

# Recent Highlights: Onco Viral Exploitation of the SUMO System

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## Abstract

Small ubiquitin-like modifier (SUMO)ylation is a crucial post-translational modification that controls functions of a wide collection of proteins and biological processes. Hence, given its pleiotropic role, viruses have developed many approaches to usurp SUMO conjugation to exploit the cellular host environment for their own benefit. Consistently, cancer cells also frequently impact on SUMO to force cellular transformation, underlining the importance of SUMO in health and diseases. Therefore, after a brief introduction to the multistep SUMOylation pathway, in this review we will focus our attention on several examples of strategies adopted by oncogenic viruses to hijack SUMOylation in order to promote infection, persistence and malignant transformation of host cells.

## Introduction

The Small Ubiquitin-like Modifier (SUMO) proteins are involved in post translational modification (PTM) of target proteins (Matunis *et al.*, 1996; Kamitani *et al.*, 1997). The name SUMO comes from a structural similarity with ubiquitin and from the similar mechanism by which it is attached to target proteins (Mahajan *et al.*, 1997). Indeed, both ubiquitination and SUMOylation

are reversible processes catalysed by a cascade of enzymes, namely E1, E2 and E3 proteins (Gong *et al.*, 1997; Mahajan *et al.*, 1997; Johnson and Gupta, 2001).

## SUMO proteins

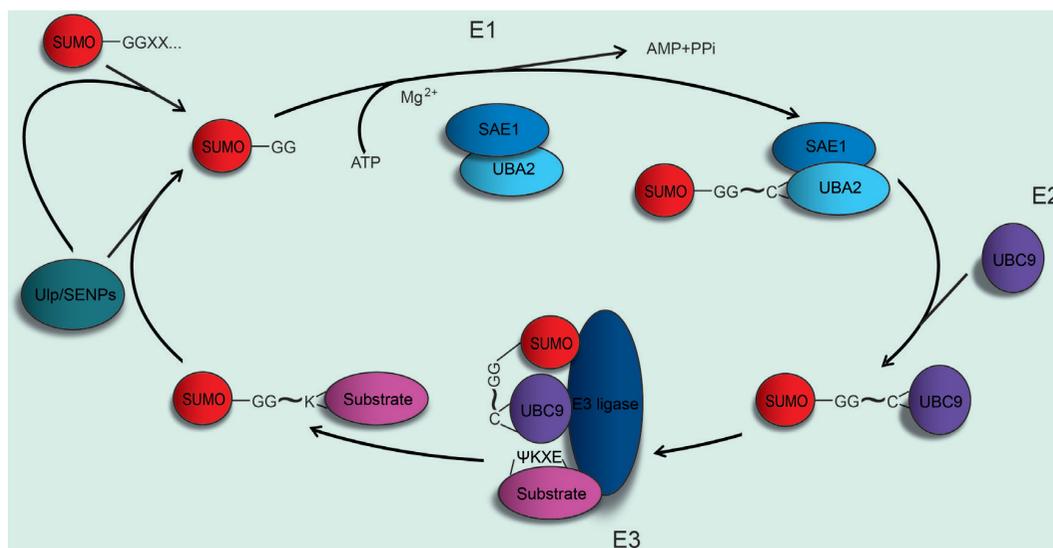
The expression of SUMO proteins is conserved among eukaryotes. Lower eukaryotes have only one SUMO, while higher eukaryotes express three or more SUMO paralogs. In particular, in humans five different isoforms of SUMO are present, differing for response to physiological or stress conditions, tissue-specificity, and the ability to form SUMO chains [recently reviewed in Yang *et al.*, (2017)]. SUMO1 is 101 amino acids protein found almost always conjugated to targets, and therefore often associated to physiological processes (Shen *et al.*, 1996; Yang *et al.*, 2017). SUMO2 consists of 95 amino acids and shares 95% homology with SUMO3 (103 amino acids), differing for only three N-terminal residues, and showing the same molecular functions, therefore often referred as SUMO2/3. They show only 45% homology with SUMO1 but they present a very similar tridimensional structure. SUMO2/3 are conjugated mostly under stress conditions and they are able to form chains (Mannen *et al.*, 1996; Lapenta *et al.*, 1997). SUMO4 seems to be expressed only in lymph nodes, kidneys and

spleen. SUMO4 has not been well characterized yet and its role still needs to be elucidated. It is probably non-conjugated under physiological conditions and it has been associated to diabetes (Wang *et al.*, 2006). Finally, the expression of a fifth SUMO isoform has been recently reported (Liang *et al.*, 2016). SUMO5 seems to be a conserved 84 amino acids protein whose mRNA is expressed at high levels in testes and peripheral blood lymphocytes, and at lower levels also in placenta, lungs and liver. Conjugation with this novel SUMO variant facilitates the formation of Promyelocytic Leukaemia Nuclear Bodies (PML-NB), structures rich in SUMOylated proteins that regulate a variety of cell functions.

### The SUMO machinery

The SUMOylation process is carried out in different steps (Fig. 3.1). Initially, SUMO is processed by a protease (belonging to the SENP family, as described in more details below), that generates the mature form consisting of a C-terminal diglycine (Hickey *et al.*, 2012). This motif is required for the following step, in which the SUMO E1 enzyme activates SUMO. There is one only SUMO

E1 enzyme expressed in mammalian cells, a heterodimer composed by SUMO-activating enzyme subunit 1 (SAE1) and ubiquitin-like activating enzyme subunit 2 (SAE2/UBA2) (Desterro *et al.*, 1999). SUMO is adenylated by the E1 complex in an ATP-Mg<sup>2+</sup>-dependent reaction and transferred to the catalytic Cys of the UBA2 subunit by an E1~SUMO thioester bond. Then, the unique SUMO E2 conjugating enzyme, ubiquitin-like conjugating 9 (UBC9), receives SUMO on a conserved catalytic cysteine, forming an E2~SUMO thioester complex (Tong *et al.*, 1997; Duan *et al.*, 2009). The E2 enzyme can attach SUMO to substrates, with the formation of an isopeptide bond between the carboxy-terminal carboxyl group of SUMO and a ε-amino group of the substrate acceptor Lys residue. UBC9 can be itself modified by different PTMs which increase or decrease its activity and localization, and confer substrate specificity (Knipscheer *et al.*, 2008; Su *et al.*, 2012). UBC9 can interact directly with some SUMO substrates but more often it needs the help of SUMO E3 enzymes, ligases that are able to give specificity to the targets (Sachdev *et al.*, 2001; Tatham *et al.*, 2005). Opposite to the unique E2, there are different SUMO



**Figure 3.1** The SUMO conjugation system. Ulp1/SENP proteases catalyse cleavage of the C-terminal domain of SUMO proteins, exposing a diglycine motif. Processed SUMO is transferred to a cysteine of the heterodimeric E1 enzyme Uba2/SAE1. SUMO is then conjugated to a cysteine of UBC9, the E2 enzyme and attached to a lysine residue of the consensus motif on target proteins. The conjugation is often facilitated by an E3-ligase, which enforces the interactions among the involved components. SUMOylation is a reversible pathway where the Ulp1/SENPs proteases dictate the de-SUMOylation process.

E3 ligases, selective for SUMO1 or SUMO2/3 [described in Mattoscio and Chiocca (2015)]. Some E3 ligases act by orientating the E2~SUMO thioester in an optimal conformation for catalysis without directly contacting the substrate, while others facilitate the release of SUMO from E2 [reviewed in Wilkinson and Henley (2010)]. As mentioned before, SUMOylation is a reversible process governed by the action of two families of proteases that deconjugate SUMO from substrates. They include Ubl-specific proteases and sentrin-specific proteases (Ulp1 and SENPs, respectively) (Li and Hochstrasser, 2000).

### SENPs

The human SENP family is composed of seven members: SENP1, SENP2, SENP3, SENP5, SENP6, SENP7 and SENP8, even if SENP8 is not specific for SUMO but acts on Nedd8, another ubiquitin-like protein (Hickey *et al.*, 2012). SENPs are cysteine proteases with a papain-like folded catalytic domain and specific N-terminal domains crucial for their own regulation and for substrate selection (Hay, 2007).

SENP proteases regulate both the level of processed SUMO and the rate of substrate modification by counterbalancing SUMO conjugation [recently reviewed in Kunz *et al.* (2018)]. In the maturation process they hydrolyse a peptide bond close to the C-terminus of SUMO precursors, eliminating the very C-terminal amino acids from SUMO1, SUMO2 and SUMO3 and exposing two glycine residues. In SUMO1–3, the diGly motif is preceded by a glutamine (Q) and threonine (T), while SUMO4 exhibits a PTGG motif, in which the proline residue confers resistance to SENP-mediated cleavage (Owerbach *et al.*, 2005). In the deconjugation process, SENPs cleave an isopeptide bond that links SUMO moieties to the  $\epsilon$ -amino group of lysine residues.

The mechanism by which SENP1 and SENP2 exert their functions has been described by X-ray crystallography. *In vitro* protease assays with the isolated catalytic domains demonstrate the processing activity of SENP1 and SENP2 on all three SUMO precursors (Reverter and Lima, 2006; Shen *et al.*, 2006a). However, they exert differential activities towards distinct precursors. In particular, SENP2 is most active on SUMO2, then SUMO3 and SUMO1, while SENP1 prefers SUMO1.

Probably these differences are due to the amino acid sequences of the C-terminal tail. SENP5 has been found to have a marked preference for SUMO2 cleavage, while SENP6 and SENP7 are not able to process SUMO for maturation.

The SUMO system leaves a dilemma on how it is possible to achieve specificity on SUMOylation of a myriad of proteins with the small numbers of conjugating and deconjugating enzymes available. In some cases, specific biological processes are regulated by distinct deconjugation events. Though, in many cases, a single SENP may act on larger groups of SUMOylated proteins (Psakhye and Jentsch, 2012; Jentsch and Psakhye, 2013). Moreover, alternative splicing and PTMs of SENPs are important to determine their localization and their protease activity. To summarize, the special control of SENPs is a fundamental principle for deSUMOylation regulation (Kunz *et al.*, 2018).

### The SUMO consensus motifs

The main SUMO consensus motif existing in the primary structure of SUMOylated protein is  $\psi$ KX(D/E), where  $\psi$  is a large hydrophobic residue, X is any amino acid and K is the acceptor lysine. These residues directly bind UBC9 and are crucial for a stable interaction between the E2 enzyme and the substrate (Rodriguez *et al.*, 2001). In addition to the canonical four amino acid SUMO consensus motifs, longer sequences that include both SUMO consensus motifs and additional elements have been identified in some SUMO substrates (Gareau and Lima, 2010). Among these, phosphorylation-dependent SUMO motifs (PDSMs) and negatively charged amino acid-dependent SUMO motifs (NDSMs) are present. PDSMs present a SUMO consensus motif located adjacent to a phosphorylation site,  $\psi$ KX(D/E)XXSP. Phosphorylation increases SUMO conjugation levels because the phosphorylated Ser side chain interacts with a basic patch on the E2 surface, extending interactions with the E2 enzyme beyond recognition of the SUMO consensus motif. This mechanism is probably shared with proteins that contain NDSMs, which comprise negatively charged residues that are C-terminal to the SUMO consensus site in the place of the phosphorylation site of PDSMs, although NDSMs may interact with a different subset of Lys residues on the UBC9 surface (Yang *et al.*, 2006; Mohideen *et al.*, 2009). Recent studies

revealed new motifs for SUMO conjugation, including inverted consensus motifs and motifs with an N-terminal hydrophobic cluster. These alternative motifs are probably important to give specificity to E2–substrate interactions through direct interaction with the E2 (Impens *et al.*, 2014).

### SIM

SUMO interacting motifs (SIMs) establish non-covalent hydrophobic interaction between SUMO and the target proteins. Canonical SIMs contain a core of hydrophobic residues that can be preceded or followed by negatively charged amino acids that take contact with hydrophobic groove on SUMO with following basic residues. This interaction is generally weak but can be increased by the binding of multiple SIMs to SUMO chains. Crucial hydrophobic and basic residues involved in SIM binding are conserved among the SUMO paralogues. However, the isoforms might differ in the placement of their hydrophobic groove suggesting that the arrangement of hydrophobic and acidic residues in SIMs might dictate their ability to bind specific SUMO isoforms (Hecker *et al.*, 2006; Kerscher, 2007).

### STUbLs

SUMO Targeted Ubiquitin Ligases (STUbLs) are Ubiquitin E3 enzymes able to recognize SUMOylated proteins and to interact with them through SIMs. They attach ubiquitin chains to SUMOylated proteins to target them for degradation by the proteasome. STUbLs constitute an important regulatory mechanism to control the levels of the SUMO conjugated form of a protein (Sriramachandran and Dohmen, 2014). The human RING Finger protein 4 (RNF4) is one of the best studied STUbL, containing at least three SIMs. These motifs mediate a similar non-covalent interaction with SUMO1 and SUMO2, with a preference for chains of a length of at least three SUMO moieties. RNF4 works as homodimer, in which the RING domains of both subunits take contact with a single ubiquitin-charged E2 (Sun *et al.*, 2007).

### SUMO functions

SUMOylation is involved in many different biological processes and can confer different properties to substrate proteins. A high number

of known SUMOylation targets are nuclear proteins, involved in DNA repair, regulation of transcription and chromatin structure. Many important nuclear targets of signalling pathways can be SUMOylated. SUMOylation is important for subcellular localization of proteins, competes with other PTMs, and also participates to protein–protein interaction. SUMOylation can also change the interaction between DNA and RNA, alter enzymatic activity and protein conformation, and modulate other modifications (recently reviewed in (Zhao, 2018)). In the following paragraphs we will describe some paradigmatic example of how SUMOylation can impact on the activity of important selected targets.

### RanGAP

RanGAP, the first protein shown to be SUMO modified, is important for nuclear import. Unmodified RanGAP is cytoplasmic, whereas SUMO-modified RanGAP is associated with the nuclear pore. SUMOylation of RanGAP increases its interaction with the SUMO E3 ligase Ran binding protein 2, a component of the nuclear pore complex. Localization of the RanBP2 SUMO E3 ligase at the nuclear pore could be important for a broad role for SUMO in regulation of nuclear trafficking (Matunis *et al.*, 1996).

### Promyelocytic leukaemia protein

Promyelocytic leukaemia protein (PML) is post-translationally modified by SUMO and is localized in subnuclear structures named PML nuclear bodies, structures highly enriched in SUMOylated proteins. PML bodies host more than 150 proteins with a wide range of functions, such as DNA repair, stress response, senescence, anti-viral immunity, and tumour suppression. Notably, a variety of SUMO-modified proteins including transcription factors, chromatin modifiers, and proteins involved in genomic maintenance, are expressed in PML nuclear bodies together with SUMO E3 ligases and SUMO-specific proteases. SUMO-modified PML probably supports some protein–protein interactions important for assembly or stability of this subnuclear domain. Focused studies of cellular membrane-less structures suggest that proteins able to form inter-molecular multivalent interactions can constitute large oligomers and phase separate

from the surrounding solution. These proteins can use their interaction domains or intrinsically disordered regions to recruit additional macromolecules even in high concentrations, maybe promoting certain biological processes (Shen *et al.*, 2006b).

### SUMO modulation of chromosomes and chromatin

SUMO deficiency drastically changes chromosome integrity and segregation. Indeed, SUMO enzymes are enriched at centrosomes, very important structures that support kinetochores for microtubules attachment during cell division (Lapenta *et al.*, 1997). Topoisomerase II is recruited to centromeres on SUMOylation of its non-catalytic C-terminus, to uncoil intertwined DNA before anaphase and facilitate centromeric segregation. Centromeric histones and chromatin regulators are also regulated by SUMO conjugation. In particular, SUMOylated Orc2 recruits the histone demethylase KDM5A to demethylate H3K4me3 into H3K4me2, enhancing non-coding RNA synthesis from the locus and subsequent heterochromatin maintenance. Moreover, Aurora B kinase deSUMOylation facilitates its localization to the spindle mid-zone, essential step during mitosis [recently reviewed in Zhao (2018)].

The SUMO pathway does not regulate only centromeric regions. For example, the heterochromatin assembly factor HP1 is SUMOylated to promote its association with RNA transcripts located at these regions. In addition, SENP7 activity is important for HP1 regulation in order to retain it at heterochromatin, even if the molecular details are still unclear (Maison *et al.*, 2012). SUMOylation also affects chromatin modifiers such as histone deacetylase 1 (HDAC1), an essential epigenetic regulator of a conserved family of deacetylases frequently involved in cancer progression (Ropero and Esteller, 2007). Indeed, in non-tumourigenic cells, SUMOylation of HDAC1 by SUMO1 promoted by the overexpressed PIASy triggers its ubiquitination and degradation in a proteasome-dependent manner, thus reducing HDAC1 expression. Conversely, in breast cancer cell lines, HDAC1 is preferentially conjugated by SUMO2 that protects HDAC1 from ubiquitin conjugation and degradation. Therefore, SUMOylation

significantly affects the expression and activity of an important chromatin modifier involved in breast cancer progression (Citro *et al.*, 2013).

In addition, SUMO plays a fundamental role in DNA double-strand breaks (DSB), where SUMOylation enables broken DNA ends to move outside to prevent illegitimate repair of repetitive sequences. Similarly, SUMO promotes movement of target eroded telomeres and DSB to nuclear periphery, in a STUB1-mediated mechanism that interacts with SUMOylated DNA repair proteins leading to their proteasomal degradation [recently reviewed in Garvin and Morris (2017)].

### SUMO in DNA damage

SUMOylation is important in the DNA damage checkpoint pathway. In both yeast and human cells, SUMOylation of DNA damage proteins occurs in parallel with checkpoint mediated phosphorylation. Interestingly, changes in the checkpoint pathway can modify SUMOylation events: decreasing Ataxia telangiectasia and Rad3 related (ATR) checkpoint kinase increases protein SUMOylation. Moreover, Ataxia-telangiectasia mutated (ATM) checkpoint kinase is able to increase SENP2 transcription in particular contexts, but also ATM seems to promote SUMOylation in the absence of ATR. This phenomenon suggests a context-dependent crosstalk between these pathways (Munk *et al.*, 2017).

As described, SUMOylation affects almost all cellular activities, resulting as a key pathway regulating cells physiology. However, conversely, it is evident that alterations in normal SUMOylation could completely subvert cell functions [reviewed in Flotho and Melchior (2013)]. Therefore, SUMO pathway components are frequently altered in human diseases such as cancer (Mattosio and Chiocca, 2015; Seeler and Dejean, 2017), and often exploited by viruses. Interestingly, oncogenic viral infections can also increase metabolic and proangiogenic markers through expression of a very specific domain that also controls SUMO enzymes expression (Pozzebbon *et al.*, 2013). Viral exploitation of SUMOylation has been recently detailed in elegant reviews (Mattosio *et al.*, 2013; Lowrey *et al.*, 2017; Wilson, 2017), to which readers can refer. In the following sections we will provide some classic examples on how oncogenic viruses

impact SUMOylation to increase their ability to infect, persist, and transform host cells.

### Oncoviruses exploitation of the SUMO pathway

Infection with oncogenic viruses is also involved in cancer pathogenesis, accounting for about 15% of total malignancies in 2012 (Plummer *et al.*, 2016). Seven viruses are associated with human cancers, including hepatitis B virus (HBV), hepatitis C virus (HCV), high-risk human papillomaviruses (HPV), Epstein–Barr virus (EBV), Kaposi's sarcoma herpesvirus (KSHV, also known as human herpesvirus type 8 HHV-8), human T-cell leukaemia virus type 1 (HTLV-1), and the recently emerged Merkel cell polyomavirus (MCPyV) (Mesri *et al.*, 2014; Spurgeon and Lambert, 2013).

#### Hepatitis B virus

HBV is a partially double-stranded circular DNA virus belonging to the *Hepadnaviridae* family. Persistent infection with HBV is associated with several liver diseases such as hepatocellular carcinoma (HCC), the most common cancer of the liver [reviewed in Di Bisceglie (2009)]. HCC pathogenesis is a combination of both indirect effects as a consequence of the chronic inflammatory condition due to the persistent HBV presence in liver cells, and directly through viral proteins expression. In particular, HBV X antigen (HBx), a viral product that acts as transcriptional cofactor during viral replication, is also able to promote cellular transformation altering crucial cellular pathways involved in cell growth, DNA repair, apoptosis, and cell cycle progression [recently reviewed in Xie (2017)].

Notably, several of these modifications are mediated by exploitation of SUMO pathway by HBx. Indeed, in HBV infected cells, HBx promotes deSUMOylation and relocalization of the host transcription factor Sp110, usually conjugated to SUMO1 and expressed inside PML-NBs. The detachment of SUMO1 moiety and the resulting Sp110 differential distribution in infected cells increases viral DNA load, decreases apoptosis and increases viability of hepatocytes, and markedly affects expression levels of genes involved in type I interferon pathway, a common response mechanism to viral infections. Mechanistically, HBx may

promote the formation of Sp110–SENP1–HBx complex able to catalyse SUMO1 removal from Sp110 and to translocate HBx to Sp110 gene promoters in order to reprogram host gene expression and to trigger viral proliferation (Sengupta *et al.*, 2017). These findings highlight the importance of the SUMOylation and deSUMOylation switch in the infection lifecycle and tumorigenesis triggered by HBV.

In addition, HBx expression in mice and human cell lines prompts cell growth altering the SUMOylation status of E-cadherin, a membrane protein crucially involved in epithelial-mesenchymal transition (EMT). Opposite to SUMOylation of Sp110, HBx expression promotes SUMO1 and 2/3-conjugation to E-cadherin, leading to E-cadherin degradation, EMT-transition, loss of cell-to-cell contact, and overgrowth of hepatocytes (Ha *et al.*, 2016). Notably, SUMO1, SUMO2/3, SAE1/2, UBC9, and SENP2 are differentially expressed in HCC and play key roles in HCC pathogenesis [(Liu *et al.*, 2015), as recently reviewed in Tomasi and Ramani (2018)], further underlining the importance of SUMOylation in liver cells transformation. However, if these alterations are directly mediated by HBV proteins or are a consequence of cancer growth is still an unresolved issue and will not be further described in this review.

#### Hepatitis C virus

Together with HBV, HCV is another important aetiological agent of HCC (Di Bisceglie, 1995). HCV is an enveloped, single-stranded RNA virus belonging to the *Flaviviridae* family. Similarly to HBV, HCV can promote HCC development as a consequence of the chronic inflammatory condition associated with its persistence in hepatocytes, or through direct effects mainly mediated by the viral core, non-structural proteins 3 (NS3), and NSSA, crucial players in viral replication and in alteration of the host gene expression landscape [reviewed in Irshad *et al.* (2017)]. In particular, NSSA affects cellular pathways involved in liver cell proliferation, apoptosis immune response, and DNA repair (Irshad *et al.*, 2017), and requires SUMOylation to increase its stability in host cells and to promote HCV replication. Indeed, NSSA is SUMOylated in the context of HCV infection by both SUMO1 and SUMO2/3, perturbing ubiquitination occurring at the same target lysine, and suppressing NSSA

proteasomal degradation. In addition to stability, SUMOylation also regulates the interaction of NSSA with NSSB occurring during replication complex formation (Lee *et al.*, 2014), a key event for viral replication. Indeed, SUMO1 is overexpressed in infected Huh7.5 cells (Akil *et al.*, 2016), and abrogation of SUMO conjugation by *Ubc9* silencing markedly impairs HCV RNA replication (Lee *et al.*, 2014; Akil *et al.*, 2016), suggesting the importance of SUMOylation for HCV lifecycle in hepatocytes. However, on the contrary, a recent report showed that SUMO removal obtained by silencing of *PIAS2* during HCV infection enhances stability and expression of NS3 and NSSA, and increases HCV replication, in a SUMO1-dependent manner (Guo *et al.*, 2017). Reasons for these apparent discrepancies are currently unknown and future studies are therefore needed to better clarify whether HCV-mediated tumorigenesis benefits or is dampened by SUMO.

### Human papilloma virus

High-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) (Bouvard *et al.*, 2009) are the aetiological agent of cervical cancer and are also associated with other anogenital malignancies, such as vulvar, vaginal, anal, and penile cancers, and with a significant proportion of oropharyngeal tumours [reviewed in (zur Hausen, 2009)]. HPVs are double-stranded DNA viruses that promote malignant transformation in chronically infected keratinocytes of epithelia mainly due to the viral oncoproteins E6 and E7 that, through degradation of tumour suppressors p53 and retinoblastoma (pRb), modify fundamental cellular pathways involved in cell cycle, apoptosis, DNA repair, and senescence [reviewed in Tommasino (2014)]. In addition to E6 and E7, HPV infection in keratinocytes entails the concerted and sequential action of other early non-structural proteins E1, E2, E4 and E5, and viral capsid protein L1 and L2 [reviewed in Woodman *et al.* (2007)]. Most of these viral proteins exploit SUMOylation to subvert cellular pathways and promote viral persistence in the host.

E2 is a multifunctional regulatory protein that binds to viral DNA and interacts with cellular proteins to regulate viral gene expression, partitioning and replication, and to modify host transcriptome (reviewed in McBride, 2013). HPV18 E2 is a substrate for mono SUMOylation *in vitro*, in an *E. coli*

expression system, and in HeLa cells after overexpression of HPV16 E2, Ubc9, and either SUMO1, 2, or 3, despite a preference for SUMO2/3. Notably, the defective E2 SUMO mutant shows defects in transcriptional ability, suggesting a crucial role for SUMOylation in mediating E2 activities during HPV-mediated transformation (Wu *et al.*, 2008). In addition, SUMOylation at K292 increases E2 expression levels after exogenous overexpression of SUMO components and by endogenous elevation of SUMOylation obtained after heat shock, due to a SUMO-mediated inhibition of E2 ubiquitination and degradation (Wu *et al.*, 2009). Combining these results with the observation that SUMO2/3 is progressively up-regulated during keratinocytes differentiation (Deyrieux *et al.*, 2007), the emerging scenario depicts that the increased SUMO2/3 expression in suprabasal layer of epithelium stabilizes E2, increases E2 concentration and activity, and promotes viral production (Wu *et al.*, 2007).

The E6 and E7 viral oncoproteins drive malignant transformation mostly due to the degradation of p53 and pRb, respectively. However, in addition to these two well characterized pathways, a number of other cellular proteins are affected by E6 and E7 during viral infection and transformation [reviewed in Moody and Laimins (2010)]. Among these, clever strategies are adopted by HPV oncoproteins to hijack SUMO during infection and tumorigenesis. HPV16 E6 and E7 overexpression in the natural host of the virus, primary human keratinocytes, significantly increases the accumulation of UBC9 and SUMO1-conjugated species (Mattosio *et al.*, 2017). Notably, similar results were also found in human samples during the natural evolution of cervical and oropharyngeal cancer (Mattosio *et al.*, 2015, 2017), underlining the importance of SUMO alterations during HPV transformation. Mechanistically, HPV16 E6/E7 prevents the autophagy-dependent UBC9 degradation obstructing the final step of autophagic pathway in E6/p53-dependent manner (Mattosio *et al.*, 2017, 2018). The resulting increased UBC9 level confers apoptosis resistance to the infected keratinocyte (Mattosio *et al.*, 2017), thus extending HPV persistence in the host and triggering cellular transformation. However, this E6-mediated UBC9 accumulation seems to be a cell-specific mechanism dependent on the cellular background of analysed cells. Indeed, E6 overexpression in

immortalized, p53 defective, cell lines drive UBC9 degradation through proteasomal-dependent pathway (Heaton *et al.*, 2011) while in primary, p53 competent cells, E6 triggers UBC9 accumulation following autophagy defects. These and other results (Boggio *et al.*, 2004, 2007) highlight a dual way to control UBC9 levels, further underlining the fundamental role of the SUMO E2 enzyme in cellular physiology.

In addition to impact on UBC9 expression, E6 also co-opts SUMOylation to re-direct activities of cellular transcription factors. Indeed, the transcriptional co-activator hADA3 (human alteration/deficiency in activation3), is down-regulated by HPV16 E6 in cervical cancer cells. In contrast to HPV E2 that exploits SUMO-conjugation to protect its own ubiquitin-dependent proteasomal degradation, HPV16 E6 triggers SUMOylation to induce ubiquitin attachment and hADA3 degradation. SUMO-dependent hADA3 deprivation encourages cell proliferation, migration, and anchorage independent growth of cervical cancer SiHa cells, pointing to the important role of SUMOylation in malignant transformation (Chand *et al.*, 2014).

Similarly, in addition to contributing to UBC9 overexpression, E7 also usurps SUMOylation to regulate levels and activity of a transcription factor crucially involved in cell cycle progression, cell proliferation, and DNA damage response, Forkhead box M1b (FoxM1b). FoxM1 de-regulation occurs in a variety of malignancies [reviewed in Myatt and Lam (2007)], where its activity and expression are frequently modified by PTMs [reviewed in van der Horst and Burgering (2007)], including SUMOylation. Indeed, *in vivo* SUMOylation assays in HEK293T cells show that FoxM1 could be modified by all three SUMO paralogues after physical interaction with UBC9 and PIAS1. Similar results were obtained also in MCF-7 cells (Myatt *et al.*, 2014). After SUMO conjugation, FoxM1 is rapidly degraded and re-localizes from nucleus to cytoplasm, suggestive of a negative regulatory loop mediated by SUMOylation to turn off its transcriptional activity (Myatt *et al.*, 2014; Jaiswal *et al.*, 2015). Notably, HPV16 E7 interferes with SUMO loading on FoxM1 by inhibiting its association with UBC9, in turn reducing FoxM1 SUMOylation and protecting it from re-localization and degradation (Jaiswal *et al.*, 2015). SUMOylated FoxM1

increases cell proliferation and delays mitotic progression (Myatt *et al.*, 2014), indicating the importance of the E7-mediated SUMO manipulation in the context of HPV-mediated cellular transformation.

Finally, SUMOylation of the late structural capsid protein L2 also plays crucial role in HPV infectivity and cellular transformation. Indeed, modification with SUMO2/3 increases L2 half-life and inhibits interaction with the other capsid protein L1, suggesting that capsid assembly could be modulated by SUMOylation during HPV infection (Marusic *et al.*, 2010).

### Epstein–Barr virus

EBV was the first virus clearly connected with human malignancies, since it was isolated in 1964 in cultured lymphoblasts from Burkitt's lymphoma cells (Epstein *et al.*, 1964). Since then, EBV infection was also consistently associated with a number of other malignancies such as nasopharyngeal cancer, Hodgkin's and non-Hodgkin's lymphomas, and a subset of gastric cancers [reviewed in Thompson and Kurzrock (2004)]. EBV is a double-stranded DNA Herpesvirus that could establish latent and lytic infection in lymphoblastoid cells, characterized by restricted viral gene expression and life-long persistence, and with virions production, respectively, in lymphocytes and epithelial cells. Several proteins are involved and expressed in lytic reactivation, to promote cell proliferation, virus production, and oncogenesis [reviewed in Tsurumi *et al.* (2005)]. Among them, the transcriptional activator Zta could be modified by both SUMO1 and SUMO2/3 (Adamson and Kenney, 2001; Hagemeyer *et al.*, 2010) at K12. SUMOylated Zta associates with and carries HDAC3 on its targeted promoters which, in this way, acetylates and exerts an inhibitory activity at Zta-responsive genes (Murata *et al.*, 2010). Consistently with the role of SUMOylation in mediating repression of Zta, the SUMO defective mutant increases gene expression and re-activation of latent EBV. Notably, the SUMO-mediated repression of Zta *in vivo* could be reverted by both the viral encoded EBV kinase (EBV-PK) and RanBPM during infection, reducing Zta SUMO conjugation, promoting transcription of Zta genes and replication of the viral genome (Hagemeyer *et al.*, 2010; Yang, Y.C. *et al.*, 2015). SUMOylation and activity of Zta are also

finely regulated by interaction of SUMOylated Zta with SIM motifs of the EBV protein kinase BGLF4. After SUMO-mediated Zta–BGLF4 interaction, the kinase activity of BGLF4 abolishes Zta SUMOylation, activating virus production (Li *et al.*, 2012).

Similar to Zta, the activity of the other EBV protein involved in lytic reactivation, Rta (Tsurumi *et al.*, 2005), is crucially regulated by SUMO. Yeast-two-hybrid screen identifies UBC9 and PIAS1 as binding partners of Rta, that is SUMOylated both *in vitro* and *in vivo* during the early stages after lytic induction of EBV infection. However, contrary to Zta, SUMO1 conjugation increases the transcriptional ability of Rta, suggesting a crucial role for SUMOylation for EBV lytic reactivation. Indeed, since Rta mediates Zta transcription (Adamson and Kenney, 2001), SUMO could both serve initially as activator of lytic phase by conjugating to Rta and promoting transcription of genes such as Zta, and then as modulator of EBV reactivation through SUMOylation-mediated Zta repression triggered by viral EBV-PK and cellular RanBPM.

Latent membrane protein 1 (LMP1) is primarily involved in EBV oncogenesis due to its ability to mimic CD40 receptor and to constitutively transduce growth signals that trigger tumorigenesis in infected cells (Gires *et al.*, 1997). LMP1 physically interacts with UBC9 to increase protein SUMOylation in latent infected cells (Bentz *et al.*, 2011). Among SUMOylated proteins, LMP1–UBC9 interaction promotes SUMO conjugation of Interferon Regulatory Factor 7 (IRF7) to promote its nuclear localization and increases its stability in EBV infected cells. However, despite nuclear accumulation, SUMOylation inhibits IRF7 association with chromatin, thus reducing its transcriptional activity and the ability to induce innate immune response (Bentz *et al.*, 2012). Moreover, LMP1 aids to preserve viral latency (Adler *et al.*, 2002) and SUMOylation plays pivotal roles also in EBV lytic reactivation. Indeed, LMP1 triggers SUMOylation of the transcriptional repressor KRAB-associated protein-1 (KAP1). In EBV-transformed lymphoblastoid cell line, SUMOylated KAP1 associates with viral EBV lytic promoters OriLyt, ZTA and RTA, promoting the transcriptional repression that contributes to the maintenance of viral latency (Bentz *et al.*, 2015).

In addition to LMP1, a recently reported genome-wide screening identifies other EBV proteins having global effects on host SUMOylation. In particular, overexpression of the transcriptional activator BRLF1 consistently decreased levels of both SUMO1 and SUMO2 conjugated proteins in transfected 293T and HeLa cells, while six EBV proteins up-regulated SUMOylation. Among them, expression of SM, an mRNA binding protein, increases levels of SUMO1 and to less extent SUMO2 conjugated proteins. This effect is due to the ability of SM to interact and bind UBC9 and SUMO, thus acting as an E3 ligase that promotes SUMO conjugation of cellular proteins such as p53. Consistently, SM depletion in AGS-EBV infected cells reduces global SUMOylation levels, suggesting the ability of SM to affect SUMOylation during viral lytic infection (De La Cruz-Herrera *et al.*, 2018).

In addition to proteins, EBV also encodes a variety of microRNAs (miRNAs) during viral infection and oncogenesis. Bioinformatic analysis based on miRNA target prediction identified 575 proteins of the SUMO interactome that could be potentially targeted and modulated by EBV miRNAs and a set of 14 predicted 3' UTR were also experimentally validated in luciferase reporter assays. SUMO proteins targeted by EBV miRNAs are mainly involved in cancer-related functions such as proliferation, apoptosis, growth signalling, and intercellular communication, suggesting that miRNAs play fundamental roles during EBV carcinogenesis (Callegari *et al.*, 2014). Accordingly, the EBV-encoded miR-BHRF-1 promotes accumulation of SUMO2/3 conjugated proteins during lytic infection due to down-regulation of RNF4 (Li *et al.*, 2017).

### **Kaposi's sarcoma-associated herpesvirus**

KSHV is responsible for Kaposi's sarcoma, a malignancy commonly occurring in AIDS patients. In addition to Kaposi's sarcoma, KSHV has been detected in primary effusion lymphoma and in multicentric Castleman's disease [reviewed in Ganem (2006)]. KSHV is a double-stranded DNA herpes virus that primarily infects endothelial and B cells that frequently exploits SUMOylation to promote its replication [recently reviewed in Chang and Kung (2014)]. Similar to EBV, KSHV infection

cycle can be divided in lytic and latent phases. Viral reactivation can be triggered by a number of specific environmental stimuli and by the viral protein K-Rta. The lytic phase is characterized by a short period where viral genes are expressed, where during the latency there is the expression of a limited number of viral genes without production of viral particles (Aneja and Yuan, 2017). One of the most abundantly genes expressed during latent phase, the latency-associated nuclear antigen (LANA) protein, is a crucial regulator of dormant infections, viral reactivation and cellular transformation [reviewed in Uppal *et al.* (2014)]. To exert its functions, LANA extensively exploits SUMOylation machinery. Indeed, LANA contains a SIM to allow interaction with SUMO2 modified proteins such as KAP1 which is in turn recruited to specific chromatin sites to silence viral gene expression (Cai *et al.*, 2013). Therefore, the LANA SIM motif plays a fundamental role in KSHV latency, even if data point to a direct binding of LANA with KAP1 independently of SIM (Sun *et al.*, 2014). Furthermore, LANA is SUMOylated itself and its expression levels in KSHV infected SLK cells are regulated by a finely tuned deSUMOylation activity mediated by SENP6. Chromatin immunoprecipitation sequencing experiments identified that LANA binds SENP6 promoter, with subsequent repression of SENP6 expression. Given that SENP6 protease removes SUMO moieties from LANA to decrease its expression and to promote viral gene expression, these results suggest that LANA inhibits SENP6 to regulate its own SUMOylation and expression levels in infected cells, and to maintain KSHV latency (Lin *et al.*, 2017).

KSHV encodes two additional transcription factors, K-bZIP (KSHV basic leucine-zipper) and K-Rta that are crucially regulated by SUMO. K-bZIP is an early lytic gene rapidly expressed after acute infection or during reactivation from latency (Lin *et al.*, 1999), that acts as transcriptional repressor through inhibition of the viral transactivator K-Rta (Izumiya *et al.*, 2003). Similar to LANA, also K-bZIP needs SUMOylation to increase its activity, since expression of the SUMO specific protease SENP1 attenuates transcriptional repression of K-Rta. K-bZIP could be conjugated by both SUMO1 and SUMO2/3 and requires interaction with UBC9 at viral promoters to mediate its repression activity (Izumiya

*et al.*, 2005). Consistently, CHIP-seq studies revealed deposition of SUMO2/3 throughout KSHV genome after viral reactivation, mirrored by decreased expression of KSHV genes (Yang, W.S. *et al.*, 2015), suggesting that SUMOylation may be involved in chromatin remodelling during viral reactivation. Notably, K-bZIP also shows SUMO E3 ligase activity with specificity towards SUMO2/3 that catalyses SUMOylation of interacting partners such as p53 and pRb (Chang *et al.*, 2010), and deposition of SUMO2/3 in chromatin locus enriched for SUMO2/3 (Yang, W.S. *et al.*, 2015). Indeed, experimental KSHV reactivation in infected B lymphoma cell line is complemented by a specific increase of SUMO2/3 conjugation and inactivation of promoter regions of genes involved in immune response such as IRF-1, IRF-2, and IRF-7 (Chang *et al.*, 2013). Collectively, these results suggest that SUMOylated K-bZIP interacts with UBC9, mediates SUMO2/3 modification of viral and cellular chromatin through its E3 SUMO ligase activity and shut-off of KSHV gene expression, and dampens the host immune activation, thus contributing to hide the virus from host responses during viral reactivation. Therefore, KSHV could regulate gene expression and viral replication manipulating SUMOylation. Indeed, modulation of global SUMO conjugation quickly occurs after induction of K-bZIP and K-Rta expression in chronically EBV-infected TRE × BCBL-1 K-Rta cell lines and is accompanied by modulation of viral gene expression (Wang *et al.*, 2017). While K-bZIP promotes accumulation of SUMO-conjugated proteins, the viral activator K-Rta decreases global SUMOylation through its SUMO-targeting E3 ubiquitin ligase (STUbL)-like activity. STUbL proteins contain SIMs to interact with SUMO to ubiquitylate their targets (Perry *et al.*, 2008). Indeed, K-Rta contains SIM domains able to bind SUMO moiety and to catalyse attachment of ubiquitin and proteasome-dependent degradation on targeted SUMOylated proteins. Among them, K-Rta promotes degradation of viral proteins like K-bZIP, and cellular proteins such as PML in order to create a conducive environment for viral replication (Izumiya *et al.*, 2013). Therefore, KSHV expresses two different early genes acting as SUMO E3 ligases (K-bZIP) or STUbL (K-Rta) that differently affect SUMOylation status of infected cells in diverse phases of viral infection

cycle. These examples are explanatory of how the dynamic and reversible alteration of SUMO conjugation represents a convenient strategy that oncogenic viruses exploit to alter and adapt host environment for viral purposes.

### Human T-cell leukaemia virus type 1

HTLV-1 is the aetiological agent of adult T-cell leukaemia (Poiesz *et al.*, 1980). Transforming ability of HTLV-1 mainly relies on the oncoviral protein Tax, a transcriptional activator able to initiate T-cell proliferation and differentiation (Jeang *et al.*, 2004). Activation of the NF- $\kappa$ B pathway, a crucial step towards transformation of a T-cell in a leukaemic cell, is finely regulated by concerted SUMO/ubiquitin conjugation steps that specifically shuttle Tax between cytoplasm and nuclear bodies. In particular, SUMOylated Tax is conjugated by ubiquitin through the STUbLs-mediated activity of RNF4 (Fryrear *et al.*, 2012) and migrates to cytoplasm to allow interaction with the regulatory subunits of I $\kappa$ B kinase, NEMO, and the subsequent relocation of the NF- $\kappa$ B subunit RelA to the nucleus. Then, deubiquitinated Tax translocates to nuclear bodies where it is SUMOylated on the same lysine residue by the resident UBC9 and SUMO, associates with RelA and NEMO, and starts the transcription of Tax-responsive genes mediated by NF- $\kappa$ B (Lamsoul *et al.*, 2005; Nasr *et al.*, 2006; Kfoury *et al.*, 2011)

### Merkel cell polyomavirus

MCPyV is the most recently emerged oncovirus, since it has been detected in about 80% of Merkel cell carcinoma, a neuroendocrine disorder of the skin frequently found in immune depressed patients [recently reviewed in (DeCaprio, 2017)]. MCPyV transforming ability mainly resides on the expression of Large T antigen proteins (Large- LT, and Short- ST) (Houben *et al.*, 2010), even if molecular details driving MCPyV-mediated cell transformation are still not fully elucidated, although a role for ST is emerging (Shuda *et al.*, 2011). Similarly, a role for viral exploitation of the SUMO pathway during Merkel cell carcinoma has not yet been investigated, even if a recent report shows that MCPyV replication depends on PML-NBs, suggesting a possible involvement of SUMOylation in regulating MCPyV transformation (Neumann *et al.*, 2016).

## Conclusions

Post-translational modification by SUMO plays central roles during oncogenic viral infections. SUMOylation is a physiological pathway regulating proteins activity, altering localization, interaction with DNA and other proteins [reviewed in Wilkinson and Henley (2010)]. At cellular levels, SUMOylation regulates processes such as cell division, DNA replication and repair, cell signalling, chromatin remodelling, apoptosis and proliferation [reviewed in Wilson (2009)]. Since the wide impact on cell physiology, alteration of SUMOylation is a convenient way that oncoviruses frequently exploit to mediate persistence in the host. Indeed, with the exception of the recently discovered MCPyV, human oncovirus extensively manipulate SUMO to modify both viral and cellular proteins. Specifically, viral oncoproteins from HCV (NSSA), KHSV (LANA), and HTLV-1 (Tax), as well as viral structural and transcription factors such as HPV E2 and L2, EBV Zta and Rta, KHSV K-bZIP and K-Rta are all modified by one or more SUMO paralogues to alter their localization, transcriptional ability, protein-protein, protein-DNA interaction, and stability. Also, proteins from oncogenic viruses could act as specific SUMO E3 ligase to catalyse the addition of SUMO moiety to cellular target (K-bZIP) or as STUbL to clear SUMOylated proteins through the ubiquitin-mediated proteasomal degradation (K-Rta). Notably, these two apparently contrasting activities are mediated by the same oncovirus (KHSV) in different steps of viral infections, suggesting the importance of SUMOylation to quickly and completely revert cellular activities for virus purposes. Furthermore, several oncoviruses proteins, such as HBx, E6, E7, and LMP1, mediate SUMOylation of specific transcription factors to trigger or dampen expression of genes that finally promote virus infectivity and oncogenesis. Similarly, viral proteins and miRNAs could also globally affect SUMOylation in infected cells, both increasing (E6/E7, LMP1, SM, miR-BHRF1-1) or decreasing (BRLF1) SUMOylation of specific SUMO paralogues. Strikingly, the ability of altering global SUMOylation could also be contingent on the manipulation of the sole SUMO E2-conjugating enzyme, UBC9, whose alteration could completely revert host functions to advantage virus endurance, as exemplified by HPV E6. Collectively, findings summarized here clearly

suggest that SUMOylation is a pivotal pathway during infection and transformation triggered by oncoviruses. Therefore, strategies aimed at interfering with viral manipulation of SUMO components could be beneficial in the attempt to reduce cancer burden arising from viral infection.

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