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Modification of Human Papillomavirus Minor Capsid Protein L2 by Sumoylation[▽]

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The human papillomavirus (HPV) minor capsid protein L2 plays important roles in the generation of infectious viral particles and in the initial steps of infection. Here we show that HPV-16 L2 protein is sumoylated at lysine 35 and that sumoylation affects its stability. Interestingly, the sumoylated form of L2 cannot bind to the major capsid protein L1, suggesting a mechanism by which capsid assembly may be modulated in an infected cell. Additionally, L2 appears to modulate the overall sumoylation status of the host cell. These observations indicate a complex interplay between the HPV L2 protein and the host sumoylation machinery.

Posttranslational modification is a central method of diversifying protein function. Ubiquitin-like proteins such as SUMO (small ubiquitin-related modifier) are known to be key regulators of several biological functions (31). In humans, at least three SUMO forms (SUMO1, -2, and -3) are expressed. SUMO2 and SUMO3 (here called SUMO2/3) are closely related, sharing 97% identity, whereas SUMO1 shares 43% identity with SUMO2/3. SUMO modification exerts a variety of effects on its targets, altering a target's cellular localization, its stability, its ability to interact with other proteins, and its activity (31). Most known sumoylation targets are transcription factors or other proteins involved in chromatin structure, regulation, and expression (12), supporting a fundamental role for this modification system in regulating cellular homeostasis. Hence, it is no surprise that viral proteins can exploit the host sumoylation system; proteins from both RNA and DNA viruses have been shown to be sumoylated and/or to interact with the sumoylation machinery (8). The viruses that exhibit interplay between sumoylation and viral proteins can be divided into two groups: viruses that have their proteins sumoylated and viruses whose proteins directly modify host sumoylation. In both cases, the outcome is a cellular environment more favorable for viral replication (5, 8).

Human papillomavirus (HPV) infects both mucosal and cutaneous epithelia, and certain high-risk HPV types are the causative agents of cervical cancer (11). Two structural proteins, L1 and L2, form the papillomavirus capsid (16). The minor capsid protein L2 plays a critical role in the generation of infectious viral particles and in early events of HPV infection (13), although its precise functions in HPV entry, intracellular trafficking, endosomal escape, and the nuclear import of the HPV genome have not been fully elucidated (21).

Previous studies have shown sumoylation to be important in the HPV life cycle. The functions of two early proteins, E1 and E2, are modified directly by sumoylation (17, 19, 26, 28), and three others, E2 (27), E6 (3), and E7 (14), affect host sumoylation pathways.

HPV type 16 (HPV-16) L2's potential involvement with the sumoylation machinery has not been documented, although a consensus sequence, ΨKXE, where Ψ is a large hydrophobic amino acid, corresponding to the SUMO acceptor site in most known SUMO substrate proteins (1), is found in the HPV-16 L2 sequence. To investigate whether this is indeed a SUMO acceptor site, HPV-16 L2 was subcloned into a Flag-hemagglutinin (HA)-tagged expression vector (23) and transfected into 293 cells alone or in combination with HA-SUMO1, HA-SUMO2, or HA-SUMO3 expression plasmids. After 24 h, cells were lysed in E1a buffer (50 mM HEPES, pH 7.0, 0.5% NP-40, 250 mM NaCl) and samples were analyzed by Western blotting with anti-HA antibody (Roche). In addition to native L2, which migrates at about 80 kDa, a slower-migrating protein of about 100 kDa was recognized in the presence of SUMO1, SUMO2, or SUMO3 (Fig. 1A). To confirm that these novel L2 species were SUMO-modified HPV-16 L2, a SUMO protease, SENP2 (Addgene), was coexpressed in 293 cells with L2 and HA-SUMO1, -2, or -3. SENP2 completely eliminated the 100-kDa bands (Fig. 1B). We conclude from these data that HPV-16 L2 is sumoylated *in vivo* and can be modified by either SUMO1, -2, or -3. Interestingly, L2 showed a strong preference for SUMO2/3; only weak sumoylation was carried out by SUMO1 (Fig. 1A).

The potential SUMO acceptor site PKVE is located at positions 34 to 37 of HPV-16 L2. To investigate whether lysine 35 might serve as an acceptor for SUMO conjugation, we generated an L2 mutant, replacing lysine 35 with arginine (L2-K35R). 293 cells were then transfected with plasmids expressing HA-SUMO1, HA-SUMO2, or HA-SUMO3 and either wild-type L2 or the L2-K35R mutant. Mutation of lysine 35 to arginine effectively abolished the appearance of the 100-kDa bands (Fig. 1C). Taken together, our data indicate that lysine 35 is the major sumoylation site in HPV-16 L2 *in vivo*.

As sumoylation often impairs protein degradation, we asked whether sumoylation controls L2 protein stability. To investi-

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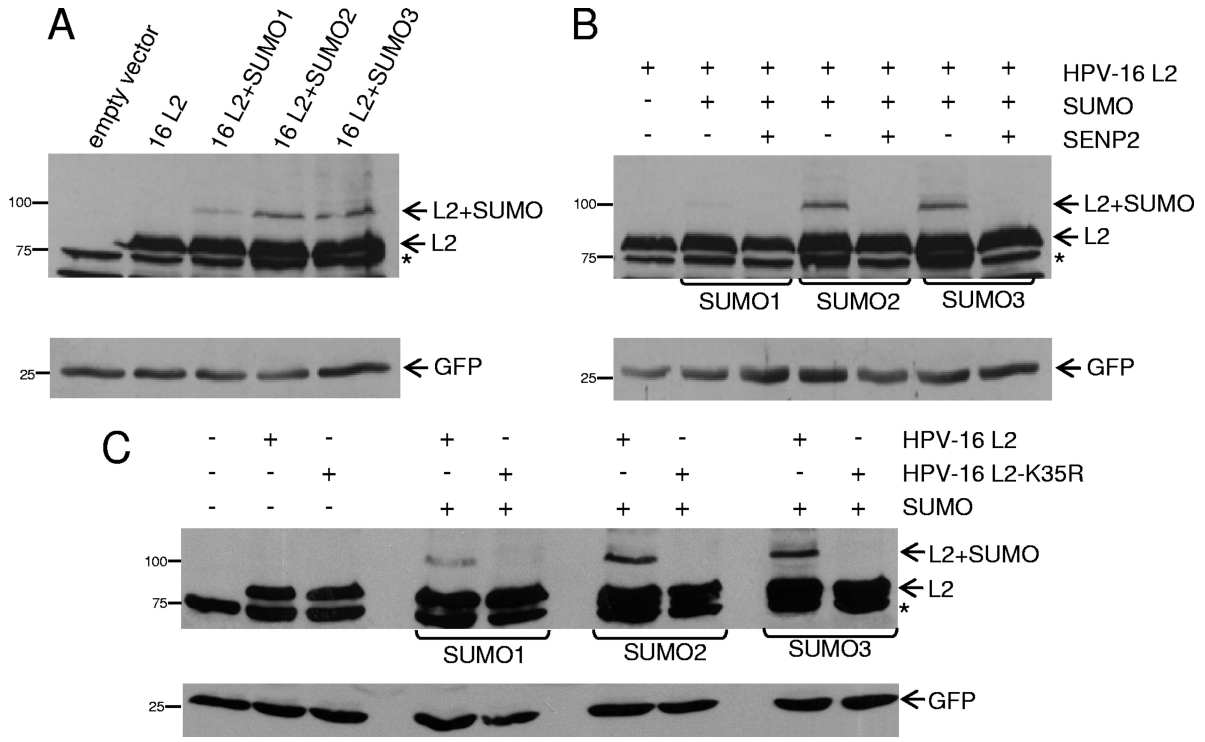

FIG. 1. HPV-16 L2 is sumoylated *in vivo* by SUMO1, -2, and -3 at lysine 35. (A) 293 cells were transiently transfected either with a Flag-HA-tagged HPV-16 L2 expression plasmid alone or in combination with a SUMO expression plasmid (SUMO1, -2, or -3). (B) Where indicated, a SUMO protease, SENP2, was coexpressed in 293 cells, transfected as described for panel A. (C) Lysine 35 of the SUMO predicted-site PKVE was substituted for the arginine (L2-K35R mutant). 293 cells were then transiently transfected either with wild-type Flag-HA-tagged HPV-16 L2 or the L2-K35R mutant alone or in combination with the SUMO1, SUMO2, or SUMO3 expression plasmid. After 24 h, cells were extracted and HPV-16 L2 was detected by immunoblotting using anti-HA antibody (12CA5; Roche). Green fluorescent protein (GFP) was used as an internal control for the monitoring of transfection efficiency. Asterisks denote a nonspecific band recognized by the anti-HA antibodies. Numbers at the left of the gels are molecular sizes in kilodaltons.

gate this, the stability of wild-type L2 and the L2-K35R mutant was determined. 293 cells were transfected with the two L2 expression plasmids, and the cells were then treated with cycloheximide (CHX; 50 μg/ml) to stop further protein synthesis. Cells were harvested at various times, and levels of L2 were analyzed by Western blotting (Fig. 2B). As can be seen in Fig. 2, wild-type L2 and L2-K35R have significantly different rates of turnover, with the half-life of wild-type L2 being almost

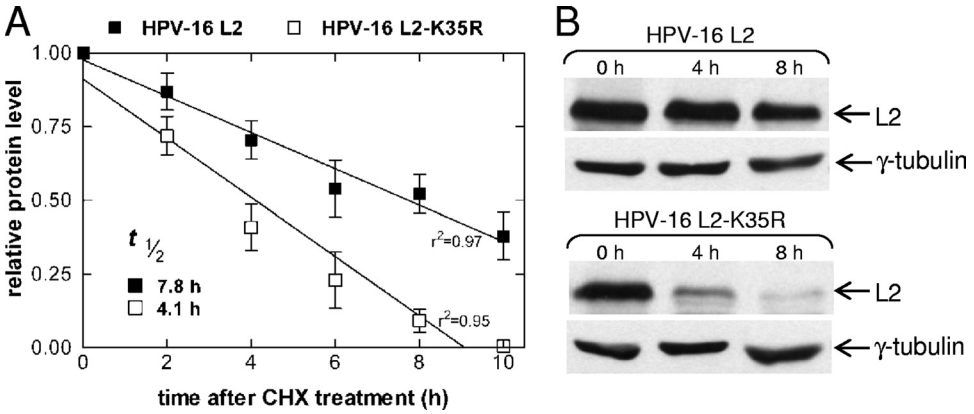

FIG. 2. Loss of sumoylation capacity decreases the stability of HPV-16 L2 protein. 293 cells were transiently transfected with either wild-type Flag-HA-tagged HPV-16 L2 or the L2-K35R mutant. After 24 h, cells were washed and treated with cycloheximide (CHX) for the indicated periods. Cell lysates were then subjected to immunoblotting. (A) The level of L2 protein was quantitated by densitometry, and the amounts of L2 were normalized to the amount of γ-tubulin. Results are expressed as the means ± standard errors (SE) of results from four independent experiments. Protein half-lives ($t_{1/2}$) represent values determined from the linear regression curves (r^2 = 0.97 for wild-type L2 and 0.95 for L2-K35R). The slopes of both curves differ significantly (P = 0.001). (B) Western blot analysis of the L2 protein level at some of the selected time points after CHX treatment using an anti-HA antibody (12CA5; Roche). γ-Tubulin (Sigma) was used as a loading control.

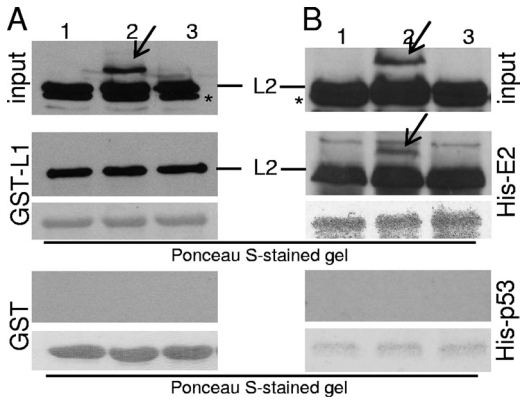

FIG. 3. Sumoylation negatively regulates HPV-16 L2 interaction with the major capsid protein L1. 293 cells were transiently transfected with either wild-type Flag-HA-tagged HPV-16 L2 (lane 1) or the L2-K35R mutant (lane 3) alone or with wild-type HPV-16 L2 in combination with the SUMO2 expression plasmid (lane 2). After 24 h, cells were harvested and subjected to a pulldown assay with GST–HPV-16 L1 (A) or His–HPV-16 E2 (B) proteins. Purified unfused GST and His-p53 proteins (lower panels) were used as the respective negative controls. Bound proteins and cell extracts were analyzed by immunoblotting using an anti-HA antibody (12CA5; Roche). Inputs (top panels) represent 10% of the extracts used for the pulldown assays. Arrows point to the sumoylated form of the HPV-16 L2 protein. Note that sumoylated L2 binds only to E2 but that both unmodified L2 and the K35R mutant bind to E2 and L1. No interaction is seen with GST alone or His-p53. The Ponceau stains show equal levels of purified proteins used in the pulldown assays. Asterisks denote a nonspecific band recognized by the anti-HA antibody in the input lanes.

twice that of the L2-K35R mutant (7.8 h versus 4.1 h) (Fig. 2A). Taken together, these experiments show that sumoylation can affect L2 stability, since nonsumoylatable L2 is turned over more rapidly.

To investigate the possible physiological role of L2 sumoylation, we analyzed the capacity of sumoylated L2 to interact with two previously described HPV-16 L2-interacting partners, L1 and E2 (10, 15). HA-tagged L2 was expressed in 293 cells, either alone or in combination with SUMO2. Cell extracts were made after 24 h, which were then used in pulldown assays with purified glutathione *S*-transferase (GST)–L1 and His-E2 proteins, with purified GST and His-p53 acting as negative controls in the L2 pulldown assays. The bound L2 was detected by Western blotting, and the results show that both the sumoylated and nonsumoylated forms of L2 were capable of binding to E2 (Fig. 3B). In contrast, only the nonsumoylated form of L2 was capable of binding to L1 (Fig. 3A), demonstrating that sumoylation of L2 negatively affects its capacity to interact with L1.

In some cases, sumoylation has been shown to alter the subcellular localization of proteins (31). To investigate whether sumoylation of L2 could affect its intracellular localization, we expressed either wild-type L2 or the L2-K35R mutant in U2OS cells. After 24 h, cells were subjected to an indirect immunofluorescence assay with anti-Flag antibodies (Sigma) to detect L2 and anti-SUMO1 or anti-SUMO2/3 (Santa Cruz) to detect endogenous SUMO proteins. Both wild-type L2 and the L2-K35R mutant localized to the nucleus in a diffuse pattern, with no apparent differences in distributions (Fig. 4C). Thus, since mutation of the sumoylation target site does not influence the subcellular localization of L2, it appears that sumoylation is not required for nuclear entry of L2. Interestingly, upon L2 expression, there was a marked increase in the SUMO2/3 signal in the nuclei of U2OS cells (Fig. 4A) and HaCaT keratinocytes (data not shown), but no such effect was observed with SUMO1 (Fig. 4B). Similar results were observed with the L2-K35R mutant (Fig. 4A and B). Western blot analysis using anti-SUMO antibodies revealed that L2 specifically upregulates endogenous sumoylation of high-molecular-mass host proteins (100 to 250 kDa) by SUMO2/3 but only weakly with SUMO1 (Fig. 4D).

This study shows for the first time that HPV-16 L2 capsid protein is a substrate for sumoylation *in vivo*. Interestingly, L2 shows a strong preference for SUMO2/3, with little detectable sumoylation by SUMO1. Several lines of evidence indicate that SUMO2 and SUMO3 have protein targets, signaling properties, and functions distinct from those of SUMO1 (20, 24). SUMO2/3 conjugation is preferentially upregulated in response to cell stress, plays a key role in mitosis (30), and affects cell growth and differentiation (29). Upregulation of SUMO2/3 has been demonstrated during keratinocyte differentiation, whereas SUMO1 expression is relatively unchanged (9), suggesting that SUMO2/3 modification of L2 might occur during keratinocyte differentiation.

The loss of the acceptor lysine for sumoylation results in the destabilization of L2. For some proteins, it has been shown that attachment of SUMO1 prevents ubiquitinylation, reducing proteasomal degradation of those proteins (2, 7, 25). Interestingly, the sumoylation-deficient L2 mutant revealed a much lower steady-state level than wild-type L2, reflected in its reduced half-life. This suggests that sumoylation of L2 indirectly contributes to its stability, although we cannot formally exclude the possibility that the mutation itself might adversely affect L2 stability. The underlying mechanisms are unclear but are unlikely to involve SUMO competing with ubiquitin for ligation to the K35 residue, since in that case the mutant is expected to be more stable.

It is well known that sumoylation influences the interaction of many target proteins with other proteins and might serve as an adaptor for protein-protein interactions. We show that sumoylation prevents binding of L2 to the major capsid protein L1, suggesting a mechanism by which capsid assembly may be modulated in an infected cell. In contrast, it appears that sumoylation of L2 does not interfere with its binding to E2. It will now be interesting to determine to what degree L2 is sumoylated during the course of a normal viral infection and whether this might vary during different stages of the viral life cycle. However, it is intriguing to note that the sumoylated lysine 35 in L2 is highly conserved across mammalian and avian PV types and that it is located in the amino-terminal region of L2, where many important domains are located, including those for DNA binding (32), nuclear transport (22), furin cleavage (18), and tSNARE syntaxin 18 interaction (6). It will be important to determine whether the sumoylation of L2 can influence these interactions and whether sumoylation is an important feature of L2 regulation across diverse PV types.

Finally, we show that L2 appears to modulate the overall sumoylation status of the host cell by specifically upregulating endogenous sumoylation of the host proteins by SUMO2/3. Two other viral proteins have been shown to have an effect on global sumoylation activities, GAM1 (4) and HPV E6 (3), both of which

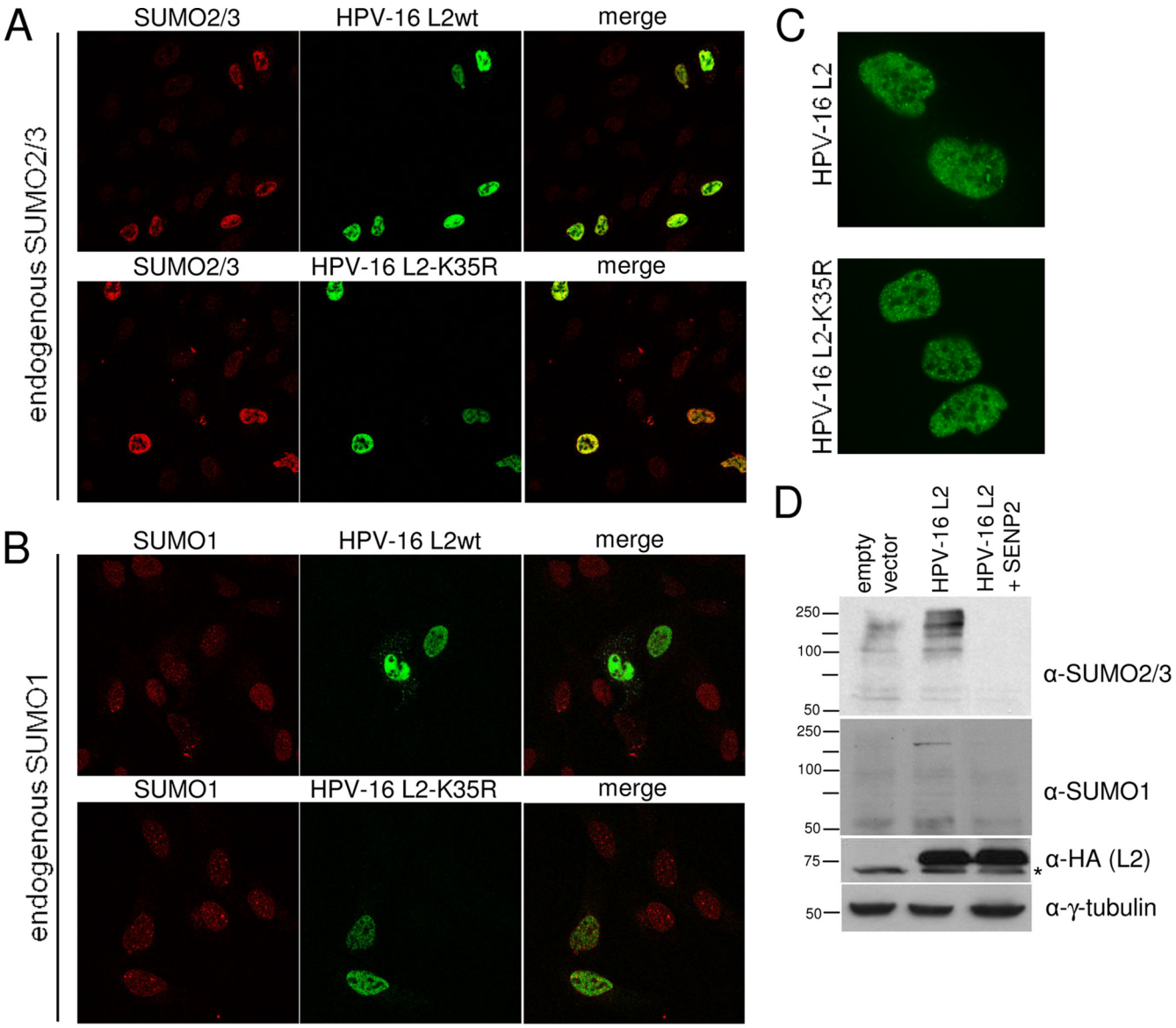

FIG. 4. HPV-16 L2 upregulates endogenous sumoylation by SUMO2/3 in the host cells. U2OS cells were transiently transfected with either wild-type (wt) Flag-HA-tagged HPV-16 L2 or the L2-K35R mutant. Twenty-four hours later, cells were fixed and stained for L2 (anti-Flag, M2; Sigma) and endogenous SUMO2/3 (anti-SUMO2/3; Santa Cruz Biotechnology) (A), or SUMO1 (anti-SUMO1, Santa Cruz Biotechnology) (B), or for L2 alone (C). The cellular localization of L2 and SUMO proteins was analyzed by indirect immunofluorescence using a confocal microscope (Zeiss Axiovert 100 M). The panels show SUMO proteins alone (red), L2 alone (green), and the merged images. (D) Western blot analysis of the overall cell sumoylation status 24 h after transfection with the wild-type Flag-HA-tagged HPV-16 L2 plasmid using anti-SUMO2/3 antibodies for the detection of SUMO2/3-sumoylated proteins, anti-SUMO1 antibodies for the detection of SUMO1-sumoylated proteins, anti-HA antibodies (12CA5; Roche) for the detection of HPV-16 L2, and anti-γ-tubulin (Sigma) as a loading control. The asterisk denotes a nonspecific band recognized by the anti-HA antibodies.

interact with a component of the sumoylation system, resulting in an overall inhibition. However, L2 is the first example of a viral protein that specifically upregulates the sumoylation of host proteins. Interestingly, upregulation of SUMO expression has also been shown during keratinocyte differentiation (9), the natural environment for the HPV life cycle (11), and it will be of interest to determine whether SUMO levels can be further increased by HPV L2 during infection. Indeed, it has been speculated that increased SUMO2/3 sumoylation in infected keratinocytes may lead to HPV E2 stabilization and the higher protein concentrations required for productive viral genome amplification (28). However, the increase in SUMO2/3 expression appears to be short-lived, and it is therefore possible that one of L2's functions is to contribute to the high cellular SUMO2/3 sumoylation level and thereby support the late stages of viral replication.

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REFERENCES

1. **Anckar, J., and L. Sistonen.** 2007. SUMO: getting it on. Biochem. Soc. Trans. **35:**1409–1413.
2. **Bies, J., J. Markus, and L. Wolff.** 2002. Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. J. Biol. Chem. **15:**8999–9009.
3. **Bischof, O., K. Schwamborn, N. Martin, A. Werner, C. Sustmann, R. Grosschedl, and A. Dejean.** 2006. The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis. Mol. Cell **23:**783–794.
4. **Boggio, R., and S. Chiocca.** 2005. Gam1 and the SUMO pathway. Cell Cycle **4:**533–535.
5. **Boggio, R., and S. Chiocca.** 2006. Viruses and sumoylation: recent highlights. Curr. Opin. Microbiol. **9:**430–436.
6. **Bossis, I., R. B. Roden, R. Gambhira, R. Yang, M. Tagaya, P. M. Howley, and P. I. Meneses.** 2005. Interaction of tSNARE syntaxin 18 with the papillomavirus minor capsid protein mediates infection. J. Virol. **79:**6723–6731.
7. **Desterro, J. M., M. S. Rodriguez, and R. T. Hay.** 1998. SUMO-1 modification of IκBα inhibits NF-κB activation. Mol. Cell **2:**233–239.
8. **Deyrieux, A. F., and V. G. Wilson.** 2009. Viral interplay with the host sumoylation system, p. 315–329. *In* V. G. Wilson (ed.), SUMO regulation of cellular processes. Springer, Dordrecht, Netherlands.
9. **Deyrieux, A. F., G. Rosas-Acosta, M. A. Ozbun, and V. G. Wilson.** 2007. Sumoylation dynamics during keratinocyte differentiation. J. Cell Sci. **1:**125–136.
10. **Finnen, R. L., K. D. Erickson, X. S. Chen, and R. L. Garcea.** 2003. Interaction between papillomavirus L1 and L2 capsid proteins. J. Virol. **77:**4818–4826.
11. **Hebner, C. M., and L. A. Laimins.** 2006. Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity. Rev. Med. Virol. **16:**83–97.
12. **Heun, P.** 2007. SUMOrganization of the nucleus. Curr. Opin. Cell Biol. **19:**350–355.
13. **Holmgren, S. C., N. A. Patterson, M. A. Ozbun, and P. F. Lambert.** 2005. The minor capsid protein L2 contributes to two steps in the human papillomavirus type 31 life cycle. J. Virol. **79:**3938–3948.
14. **Ledl, A., D. Schmidt, and S. Müller.** 2005. Viral oncoproteins E1A and E7 and cellular LxCxE proteins repress SUMO modification of the retinoblastoma tumor suppressor. Oncogene **26:**3810–3818.
15. **Okoye, A., P. Cordano, E. R. Taylor, I. M. Morgan, R. Everett, and M. S. Campo.** 2005. Human papillomavirus 16 L2 inhibits the transcriptional activation function, but not the DNA replication function, of HPV-16 E2. Virus Res. **108:**1–14.
16. **Pereira, R., I. I. Hitzeroth, and E. P. Rybicki.** 2009. Insights into the role and function of L2, the minor capsid protein of papillomaviruses. Arch. Virol. **154:**187–197.
17. **Rangasamy, D., K. Woytek, S. A. Khan, and V. G. Wilson.** 2000. SUMO-1 modification of bovine papillomavirus E1 protein is required for intranuclear accumulation. J. Biol. Chem. **275:**37999–38004.
18. **Richards, R. M., D. R. Lowy, J. T. Schiller, and P. M. Day.** 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc. Natl. Acad. Sci. U. S. A. **103:**1522–1527.
19. **Rosas-Acosta, G., M. A. Langereis, A. Deyrieux, and V. G. Wilson.** 2005. Proteins of the PIAS family enhance the sumoylation of the papillomavirus E1 protein. Virology **331:**190–203.
20. **Rosas-Acosta, G., W. K. Russell, A. Deyrieux, D. H. Russell, and V. G. Wilson.** 2005. A universal strategy for proteomic studies of SUMO and other ubiquitin-like modifiers. Mol. Cell. Proteomics **4:**56–72.
21. **Sapp, M., and M. Bienkowska-Haba.** 2009. Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. FEBS J. **276:**7206–7216.
22. **Sun, X. Y., I. Frazer, M. Müller, L. Gissmann, and J. Zhou.** 1995. Sequences required for the nuclear targeting and accumulation of human papillomavirus type 6B L2 protein. Virology **213:**321–327.
23. **Tomaić, V., D. Pim, and L. Banks.** 2009. The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. Virology **393:**7–10.
24. **Vertegaal, A. C., J. S. Andersen, S. C. Ogg, R. T. Hay, M. Mann, and A. I. Lamond.** 2006. Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. Mol. Cell. Proteomics **5:**2298–2310.
25. **Wei, F., H. R. Schöler, and M. L. Atchison.** 2007. Sumoylation of Oct4 enhances its stability, DNA binding, and transactivation. J. Biol. Chem. **282:**21551–21560.
26. **Wu, Y. C., A. A. Roark, X. L. Bian, and V. G. Wilson.** 2008. Modification of papillomavirus E2 proteins by the small ubiquitin-like modifier family members (SUMOs). Virology **378:**329–338.
27. **Wu, Y. C., A. F. Deyrieux, and V. G. Wilson.** 2007. Papillomaviruses and the host SUMOylation system. Biochem. Soc. Trans. **35:**1433–1435.
28. **Wu, Y. C., X. L. Bian, P. R. Heaton, A. F. Deyrieux, and V. G. Wilson.** 2009. Host cell sumoylation level influences papillomavirus E2 protein stability. Virology **387:**176–183.
29. **Yang, W., and W. Paschen.** 2009. Gene expression and cell growth are modified by silencing SUMO2 and SUMO3 expression. Biochem. Biophys. Res. Commun. **382:**215–218.
30. **Zhang, X. D., J. Goeres, H. Zhang, T. J. Yen, A. C. Porter, and M. J. Matunis.** 2008. SUMO-2/3 modification and binding regulate the association of CENP-E with kinetochores and progression through mitosis. Mol. Cell **29:**729–741.
31. **Zhao, J.** 2007. Sumoylation regulates diverse biological processes. Cell. Mol. Life Sci. **64:**3017–3033.
32. **Zhou, J., X. Y. Sun, K. Louis, and I. H. Frazer.** 1994. Interaction of human papillomavirus (HPV) type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. J. Virol. **68:**619–625.