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Adenovirus E1B-55K Controls SUMO-Dependent Degradation of Antiviral Cellular Restriction Factors

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Abstract

The human adenovirus type 5 (HAdV-C5) early region 1B 55-kDa protein (E1B-55K) is a multifunctional protein that promotes viral replication and adenovirus-mediated cell transformation through various mechanisms that primarily counteract host intrinsic and innate immunity. These include post-translational activities that exploit the host cell ubiquitin- and SUMO-conjugation machineries to regulate antiviral cellular restriction factors. However, despite significant advancements in this field, several underlying mechanisms governing these processes remain unidentified to date. Here, we performed SILAC-based quantitative SUMO proteomics to better understand cellular consequences of E1B-55K-mediated host cell modulation and adenovirus infection in general. We assessed cellular proteins for abundance changes and SUMO2 conjugate proteome changes during infection with wild type HAdV-C5 or E1B-55K deletion mutants. We provide evidence that changes in the SUMOylated proteome have the potential to regulate the DNA damage response, cell cycle control, chromatin assembly, and gene transcription and present these data as a resource for the research community. Strikingly, we identified a SUMO-dependent, ubiquitin-mediated degradation mechanism for some SUMO substrates, suggesting that E1B-55K may use multiple mechanisms to alter the activity of restrictive cellular pathways.

Importance

HAdVs generally cause mild and self-limiting diseases of the upper respiratory and gastrointestinal tracts but pose a serious risk to immunocompromised patients and children. Moreover, they are widely used as vectors for vaccines and vector-based gene therapy approaches. It is therefore vital to thoroughly characterize HAdV gene products and especially HAdV virulence factors. E1B-55K is a multifunctional HAdV-encoded oncoprotein involved in various viral and cellular pathways that promote viral replication

and cell transformation. We analyzed the E1B-55K-dependence of SUMOylation, a post-translational protein modification, in infected cells using quantitative proteomics. We found that HAdV increases overall cellular SUMOylation and that this increased SUMOylation can target antiviral cellular pathways that impact HAdV replication. Moreover, we showed that E1B-55K orchestrates this SUMO-dependent degradation of certain cellular antiviral factors. These results once more emphasize the key role of E1B-55K in the regulation of viral and cellular proteins in productive HAdV infections.

Introduction

Viruses reorganize and manipulate cell processes for their own benefit in order to efficiently replicate in their hosts. To achieve this, viral proteins engage in complex interactions with host proteins to alter different cellular networks. Consequently, viral proteins can modify the functionality of various cellular proteins by regulating their stability and activity. Viral proteins often interfere with reversible post-translational modifications (PTMs), which in turn, are mediated by the host cell machinery. PTMs include phosphorylation, ubiquitination, and modification by small ubiquitin-like modifiers (SUMO) (1).

Protein SUMOylation is a pivotal PTM that regulates almost all cellular pathways (2). Mammalian cells encode three different SUMO isoforms, SUMO1, SUMO2, and SUMO3, while others like SUMOs 4 and 5 are less investigated, have previously been suggested to be non-expressed pseudogenes, and remain mostly enigmatic (3-6). Initially, SUMO-specific proteases (a.k.a. sentrin-specific proteases, SENPs) maturate SUMO precursors by cleavage and subsequent exposure of their carboxy termini (7-9). Conjugation begins with the heterodimeric E1 enzyme containing SAE1/SAE2 (SUMO-activating enzyme subunit 1/2), which forms a thioester bond with maturated SUMO. In the second step, E1-

conjugated-SUMO is transferred to a cysteine residue within the SUMO E2-conjugating enzyme, ubiquitin-conjugating enzyme 9 (Ubc9). The conjugation of some SUMO-substrates requires the catalytic activity of a SUMO E3-ligase that enhances the rate of transfer from Ubc9 to target lysines and may confer substrate specificity *in vivo*. SUMOylation is reversed by sentrin proteases (SENPs), which catalyze the deconjugation of SUMO from its substrates (10).

SUMO modification regulates many nuclear-specific functions such as gene expression, genome stability, DNA damage repair, and RNA processing (7, 9, 11-14). Hence, dysregulation of SUMOylation can alter cell division and survival resulting in cancer progression and viral pathogenesis (15-19). Viruses have evolved different mechanisms to modify protein SUMOylation to ensure proper viral replication and in some cases also cell transformation. Numerous viruses suppress overall cellular SUMOylation. For example, the ICP0 protein from herpes simplex virus-1 (HSV-1) ubiquitinates SUMOylated proteins that are then targeted to the proteasome for degradation (20). Another example is the global decrease in SUMOylation induced by the viral kinase BGLF4 during Epstein-Barr virus (EBV) infection to facilitate its lytic replication (21). In contrast, the principal EBV oncoprotein LMP1 (latent membrane protein-1) mediates an increase in global cellular SUMOylation, which is important for its oncogenic potential and the regulation of the innate immune response during latency (22-24). Furthermore, global SUMOylation is increased upon influenza virus infection (25). The modulation of global substrate SUMOylation by viruses is mainly mediated by targeting the SUMOylation machinery (17).

Human adenoviruses (HAdVs) are double-stranded, non-enveloped, linear DNA viruses that generally cause mild or even asymptomatic disease in immunocompetent individuals, whereas severe disease in immunocompromised patients is a serious health concern (26). HAdVs encode proteins that can deregulate different PTMs to achieve

efficient viral DNA replication (27). Of particular importance is the viral multifunctional protein early region 1B 55-kDa (E1B-55K) from human adenovirus species C type 5 (HAdV-C5), which mediates lytic and oncogenic activities in several critical steps of infection by counteracting host intrinsic and innate immunity pathways (27-29). Its functional range is maximized by usurping different cellular post-translational machineries that additionally regulate each other. For example, in cooperation with E4orf6 (early region 4 open reading frame 6), E1B-55K forms an SCF (Skp, cullin, F-box)-like E3-ubiquitin-ligase complex by interacting with the cellular proteins cullin-5 (Cul-5), Rbx1/RCO1/Hrt1, and elongins B and C. This complex can ubiquitinate cellular proteins such as Mre11, p53, DNA ligase IV, Tip60 (a lysine acetyltransferase), integrin alpha3, ATRX, and SPOC1, and can target them for proteasomal degradation (30-36). Recent findings indicate that E1B-55K/E4orf6-mediated ubiquitination can also operate in a non-degradative fashion. This ubiquitination process is implicated in the modulation of cellular RNA-binding proteins, influencing the processing of viral RNA (37). In the early phase of HAdV-C5 infection, E1B-55K counteracts anti-proliferative processes comprising p53-dependent and -independent induction of apoptosis, cell cycle arrest, and cellular DNA damage response (38, 39). In the late phase, viral late mRNAs are preferentially accumulated within the cytoplasm by E1B-55K to ensure efficient late viral protein production (40-43), although this phenomenon has recently been shown to be, at least in part, due to an export competition between cellular and viral RNAs (44).

E1B-55K further interferes with cellular anti-apoptotic mechanisms such as those mediated by promyelocytic leukemia nuclear body (PML-NB)-associated factors Mre11 and Daxx (45-47). Studies on Daxx repression revealed a novel mechanism through which E1B-55K exploits the cellular PTM pathways to fulfill its function (46, 48, 49). Furthermore, E1B-55K interacts with the cellular SUMO-targeted ubiquitin ligase

120 (STUbL) protein RNF4, which induces ubiquitination of SUMOylated Daxx to induce its
121 proteasomal degradation (50). SUMOylation of another PML-NB component, the Sp100A
122 protein, is also induced by E1B-55K (51). This promotes the interaction between Sp100A
123 and p53 at PML-NBs, which in turn inhibits Sp100A-induced p53 activation. Besides
124 inducing SUMOylation of cellular proteins, E1B-55K induces deSUMOylation of target
125 proteins such as KAP1, a protein that regulates transcription of both cellular and viral
126 genes (52).

127 In this study, we present analyses of the SUMO2-proteome in HeLa cells infected with wild
128 type (WT) HAdV-C5 and E1B-55K-null mutant (ΔE1B) viruses. Upon infection, HAdV-C5
129 increases general cellular SUMOylation of proteins involved in DNA damage response,
130 chromatin assembly, transcription, and regulation of the cell cycle. We also provide
131 further evidence that E1B-55K is involved in a SUMO-dependent degradation of several
132 key cellular factors including all three MRN complex proteins and FAM111B. This study
133 provides insights into the role of E1B-55K in supporting efficient infection and is a starting
134 point from which to assess how the cellular SUMOylation machinery is exploited by the
135 virus.

136 **Results**

137 Adenovirus infection induces SUMOylation of cellular proteins.

138 To assess the effect of adenovirus infection on SUMO conjugation to human proteins, HeLa
139 cells stably expressing 6His-SUMO2 were infected with WT HAdV-C5 or the E1B-55K
140 deficient mutant (ΔE1B-55K) for 48 hours at an MOI of 20 and both crude cell extracts
141 and Ni^{2+}-NTA purifications prepared for western blot analysis. Anti-6His western blot
142 analysis of these samples (Fig. 1A) showed that both infections stimulated an increase in

143 the overall abundance of 6His-SUMO2-conjugated proteins in comparison to non-infected
144 cells (mock), although the mutant gave a less robust response. The choice of a "late" time
145 point (48 h p.i.) strategically compensates for the replication delay caused by the E1B-55K
146 mutant and provides a single readout endpoint (53). This approach serves as a well-
147 considered compromise, enabling a comprehensive understanding of the impacts of E1B-
148 55K during infection.

149 To measure these changes on an individual protein basis during HAdV-C5 infection and
150 investigate the influence of E1B-55K, we employed a SILAC-based quantitative proteomic
151 approach involving purification of 6His-tagged SUMO2 under denaturing conditions,
152 combined with tandem mass spectrometry (LC-MS/MS) and subsequent data analysis
153 using MaxQuant software (Fig. 1B). Denaturing lysis of cells inactivates SUMO proteases
154 and reduces non-covalent protein interactions during enrichment of 6His-SUMO2
155 conjugates using Ni^{2+} affinity chromatography (54), although many cellular proteins bind
156 non-specifically to this affinity matrix. HeLa 6His-SUMO2 cells were grown in lysine and
157 arginine isotopically normal (light) or nitrogen (^{15}N) and carbon (^{13}C) isotopically heavy
158 SILAC media. SILAC-labeled light cells were infected with either WT HAdV-C5 or the
159 ΔE1B-55K variant and extracts were mixed with heavy (H) labeled mock extract as
160 indicated in Figure 1B, to give two separate SILAC mixes. Both shared the same heavy,
161 mock-infected condition, which could be used as a reference to compare data between the
162 two viral responses. Both the “crude” whole-cell extracts and the “pure” 6His-SUMO2
163 conjugate fractions were analyzed to assess virus-induced changes to total protein
164 abundance as well as SUMO2 conjugation state, respectively. Confirming the experimental
165 design, viral proteins were detected in the light (infected) isotopic conditions of the crude
166 cell extracts, and E1B-55K was detected only in the WT-infected samples (Fig. S1A). In
167 general, abundances of virus proteins were slightly higher in the WT infection compared

to the E1B-55K null mutant (Fig. S1B), indicating a temporally more advanced infection with the intact virus. There was evidence that E4orf6 levels were higher during the E1B-55K null mutant virus infection (Fig. S1B). E4orf6 partners with E1B-55K along with cullin-based cellular machinery in the formation of an SCF-like ubiquitin E3 ligase complex to degrade host cell proteins (33). Therefore, higher levels of E4orf6 may be a consequence of reduced stability in absence of E1B-55K.

In whole-cell extracts, over 5600 proteins in total (~5300 proteins common to both infections) were detected and quantified (see Supplementary Datafile 1 for details). According to data derived from crude cell extracts, changes in cellular protein levels during infection with both viruses were broadly modest (Fig. 2A & B – x-axes). The data from Ni^{2+}-NTA affinity purifications describing changes to the SUMO conjugation status of proteins (Fig. 2A & B – y-axes), showed a much wider range in protein ratios and did not correlate well with total protein data (Fig. 2A & B – x-axes). Only a small number of proteins showed similar ratio changes in both fractions (Fig 2A & B - dotted circles), confirming that changes in SUMO2 conjugation are for most proteins, not explained by altered protein abundance in the cells. Consistent with the finding that the WT virus triggers a greater SUMOylation response than the ΔE1B-55K mutant (Fig. 1A), there appeared to be more proteins experiencing increased SUMOylation during WT infection than during infection with the mutant (Fig. 2A & B pink areas). We also found a smaller number of proteins that apparently showed loss of SUMO during infection (Fig. 2A & B blue areas). These data show that SUMOylation changes are on a larger scale than that of the background proteome, and represent a potentially significant biological response to adenovirus infection.

Adenovirus-induced SUMOylation changes of proteins involved in gene transcription, protein ubiquitination, and DNA damage response

The 50 proteins apparently most increased or reduced in SUMO2 conjugation during WT HAdV-C5 infection can be found in Figure S2A & B. Some interesting antiviral factors such as dermcidin (DCN) and lysozyme C (LZC) appear in these lists, although they have not been described as SUMO substrates previously, and data for total cellular abundance change were not available. Therefore, these have a high probability of being non-specific column contaminants and have not been followed up in this study. Several well-characterized SUMO substrates including the Werner syndrome helicase (WRN) (55), histone deacetylase HDAC4 (56) and the PML body component Daxx (57) appear to show increased SUMOylation during WT virus infection, while BCLAF1, a transcription repressor involved in interferon signaling (58), showed a reduction. Mre11 and Nibrin are two members of the MRN (Mre11-RAD50-Nibrin) DNA double-strand break repair complex and experienced between 6 and 15-fold increases in SUMO2 conjugation. The MRN complex represses viral replication by promoting unproductive viral DNA concatemer formation and is subject to multiple levels of regulation by adenovirus proteins. These data indicate that MRN complex SUMOylation increases during adenovirus infection, although whether this is a pro-viral or pro-host response is unclear.

To search for broad biological patterns in the adenovirus-induced SUMOylation data, bioinformatics analysis was undertaken. We performed a STRING 'Proteins with values/ranks' analysis to look for gene ontology term enrichments at either extreme of the ratio range. Concentrating on the WT virus data and using the entire set of 4231 infected/uninfected ratios from 6His-SUMO2 purifications, almost 100 functional categories were returned by the analysis, most of which showed a coordinated increase in apparent SUMOylation during infection (see Supplementary Datafile 2 for details). One

such group contained proteins involved in ubiquitin and ubiquitin-like protein modifications and contained several members of the SCF ubiquitin ligase machinery (Fig. 3A). Notably, SKP2 (5.0-fold), and Cul5 (5.8-fold) were among the proteins showing the largest increase in SUMOylation in this group, although neither was measured in the mutant samples. This network also included other E3 ubiquitin ligases including TRIM33, UHRF1, RAD18, ARIH2, and several RING-finger proteins (Fig. 3A). Three SUMO-specific E3 ligases, protein inhibitors of activated STAT (PIAS) 1, 3, and 4 were strongly increased in SUMOylation (Fig. 3B). Three sub-networks related to DNA damage repair and regulation of TP53 degradation (Fig. 3C - E) also contained many factors with increases in SUMOylation upon infection. The above-mentioned MRN complex and the SMC5-SMC6 complex contained many of the proteins with the greatest increase in SUMO conjugation during WT HAdV-C5 infection (Fig. S2A). Although less robust than these, several proteins with structural similarity including zinc finger and homeobox domain-containing transcription factors and co-factors also seem to undergo a general increase in SUMO conjugation during infection (Fig. 3F - H). A large and well-connected network of ribosomal proteins showed a modest increase in SUMO2 conjugation during infection (Fig. 3I). Many of these have been defined as SUMO substrates, and although their canonical roles are as cytoplasmic constituents of ribosomes, some have functions outside of this context (59). We cannot exclude the possibility that modification of transient nuclear forms may explain these changes. RPS27A was the most highly upregulated ribosomal protein. However, RPS27A is initially expressed as a fusion with ubiquitin which is post-translationally cleaved to yield free ubiquitin, and the increase in ubiquitin seems to be a consequence of increased ubiquitination associated with SUMO2 during viral infection (explored further below). Functional groups of proteins experiencing deconjugation upon infection were less significantly enriched (Supplementary Datafile 2),

with modest deconjugation shown for proteasome proteins and factors involved in apoptosis. In summary, changes to cellular SUMOylation in response to adenovirus infection appear to influence the ubiquitin and SUMO systems, DNA damage response, gene and protein expression, and protein turnover.

Effect of E1B-55K deletion on the cellular response to adenovirus infection.

For the same duration and MOI as for WT HAdV-C5 infected cells, SUMOylation changes during infection with the mutant adenovirus deleted of E1B-55K were generally smaller (compare Fig. S3A with B). Many of the proteins with a large and significant increase in SUMOylation during WT virus infection were not detected at all in the 6His-SUMO2 enrichments from the mutant infected samples (Fig. S3C). It is possible that some of these are E1B-55K dependent targets, but due to the stochastic nature of peptide sampling in data-dependent mass-spectrometry some proteins that are actually present may not have been detected in both SILAC mixes. Therefore, it is possible that the absence of a protein was for technical, rather than biological reasons. Because the heavy isotope condition derived from mock-infected cells was common with the WT infected cells (Fig. 1B), the difference in the SUMOylation response between the virus types for common identifications can be inferred from the ratio of both H/L ratios. From this analysis, WT/ΔE1B-55K ratios were calculated for 3696 proteins (Fig. 4A & Supplementary Datafile 1). For most proteins, WT/ΔE1B-55K ratios were close to 1, with less than 1% of identifications differing in apparent SUMO conjugation status by more than a factor of 2 (Fig. 4A). The top 50 most differing between the two virus types are shown in Figure S4, with validations of selected proteins based on antibody availability and quality (Fig. 4D & Fig. S5). CREB regulated transcription coactivator 3 (CRTC3) and the histone acetyltransferase EP300, were among those showing the strongest increase in –infected cells (Fig. 4A & Fig. S4), followed by many ubiquitin or SUMO ligases including RAD18,

TRIM33, UBR1 and PIAS proteins 1 and 2. Heterogeneous nuclear ribonucleoprotein M (hnRNPM), a pre-mRNA binding protein involved in splicing, is more than twice as SUMOylated during infection with the WT virus compared to the mutant (Fig. 4A & Fig. S4). hnRNPM was in the top 1% of all protein intensities in 6His-SUMO2 purification, suggesting it is a very abundant substrate. Testament to this is that in these purifications the large and difficult to identify tryptic SUMO-hnRNPM branched peptides (at lysine 145) were also detected and displayed the same abundance pattern as the entire protein (Supplementary Datafile 3 & Fig. S6A & B). Confirmation of the reported E1B-55K effect was also obtained by western blotting (Fig. S6C).

The list of proteins showing the opposite trend of apparently greater SUMOylation in the E1B-55K null variant samples compared to the WT (Fig. S4), contained many zinc-finger proteins, including ZNF189, ZNF205, ZNF444, and ZNF800, all of which were at least twice as SUMOylated during infection with the mutant virus compared to the WT. However, a negative $\log_2$ WT/mutant ratio is not necessarily an indication that the WT triggered greater SUMO2 modification than the mutant, as for some proteins infection with the WT virus stimulated a loss of SUMO not detected in the mutant infection. Examples include NME7, ZNF205, RREB1 and PRDM2 (see Supplementary Datafile 1 for details). Notably, once again MRE1 and Nibrin appeared in this shortlist this time accompanied by RAD50 (Fig. S4). A comparison of infected/uninfected ratios from 6His-SUMO purifications for both viruses (Fig. 4B) shows these groups of substrates with preferential modification by each virus (Fig. 4B yellow and orange regions). Along with the MRN proteins, the glucuronyltransferase B3GAT3, CRMP1 (collapsin response mediator protein 1), the phosphodiesterase PDE4D, PRRC2C (proline-rich coiled-coil 2C – a.k.a. BAT2D1) and the trypsin-like peptidase FAM111B all showed apparent ΔE1B-55K preferential modification. By WT/ΔE1B-55K ratios alone, FAM111B was reported to be

over four times as SUMOylated during infection with the E1B-55K null virus than with the WT (Fig. 4A), but FAM111B SUMOylation not only increased with the mutant but also apparently reduced with the WT (Fig. 4B). Certain cellular proteins exhibiting deregulated SUMOylation patterns have been observed to undergo degradation during HAdV infection. As FAM111B has been discussed as a target for E1B-55K ubiquitination (60) we interrogated the whole-cell extract data to determine any change in total protein abundance. Indeed, the crude cell lysate data showed that the total cellular abundance of FAM111B is modestly increased with the mutant virus, but reduced with the WT (Fig. 4C, validated in Fig. 4D). The equivalent analysis for MRN proteins revealed a similar pattern of greater apparent SUMOylation and total abundance during ΔE1B-55K infection compared to WT (Fig. 4C), and was confirmed independently by western blotting (Fig. 4 E & F). Thus, adenovirus infection appeared not only to influence the SUMOylation status of these proteins but also their stability, and E1B-55K had some influence on both.

The relationship between SUMOylation change and total protein abundance highlighted the need to consider both when analyzing our data. To do this, a hierarchical clustering analysis was undertaken. This revealed 329 proteins with known SUMO modification sites, identified in both 6His-SUMO2 preps and whole-cell extracts, and with a Significance B value less than 0.01 in any ratio comparison. The result of this is shown in Figure 5 for proteins with the largest ratios and Figure S7A & B for the remainder.

The proteins with the largest changes can be broadly grouped into 4 clusters. Cluster A contains Mre11, Nibrin, FAM11B and RAD50, whose SUMOylation and cellular abundance are lower in WT infected cells compared with those infected by the mutant. Cluster B proteins contains those, which had larger SUMO modification levels for WT than for ΔE1B-55K but little evidence for a change in cellular abundance. The proteins in cluster C also showed little change in cellular abundance but appeared to have similar increases in

SUMOylation for both infections. Cluster D proteins exhibited increased SUMO induction for ΔE1B-55K infections but not WT but with modest cellular abundance changes (Fig. 5). Together, these data identify a relatively complex range of responses by groups of proteins to infection with WT HAdV-C5, which in some cases are influenced by the loss of E1B-55K.

Increased SUMO2 polymerization and ubiquitination associated with substrates during adenovirus infection.

One of the groups from the hierarchical clustering analysis, Cluster I (Fig. S7A), contained many proteins that showed modestly increased SUMOylation during WT virus infection, but little or no change with the mutant. Notably, this group contained SUMO1, SUMO3, and ubiquitin (RPS27A). Post-translational modifiers like these can be detected in 6His-SUMO2 purifications for two reasons; first, if they are themselves conjugated to the C-terminal glycine of 6His-SUMO2, or second if they modify 6His-SUMO2. In a manner akin to ubiquitin, SUMO2 is known to form polymeric chains in vivo via K11 (61). We can measure changes to SUMO polymers by monitoring SUMO-SUMO branched peptides in the mass spectrometry data (62). We found peptides diagnostic of SUMO2-SUMO2 and SUMO2-SUMO3 polymers (Fig. S8A & B), both of which more than doubled during infection with the WT virus but were not induced as much with the mutant (Fig. 6A). Furthermore, three sites diagnostic of Ub-Ub linkages via K11, K48, and K63 were identified and quantitated (Fig. 6B). These also showed an increase in Ni^{2+}-NTA fractions upon infection with WT virus which was somewhat blunted by loss of E1B-55K (Fig. 6B). This implies that viral infection triggers SUMO polymerization and induces ubiquitin polymerization on SUMO-modified substrates, and also implicates E1B-55K in this process. We validated this ubiquitin-dependent proteasomal degradation of SUMO-modified proteins in wt-infected cells using SUMO- (ML-792) and ubiquitin-inhibitors

(TAK-243) and. Here, we show that Mre11 is a target of SUMO-dependent degradation, as evidenced by substantially differing protein steady-state levels in the SUMO inhibitor-treated versus the DMSO-treated condition (Fig. S9). While the protein can be detected in mock-infected cells, it is nearly completely degraded in HAdV-C5 infections in the absence of the inhibitors, and the degradation is incomplete in ML-792-treated cells. Moreover, we observed reduced GTF2I-degradation in inhibitor-treated cells, suggesting that this protein is also degraded and undergoes SUMO-dependent proteolysis during infection. Given the reduced E1B-55K levels after ML-792 treatment, these processes may also be E1B-55K-dependent. UHRF1, in contrast, was clearly not degraded during infection, regardless of the treatment conditions (Fig. S9). TAK-243-treatment and thus, inhibition of the ubiquitin activating enzyme (UAE), results in a remarkable inhibition of viral protein expression and increased abundance of SUMO2 (63), which we will follow up on in future projects (Fig. S9).

While we detected almost the entire viral proteome in our SUMO2 enrichments, this is not evidence enough that these proteins are SUMO-modified, as nickel affinity chromatography can yield many non-specific purification contaminants. Our SUMO branched peptide search of the MS data, however, suggested the modification of E2A/DBP (at lysines 202 and 503) and IVa2 (at lysine 71; Supplementary Datafile 3). E2A/DBP has been shown to harbor SUMO conjugation sites (64), but our findings require additional experimental investigation to confirm the major acceptors and functional consequences in these proteins.

Discussion

The SUMO pathway is an attractive target for viruses. While some, such as HSV-1, trigger a reduction in total SUMO conjugation during infection (20), others including influenza

366 (25) and as shown here, adenovirus, trigger a net increase. The downstream
367 consequences of SUMO modification are often substrate-specific and have been
368 previously shown to affect protein function in many ways. Among other mechanisms,
369 SUMOylation can affect catalytic activity, subcellular localization, protein complex
370 formation, and protein stability (65, 66). However, the protein-specific consequences of
371 SUMOylation are unknown for most substrates, and the stoichiometry of modification is
372 often very low, making the prediction of biological consequences and salience difficult.

373 To begin to understand this in the context of adenovirus infection, we have used
374 quantitative proteomics to monitor changes in total protein abundance and SUMO2
375 conjugation during infection of cultured human cells with HAdV-C5. We decided to work
376 in HeLa cells due to their frequent usage in studying the impact of SUMOylation on virus
377 infection, both by other researchers and ourselves (e.g. (20, 67)). This choice allows for
378 more precise comparisons with previous studies that have examined SUMOylation during
379 HAdV infection and/or investigated the characteristics of SUMOylated HAdV proteins
380 utilizing the same cell line. We identified over a hundred proteins with increased
381 SUMOylation during HAdV-C5 infection, and offer this as a resource to the research
382 community from which future studies may develop. Notably, many of the proteins whose
383 SUMOylation is altered during adenovirus infection have already been described as
384 important restriction factors to viral replication. A particularly conspicuous example was
385 HDAC4, which experienced a more than 6-fold increase in SUMOylation during
386 adenovirus infection. HDAC4 is a class II HDAC that promotes type I IFN signaling by
387 interacting with and recruiting STAT2 to multiple ISG promoters following IFN-alpha
388 (68). Thus, HDAC4 has been shown to be a virus restriction factor and is known to be
389 targeted by vaccinia virus and HSV-1 (68). WT HAdV-C5-induced SUMOylation of HDAC4
390 may alter its activating capacity on promoters (68, 69) and, subsequently, regulate type I

IFN signaling. HDAC4-mediated transcriptional repression is apparently reduced in a SUMOylation-deficient mutant (56), implying that increased SUMOylation may enhance repression. HDAC4 is known to bind multiple transcription factors (e.g., MEF2, Runx2, SRF, and HP1) (70-72), therefore HDAC4 regulation may impact many different genes that would promote virus replication.

POLDIP3 (encodes the DNA polymerase delta interacting protein 3) is a protein that is affected by viral infection in terms of both SUMO conjugation and overall abundance, regardless of E1B-55K (Fig. 5 and Supplementary Datafile 1). Previous studies have described its degradation during HAdV infection, suggesting that this degradation may also be SUMO-dependent (73). Another intriguing cellular protein of this category is GTF2I (TFII-I), one of the most SUMO2 modified proteins within the cell. The fact that this highly modified protein undergoes a roughly 2-fold SUMOylation change is remarkable (Fig. 5 and Supplementary Datafile 1). Previous research has demonstrated that E4-region proteins (especially E4orf3) stimulate SUMOylation of TFII-I, leading to its proteasomal degradation (67, 74, 75). Future work will entangle this complex interplay of E1B-55K and other viral factors during degradation of host restriction factors. It is noteworthy that fowl adenovirus Gam1 has been demonstrated to be the first example of a viral protein that exhibits affinity towards the E1 heterodimer, thereby impeding its functionality and leading to a complete SUMOylation block (76). Additional E1B-55K-independent effects on SUMOylation of adenovirus proteins, besides E4orf3, includes the direct interaction between E1A and Ubc9 and effects on polySUMOylation (77). Thus, E1B-55K aligns with the aforementioned adenovirus proteins known to interfere with the host SUMOylation system.

A second potentially salient responder to adenovirus infection was the mRNA splicing factor hnRNPM which experienced a 3-fold increase in SUMO2 conjugation during

adenovirus infection. hnRNPM is one of the most heavily modified cellular proteins with 25 sites identified to date (May 2023 - www.phosphosite.org). Despite this, the functional consequences of SUMOylation are currently unclear. hnRNPM is a nuclear pore complex-associated protein that is also involved in cellular RNA sensing of incoming RNA viruses. Upon Sendai virus and enterovirus infection, it has been shown that hnRNPM is re-localized from the nucleoplasm to the cytoplasm where it is capable of inhibiting RNA virus-triggered innate immune responses as a proviral factor (78, 79). However, studies on Semliki Forest virus (an RNA virus) and HSV-1 (a DNA virus) showed that hnRNPM may be an antiviral factor, as its depletion results in enhanced gene expression and replication (78, 80).

It has been proposed that the biological consequences of SUMOylation may manifest through the accumulation of multiple low-level modifications to proteins that form higher-order multi-protein complexes via multiple SUMO-SIM (SUMO interaction motif) interactions (81), thus increasing the stability of these complexes. Hence, modest SUMOylation changes to multiple proteins from functionally related pathways may coordinate to result in significant biological effects. Many proteins experiencing increased SUMOylation during adenovirus infection are involved in gene expression, RNA metabolism, DNA repair, and protein expression. Therefore, it is possible that SUMOylation of these groups alters the cellular environment to either promote or inhibit the viral life cycle through the stabilization of multi-protein complexes. One large group of proteins showing a coordinated increase in conjugation during adenovirus infection was ubiquitin and SUMO metabolizing enzymes. Many ubiquitin E2 enzymes, SCF components, RNF proteins, and TRIMs were among the group of proteins experiencing increased SUMOylation during adenovirus infection. In particular, the ubiquitin E3 ligase UHRF1 showed an almost 4-fold increase in SUMOylation. UHRF1 is known to play roles

441 in both epigenome maintenance and genome integrity (82) and is modified by SUMO at
442 multiple sites (14 sites so far identified - July 2021 - www.phosphosite.org).

443 Intriguingly, cullin-5 was among the most responsive proteins to adenovirus,
444 experiencing an increase in abundance in 6His-SUMO purifications of over 6-fold. Direct
445 evidence for Cul5 SUMOylation has not yet been established, so it is possible abundance
446 changes in 6His-SUMO2 enrichments may be independent of modification. However, Cul5
447 was not detected in samples purified from cells infected by the E1B-55K mutant virus, so
448 Cul5 modification could be E1B-55K-dependent. E1B-55K is a member of the Cul5-based
449 E3 ubiquitin-ligase complex that targets substrates for polyubiquitination and
450 subsequent proteasomal degradation (33), and this putative increase in modification by
451 SUMO potentially identifies a functional link between E1B-55K and the SUMO response to
452 infection. Three SUMO E3 ligases (PIAS 1, 2, and 3) also were among the most responsive
453 SUMO substrates to adenovirus infection, with conjugation increasing approximately 3-
454 fold over uninfected cells. Increased self-modification of E3 ligases can be evidence of
455 higher rates of enzymatic turnover, and therefore this raises the possibility that PIAS
456 proteins may be involved in the shift towards increased SUMOylation during adenovirus
457 infection. PIAS proteins have been shown to negatively regulate transcription factors by
458 inhibiting their DNA-binding activity (83), recruiting transcriptional co-regulators such
459 as HDACs to inhibit transcription (84), or by recruiting p300 or CBP (cyclic-AMP-
460 responsive-element-binding protein (CREB)-binding protein) to activate transcription
461 (85). Notably, p300 (EP300) and CRTC3 both showed increased SUMO2 conjugation
462 during infection and this appeared to be positively influenced by E1B-55K. p300 and
463 CRTC3 both bind CREB1, a transcription factor that stimulates transcription at the DNA
464 cAMP response element (CRE) found in many viral and cellular promoters. Specifically,
465 EP300 is thought to be important for inducing chromatin remodeling of proviral genes

(86), although whether its SUMOylation is pro- or antiviral is unclear. PIAS 1 regulates a specific subset of IFN-responsive genes including hnRNPM providing two mechanisms by which E1B-55K-mediated hnRNPM-regulation could interfere with the cellular immune response.

Another SUMO E3 ligase that undergoes higher SUMOylation during adenovirus infection is NSMCE2. This is a member of the SMC5-SMC6 complex involved in DNA double-strand break repair, which along with four other members of this complex (NSMCE1, NDNL2, SMC5, and SMC6) are among those proteins that experience the greatest increase in conjugation during viral infection. Similarly, two of the three members of the DNA double-strand binding MRN complex, namely Nibrin and Mre11 are also more SUMOylated upon WT adenovirus infection, providing strong evidence for a DNA damage focus for SUMO changes. Interestingly, the third member, RAD50 appeared not to become more SUMOylated at the 48h time point used in our proteomic study. In fact, by monitoring both SUMOylation and total protein abundance changes we found that all three members of the MRN complex undergo an initial phase of SUMOylation followed by ubiquitination and degradation. Interestingly, it appears that the relocalization of the MRN complex via SUMOylation is also induced by E4orf3 and earlier time points post-infection (87). Additional research is warranted to comprehensively investigate the impact of adenovirus proteins on the cellular DNA damage response through the induction of SUMOylation. For viruses lacking E1B-55K, this process is slower, raising the possibility that E1B-55K is involved in the degradation of SUMOylated cellular proteins. For example, E1B-55K may recruit other factors such as the STUbL RNF4 (50), or E1B-55K may itself be a STUbL. Notably, E1B-55K contains a number of putative SUMO interaction motifs (SIMs), some of which are within hydrophobic domains conserved between E1B-55K proteins of different serotypes of different species (Fig. S10) (88, 89). Recent data indicate

conserved features of E1B-55K SUMOylation (89), but the potential influence of these SIMs on E1B-55K function remains to be established.

For the majority of proteins, changes in SUMOylation and total proteome levels during infection with the mutant virus lacking E1B-55K were similar to the WT. Only 17 proteins in our study were found to be more than twice as SUMOylated during infection with WT virus compared to the ΔE1B-55K mutant. The aforementioned EP300 and CRTC3 were the only two that showed apparently strict dependence upon E1B-55K for their SUMO modification. The remainder still showed some degree of SUMOylation increase during infection with the E1B-55K null mutant, meaning their modification is not strictly dependent on E1B-55K but influenced either directly or indirectly by it. In a manner similar to the MRN complex proteins three further proteins showed apparently greater SUMOylation with the E1B-55K null mutant; PDE4D (phosphodiesterase 4D), PRRC2C (proline-rich coiled-coil 2C), and the trypsin-like peptidase FAM111B. FAM111B is known to be antiviral and is likely a substrate for E1B-55K-mediated ubiquitination (60) although we could find no published link between adenovirus and PDE4D or PRRC2C. Importantly, the apparently greater SUMOylation observed with the mutant virus was at least in part caused by a more advanced response during WT infection. What appears to be the case is at the 48 hour time point studied here, WT infections have proceeded further than those with the mutant and these proteins had already begun to be degraded. This degradation disproportionately affected the SUMOylated forms, suggesting this is a SUMO-dependent degradation triggered by viral infection. Stracker and colleagues have shown that early in HAdV-C5 infection, the virus-encoded E4orf3 protein induces Mre11 and NBN SUMOylation, whereas at later time points E1B-55K acts in combination with E4orf6 to target these cellular proteins for proteasomal degradation (36). Therefore, we propose a model for adenovirus infection where a small number of cellular factors follow

the same process of an initial phase of SUMO modification followed by E1B-55K-mediated ubiquitination, then degradation.

In summary, we defined the changes to the cellular SUMO proteome in response to adenovirus infection using WT and ΔE1B-55K viruses, and established a mechanism for cellular protein turnover involving both SUMO- and ubiquitin-mediated degradation that influences the activities of many fundamental pathways with bearing upon viral infection and cell transformation. This study represents a resource for future work, which will aim to establish the extent to which this mechanism is employed in the regulation of cellular protein functions.

Materials and methods

Cell lines and viruses

HeLa cells stably expressing 6His-SUMO2 were generated as previously described (54). All cells were grown in Dulbecco's Modified Eagles Medium (DMEM; Gibco, CA, USA) containing 10% fetal calf serum (PAN-Biotech, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (PAN-Biotech). Additionally, HeLa 6His-SUMO2 were maintained in 2 µM puromycin selection to ensure 6His-SUMO2 expression.

The H5*pg*4100 virus strain was used as the WT HAdV-C5 virus (90). The H5*pm*4149 strain is a mutant virus in the H5*pg*4100 background, that contains four stop codons in the E1B-55K open reading frame to prevent the expression of this early adenovirus protein (in the text referred to as ΔE1B-55K, or as ΔE1B in the figures) (91). Both virus strains were propagated and titrated as previously described (90).

SILAC labeling and viral infections

Stable isotope labeling with amino acids in cell culture (SILAC) was performed as previously described (20). Briefly, HeLa cells overexpressing 6His-SUMO2 were cultured in SILAC DMEM medium (Invitrogen, CA, USA), which lacks L-lysine and L-arginine. These amino acids were replaced by either light (L) or heavy (H) stable isotope-labeled forms. Mock-infected cells were cultured in heavy DMEM SILAC medium, containing $^{13}C_6$ $^{15}N_2$-lysine, Lys^8 and $^{13}C_6$ $^{15}N_4$ arginine, Arg^{10}, whereas cells either infected with the WT or ΔE1B-55K virus were cultured in light DMEM SILAC medium containing isotopically normal lysine and arginine (Lys^0, Arg^0) (Fig. 1). DMEM SILAC cell culture medium was supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin and 2 µM puromycin. The cells were cultured in their corresponding medium for a period of six cell divisions (7 days) prior to being seeded into 40 x 150 mm cell culture dishes for each condition. The cells were infected with a multiplicity of infection (MOI) of 20 with either the WT or ΔE1B-55K mutant virus, or mock-infected. Cells were harvested at 48 hours post-infection (h p.i.).

Enrichment of putative SUMO conjugates by nickel affinity purification

The nickel (Ni^{2+}) purification of SUMO-conjugated proteins was performed as previously described (54). Briefly, cells were washed with PBS before lysis in guanidinium hydrochloride buffer (denaturing GuHCl Ni^{2+} sample buffer) and stored at -80°C until further use. Equal amounts of heavy (mock-infected) and light (WT or ΔE1B-55K) labeled protein lysates were mixed and sonicated. Cell debris was removed by centrifugation and sterile filtration (0.45 µm). 50 µl of pre-equilibrated Ni^{2+}-nitrilotriacetic acid (NTA) agarose beads were added to filtered lysates and were incubated at 4°C for 24 h. Afterwards, agarose beads were pelleted by centrifugation and washed once with

denaturing Ni^{2+} sample buffer, followed by subsequent washes with buffers at decreasing pH. Proteins that bound to the beads were eluted in 40 µl of Ni^{2+} resin elution buffer and stored at -20°C.

Proteomic analysis

The schematic workflow of the SILAC experiment is shown in Figure 1. Prior to Ni^{2+} purification, crude samples were prepared by adding 400 µl of 10% trichloroacetic acid (TCA) to 35 µl of mixed protein lysates and incubation on ice for 20 min. Precipitated proteins were pelleted by centrifugation at 14,000 rpm for 15 min at 4°C and washed in 4°C 100% ethanol. After another centrifugation step, supernatants were discarded and pellets were dried in a gyrovap centrifuge and then resuspended in 80 µl of LDS sample buffer (Invitrogen, CA, USA). 35 µl of the Ni^{2+}-NTA purified proteins, 30 µg of crude samples, and 20 µl of crude mixtures were resolved on a 10% SDS gel and Coomassie-stained. Lanes were sliced into sections 1-12 (Fig. 1) and peptides were extracted by tryptic digestion (92) including alkylation with iodoacetamide and resuspended in 35 µl acidic solution. Peptide samples were analyzed by LC-MS/MS on a Q Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA) coupled to an EASY-nLC 1000 liquid chromatography system via an EASY-Spray ion source (Thermo Fisher Scientific) running a 75 µm x 500 mm EASY-Spray column at 45°C. Data were acquired in the data-dependent mode. Full scan spectra (m/z 300–1,800) were acquired with resolution R = 70,000 at m/z 200 (after accumulation to a target value of 1,000,000 with a maximum injection time of 20 ms). The 10 most intense ions were fragmented by HCD and measured with a resolution of R = 17,500 at m/z 200 (target value of 500,000, maximum injection time of 60 ms) and intensity threshold of $2.1x10^4$. Peptide match was set to 'preferred' and a 40-second dynamic exclusion list was applied. For each experiment, two runs were performed with elution gradients of 90 min and 240 min.

All 96 raw mass spectrometry (MS) datafiles were processed together using MaxQuant (v 1.3.0.5) with the built-in Andromeda peptide search engine (93, 94). The human UniProt proteome was searched along with proteins encoded by HAdV-C5. Enzyme specificity was set to trypsin-P. Cysteine carbamidomethylation was selected as a fixed modification with methionine oxidation and protein N-terminal acetylation as variable modifications. The initial maximum allowed mass deviation was set to 20 parts per million (ppm) for peptide masses and 0.5 Da for MS/MS peaks. A false discovery rate (FDR) of 1% was required at both the protein and peptide levels. 'Requantify' was applied and the 'match between runs' option was selected with a time window of two minutes. The experimental design template was set such that gel slices from the Ni^{2+}-NTA purifications were numbered 1 to 12 for both experiments and 101 to 112 for the crude extracts so matching only occurred between adjacent slices of the same fraction type across both experiments. Outliers in the data were selected based on significance B (SigB) <0.01 (94) and $\log_2$ L/H ratio >1.

Functional enrichment and protein network analyses

275 proteins whose SUMOylation apparently changed between WT adenovirus C type 5 infected and uninfected cells were uploaded to STRING (www.STRING-db.org) for functional enrichment analysis compared to the human proteome. 4,231 proteins with a ratio reported in comparisons between WT-infected and uninfected 6His-SUMO purifications, were analyzed by STRING "Proteins with values/ranks" (95) to identify functional groups whose SUMOylation was globally regulated by infection. A summary of the uploaded data and STRING outputs is in Supplementary Datafile 2. A large interaction network of the 275 regulated proteins was created in STRING using a high minimum interaction score (0.7). Smaller sub-networks were created by the same method using proteins belonging to functional groups defined as being globally regulated by

610 SUMOylation during WT virus infection. Networks were either taken directly from
611 STRING or rendered in Cytoscape (96).

612 Confirmation of putative SUMO targets

613 To confirm the SUMOylation of some of the putative SUMO substrates, parental and 6His-
614 SUMO2 HeLa cells were WT- or ΔE1B-55K infected with an MOI of 20 or mock-infected.
615 At 48 h p.i., eighty percent of the cells were harvested in lysis buffer for purification of
616 SUMOylated proteins. Protein lysates were incubated with prewashed Ni^{2+}-NTA agarose
617 beads (Thermo Fisher Scientific) for 16 h at 4°C and subsequently eluted. The remaining
618 twenty percent of the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer
619 for analysis of steady-state levels, as previously described (91). Purified SUMO substrates
620 and crude extracts were resolved by SDS-PAGE and western blotting.

621 Western blotting and antibodies

622 Equal volumes of the crude or Ni^{2+}-NTA-purified protein lysates were used for SDS-PAGE
623 separation and western blotting. The protein concentration of the crude extracts was
624 determined using the Bradford assay (Protein Assay Dye Reagent Concentrate; BioRad,
625 CA, USA), and diluted 1:3 to 1:4, depending on the protein concentration. After SDS-PAGE
626 separation, samples were transferred to a nitrocellulose membrane. Blots were blocked
627 with 5% (w/v) non-fat milk in 1x PBS, incubated with the respective antibodies (Tab. 1),
628 and visualized as described previously (91).

629

Table 1: Antibodies used in this study.

Antibody	Dilution	Reference or company
α-β-actin mouse mAb A-5441	1:5,000	Sigma-Aldrich (MO, USA)
α-6his-tag mouse mAb 631212	1:5,000	Clontech (Japan)
α-HAdV-C5 E1B-55K mouse mAb clone 2A6	1:10	(97)
α-HAdV-C5 E2A/DBP mouse mAb clone B6-8	1:10	(98)
α-HAdV-C5 E1A mouse mAb clone M73	1:10	(99)
α-Mre11 rabbit mAb NB100-142	1:5,000	Novus (CO, USA)
α-NBS1 (Nibrin) rabbit mAb USB-350756	1:1,000	Biozol (Germany)
α-hnRNPM mouse mAb sc-20001	1:200	Santa Cruz (TX, USA)
α-KAP1 (Tif1β) rabbit pAb sc-33186	1:1,000	Santa Cruz (TX, USA)
α-FAM111B rabbit pAb HPA038637	1:2,000	Sigma-Aldrich (MO, USA)
α-UHRF (D6G8E) rabbit mAb #12387	1:1,000	Cell Signaling
α-GTF2I (PA5-17642) rabbit pAb	1:1,000	Invitrogen
HRP α-mouse 115-036-003	1:10,000	Jackson (PA, USA)
HRP α-rabbit 111-036-003	1:10,000	Jackson (PA, USA)

Inhibitor assays

Ubiquitin-mediated proteasomal activity was inhibited with TAK-243 (5 μM; Hycultec), and ML-792 was used as a selective small-molecule SUMO-inhibitor (2 μM; MedChemExpress) (100, 101). Dimethyl sulfoxide (DMSO) was included as the negative control in these experiments. Infected cells (MOI 40) were treated at 8 h p.i., harvested at 24 h p.i., and the protein steady-state levels were analyzed by western blotting as described above.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (102) with the dataset identifier PXD042236.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Figure Legends:

Fig. 1: 48h adenovirus infection triggers a net increase in SUMO2 conjugation in cultured human cells. (A) Western blots of indicated proteins from crude lysates (crude) and Ni^{2+}-NTA affinity purified (pure) samples from HAdV-C5 WT and HAdV-C5 ΔE1B-55K-infected HeLa SUMO2 cells (antibodies are listed in Tab. 1). (B) SILAC-based proteomic experiment workflow. HeLa cells overexpressing 6His-SUMO2 were grown in light (L; isotopically normal, K0R0) or heavy (H; K8R10) SILAC medium prior to infection and 6His-SUMO2 enrichment. Proteins were separated by SDS-PAGE and the gel was cut into 12 slices for subsequent processing as indicated.

Fig. 2: Changes in cellular protein levels and protein SUMOylation during HAdV-C5 WT and HAdV-C5 ΔE1B-55K infections. (A) & (B) Scatter plots comparing Infected/Uninfected ratios from crude cell extracts (x-axes) with 6His-SUMO2 purifications (y-axes) for HAdV-C5 WT (A) and HAdV-C5 ΔE1B-55K (B) infections. Pink areas show putative SUMO substrates that respond to infection with increased conjugation, blue areas are those showing an apparent reduction in SUMOylation. Dotted circles indicate the positions of proteins for which changes in abundance in crude extracts and Ni^{2+}-NTA purifications are similar. In all charts, y=x is indicated with a broken line and fold changes less than a factor of 2 are bound by broken boxes.

Fig. 3: Networks of proteins with regulated SUMOylation during WT adenovirus infection. (A to I) Data for 4231 proteins identified from 6His-SUMO2 purifications were uploaded to STRING using the log2 infected/uninfected ratio data for HAdV-C5 WT as quantification. "Proteins with values/ranks" analysis was conducted to identify any functional enrichments in proteins at either end of the ratio scale (see Supplementary Datafile 2). STRING interaction networks were made for representative functional groups and uploaded to Cytoscape for visualization. Nodes are colored by ratio (see key). Grey nodes had no data for this set. Nodes with a thick border have already had a SUMO2 conjugation site identified through published high-throughput proteomic analyses. FDR: False discovery rate for the STRING analysis.

Fig. 4: Influence of E1B-55K on the cellular SUMO proteome during infection. (A) Relationship between abundance difference and total intensity for proteins identified in both 6His-SUMO2 purifications from HAdV-C5 WT and HAdV-C5 ΔE1B-55K-infected cells. Dotted lines indicate double or half abundance thresholds. Red markers are viral proteins, blue are SigB<0.01 and grey SigB>0.01. Selected outliers are indicated. (B) Scatter plot showing the relationship between infected/uninfected ratios for HAdV-C5 WT infection (x-axis) and HAdV-C5 ΔE1B-55K infection (y-axis) in Ni^{2+}-NTA purified samples. Yellow and orange regions in B indicate areas of the greatest differences between virus types. (C) Log_2-fold changes of SUMOylated protein abundances of FAM111B and MRN complex components in crude and pure lysates from HAdV-C5 WT and HAdV-C5 ΔE1B-55K infections. (D) – (F) Western blot confirmation for FAM111B (D), Nibrin (E) and Mre11 (F) of the data summarized in part (C). Here, the cells were harvested 48 h p.i. and 10% of each sample was separated for input preparation. 90% of the cells were lysed in GuHCl and subjected to Ni^{2+}-NTA purification. Input and Ni^{2+}-NTA purified samples were analyzed by SDS-PAGE and western blotting (antibodies are listed in Tab. 1). Asterisks in A&B indicate proteins previously described as a SUMO substrate.

Fig. 5: Hierarchical clustering analysis of significantly affected SUMO substrates during adenovirus infection. Hierarchical clustering analysis of the proteomic data from HAdV-C5 WT and HAdV-C5 ΔE1B-55K-infected cells relating to both SUMOylation changes and total protein abundance changes. Data derived from 329 proteins previously identified as SUMO substrates

with data in both SILAC experiments and significantly differing according to at least one ratio comparison. Clustering only by rows. The full dataset is shown as an insert, and the region expanded in the figure is boxed in red. Individual clusters are indicated with a brief summary of the group. WCE, whole cell extract.

Fig. 6: SUMO polymerization and SUMO-substrate ubiquitination increase during infection. (A) Left - ratio data for peptides diagnostic SUMO2-SUMO2 and SUMO2-SUMO3 polymerization via lysine 11 (K11). (B) Left - ratio data for ubiquitin-ubiquitin polymerization via lysines 11, 48 and 63. Proteins abundance changes are shown to the right of (A) & (B) using ratio data for all peptides from SUMO2, SUMO3 and ubiquitin, respectively. Bars show the median normalized ratio and MaxQuant derived coefficient of variability and values indicate SigB for infected/uninfected ratios.

Supplementary Figures:

Supplementary Fig. 1: Viral protein expression during infection with WT HAdV-C5 and ΔE1B-55K mutant.

Supplementary Fig. 2 (A & B): 100 most affected proteins by ratio in 6His-SUMO2 purifications during WT adenovirus infection.

Supplementary Fig. 3: Ratio versus intensity plots for 6His-SUMO2 data for both WT and ΔE1B-55K infection.

Supplementary Fig. 4: Summary lists of top 50 abundance changers by ratio in 6His-SUMO2 purified protein samples comparing HAdV-C5 WT with HAdV-C5 ΔE1B-55K infection.

Supplementary Fig. 5: Validation of proteomic data for selected substrates.

Supplementary Fig. 6: Increased hnRNPM modification during adenovirus infection.

Supplementary Fig. 7 (A & B): Hierarchical clustering analysis of significantly affected SUMO substrates during adenovirus infection (continued).

Supplementary Fig. 8: Annotated MS/MS spectra for SUMO2-SUMO2 and SUMO2-SUMO3 branched peptides.

Supplementary Fig. 9: SUMO-dependent, ubiquitin-mediated degradation of cellular proteins.

Supplementary Fig. 10: Putative SIM domains in E1B-55K.

Supplementary Datafiles:

Supplementary Datafile 1: Quantitative data

Supplementary Datafile 2: STRING enrichments

Supplementary Datafile 3: Branched peptides (GlyGly-K containing peptides)