiol groups is described in Materials and methods.

**Fig.4.** Silencing of *Sfp53* during transfection of Ac92KO-PG DNA does not rescue the BV production defect in Ac92KO-PG.

(A) Sf9 cells were transfected with either control *cat* or *Sfp53* dsRNA, and 24 h p.t. transfected with Ac92KO-PG bacmid DNA. Cell lysates were prepared at the indicated times h p.t. and immunoblotted for Sfp53. AcWT-PG DNA was also transfected as a control. (B) Budded virus was collected and the titers were determined by TCID<sub>50</sub> endpoint dilution. Bars show means and standard errors from three independent experiments.

**Fig. 5.** Caspase activity measurement.

Sf9 cells were (co)transfected with the indicated plasmid(s). At 24 h p.t. cells were collected and the lysates were assayed for caspase activity. Fluorescence intensity is shown relative to that of cells transfected with pBlueScript II SK(+) (BS). Bars indicate standard errors of three independent experiments. Plasmid abbreviations are specified in the legend to Fig. 1.

**Fig. 6.** Localization of SfP53 and Ac92 by immunofluorescence staining.

Sf9 cells were mock- (mi) or virus-infected (AcWT-PG or Ac92GFP-PH), or transfected with the indicated viral (Ac92KO-PG) or plasmid (pAc92GFP) DNA, and SfP53 and Ac92 were localized by confocal microscopy. TO-PRO-3 was used to stain nuclei of the cells (red). SfP53 was detected by anti-SfP53 antisera (blue). (A) SfP53 localization was not affected by Ac92. GFP expression (green) is shown in virus-infected or viral DNA transfected Sf9 cells. (B) Localization of SfP53 and Ac92 in the presence or absence of baculovirus infection. Ac92 was expressed as a C-terminal EGFP fusion and its localization detected by visualizing GFP (green). The merge shows a merge of the TOPRO-3, SfP53 and Ac92GFP images, and the bright field merge includes the bright field image in the merged images. Arrows indicate occlusion bodies in the nucleus of infected cells.

**Fig. 7.** Effects of Ac92 on the accumulation of SfP53.

(A) Expression of Ac92 does not affect SfP53 levels. Sf9 cells were transfected with the indicated plasmids, and at 24 h p.t., cells were collected and lysates were immunoblotted with anti-SfP53 antisera to detect expression levels of SfP53, and with anti-FLAG to confirm the expression of Ac92 or the Ac92 cysteine mutant, Flag-Ac92M1. The asterisk indicates a protein band that can serve as a loading control. (B) Overexpression of Ac92 during baculovirus infection does not affect SfP53. Sf9 cells were transfected with pHSGFP (GFP) as a negative control or phsFHORF92 (Flag-Ac92) and at 24 h p.t., cells were infected with Ac92HARep-PG. Cells were collected and lysates were immunoblotted with anti-SfP53 antisera. Since anti-SfP53 antisera were raised against HA-tagged SfP53 recombinant protein, it also recognized HA-tagged Ac92. (C) Silencing *ac92* during baculovirus infection does not affect SfP53. Sf9 cells were transfected with either control *cat* or *ac92* dsRNA, at 24 h p.t., cells were infected with Ac92HARep-PG. Cell lysates were prepared at the indicated h p.i. or h p.t. and immunoblotted with anti-SfP53 antisera. The data shown are representative of three independent experiments.

**Fig. 8.** Overexpression of SfP53 during baculovirus infection does not affect Ac92.

(A). Sf9 cells were transfected with pHA-SfP53 (HA-SfP53) to overexpress SfP53 or pHSGFP (GFP) as a negative control. At 24 h p.t., cells were infected with Ac92HARep-PG. Cells were collected and lysates were immunoblotted with anti-SfP53 antisera. (B) Silencing *Sfp53* during baculovirus infection does not affect Ac92. Sf9 cells were transfected with either control *cat* or *Sfp53* dsRNA. At 24 h p.t., cells were infected with Ac92HARep-PG. Cell lysates were prepared at the indicated times (in hours) post-infection (h p.i.) or post-transfection (h p.t.) and immunoblotted with anti-SfP53 antisera. The data shown are representative of three independent experiments.