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TARGETING SUMO E1 TO UBIQUITIN LIGASES: A VIRAL STRATEGY TO COUNTERACT SUMOYLATION

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Running title: A viral strategy to counteract SUMOylation

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The Small Ubiquitin-related Modifier, SUMO-1, is a ubiquitin-like family member that is conjugated to its substrates through three discrete enzymatic steps: activation, involving the E1 enzyme (SAE1/SAE2); conjugation, involving the E2 enzyme; substrate modification, through the cooperation of the E2 and E3 protein ligases. The adenoviral protein Gam1 inactivates E1, both in vitro and in vivo followed by SAE1/SAE2 degradation. We show here that Gam1 possesses a C-terminal SOCS domain that allows its interaction with two cellular cullin RING ubiquitin ligases (CRLs). We demonstrate that Gam1 is necessary for the recruitment of SAE1/SAE2 into Cul2/5-EloB/C-Roc1 ubiquitin ligase complexes and for subsequent SAE1 ubiquitylation and degradation. SAE2 degradation is indirect and due to its destabilization following SAE1 disappearance. These results reveal the mechanism by which a viral protein inactivates and subsequently degrades an essential cellular enzyme, arresting a key regulatory pathway.

Post-translational modifications of proteins are resourceful tools that cells use to control the function of proteins by regulating their activity, sub-cellular localization, stability, as well as their interaction with other proteins. They are also important to promptly adjust protein functions in response to changes in a cell's state or its environment. The dynamic post-translational process that covalently transfers ubiquitin to itself or to other proteins is called ubiquitylation. Ubiquitin transfer requires distinct chemical steps catalyzed by sequential activities of different enzymes (1). The ubiquitin E3s ligases perform the rate-limiting selectivity step of direct substrate recognition and are the most numerous and diverse in the ubiquitylation pathway. They can be sub-divided into different general protein families, based on their structural features and their characteristic motifs. These include the cullin RING (Really Interesting New Gene) ligases (CRLs). Through the assembly of their modular enzymatic core with different substrate-recognition components, CRLs can promote the specific ubiquitylation of a wide array of targets (2). Belonging to CRLs, the Cul2- and Cul5-based complexes share a common heterodimeric adaptor subunit, elongin B / elongin C (EloB/C) (3), which allows specific recruitment of the substrate-receptors that contain a SOCS motif (4).

The physiological importance of the CRL pathways is emphasized considering that many receptors and adaptors of these ligases are exploited by viral and bacterial pathogens to subvert normal cellular processes (5). For example, the HIV-encoded protein Vif interacts with (EloB/C), Cul5 and Roc1 to form an E3 that eliminates the host anti-viral factor APOBEC3G (6,7). Another example is the complex formed from the adenoviral proteins E4orf6 and E1B55K and the host Cul5 complex that catalyzes the elimination of the tumor suppressor p53 to allow efficient viral replication (8,9).

Gam1 is an essential viral protein encoded by the avian adenovirus CELO (10) able to interfere with cellular sumoylation, a post-translational ubiquitin-like pathway (11). Sumoylation, the covalent addition of SUMO proteins to its substrates, similarly to ubiquitylation, exploits different enzymatic reactions involving the E1-activating enzyme, the SAE1/SAE2 heterodimer, the E2-conjugating enzyme and E3 SUMO ligases (12). In this report we show that Gam1, recruiting Cul2/5-EloB/C-Roc1 through its C-terminus degenerate SOCS

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motif, targets the SUMO E1 heterodimer to these CRLs and promotes specifically SAE1 ubiquitylation. Subsequent proteasomal degradation of SAE2 depends on SAE1 disappearance, demonstrating that the two SUMO E1 subunits are mutually stabilized in vivo.

EXPERIMENTAL PROCEDURES

Plasmid constructs - pSG9m Gam1 WT (Gam1 WT-myc), pSG9m Gam1 L258/265A (Gam1 LL/AA-myc), GST-Gam1 WT and GST- Gam1 L258/265A were previously described (13,14). pSG9m Gam1 S251A, L252A, Q253A, D254A, W255A, A256G, R257A, L258A, V260A, L272A and P278A were generated by site specific mutagenesis. pCDNA3 SAE1-SV5 and pCDNA3 SAE2-HA were kindly provided by Ron Hay (15). pGEM3 Elongin B and pGEM3 Elongin C were kindly provided by Michele Pagano.

Transfection, Western Blot analysis and Immunoprecipitation - HeLa or Phoenix cells were transfected with Calcium-phospate method using the indicated plasmids. After 24 or 48 hours, cellular extracts were obtained lysing cells in E1A buffer (50 mM Hepes pH 7.0; 250 mM NaCl; 0.1% NP-40; 5 mM EDTA; 1 mM DTT; 0.2 mM PMSF; 1 mg/ml Leupeptin and 1mg/ml Aprotinin). In the experiments depicted in Figures 4E and 4F the cellular extracts were obtained using a SDS buffer (11). Immunoprecipitations (IPs) were done using protein extracts incubated with the indicated antibodies in E1A buffer. The following antibodies were used in these assays: anti-myc epitope 9E10, anti-HA epitope (12CA5); anti-HA-probe (Y-11), anti-Vinculin, anti-EloB (FL-118), anti-EloC (R-20) and anti-Cul5 (H-300) from Santa Cruz; anti-SV5 epitope (SV5-Pk1 clone, Serotec); anti-SAE1 and anti-SAE2 (kindly provided by Dr. Ron Hay); anti-Roc1 (Biosource); anti-Cul1, anti-Cul2 and anti-Cul3 from Zymed; anti-Cul4A (Biodesign); anti-HDAC2 (Abcam).

siRNA and shRNA - The siRNA experiment (Figure 4F) was performed using the SMARTpool reagents (Dharmacon) according the experimental procedures manual. Human Cul2 RNAi: M-007277; human Cul5 RNAi: M-019553; Control (CTRL): D-001206-13. The transfections were performed using the Oligofectamine Reagent (Invitrogen). The DNA for shRNA experiment (Figure 4E) encoded a 21-nucleotide hairpin specific sequence, with a loop sequence (-ttcaagaga-) separating two complementary domains. The specific 21-nucleotide sequences were cloned in a pSUPER vector. shSAE1: GTTCTTTACAGGAGATGTT; shSAE2: AGTGGAACAGCTGGGTATC; shControl: CGTACGCGGAATACTTCGA.

In Vitro binding - Proteins were *in vitro* translated (IVT) in a rabbit reticulocyte lysate system kit (Promega), ^{35}S-Methionine-labeled (Amersham) according to manufacture's instructions. The IVTs were incubated in E1A buffer with 5 μg of GST or GST-fused proteins for 1 hr at RT on rotation. After 3 washes in E1A buffer, the samples were loaded in SDS-PAGE, stained with Coomassie staining, dried and exposed for autoradiography (Figure 3B and 4A) or loaded on SDS-PAGE and immunoblotted with the indicated antibodies (Figure 3C and B).

In Vitro Ubiquitylation assay - Phoenix cells were transfected using indicated plasmids. 48 hours after transfection, cells were lysed in E1A buffer and immunoprecipitated using an anti-myc antibody. An aliquot of each IP were checked by Western Blot analysis to verify the equal amount of immunoprecipitated proteins. The immunoprecipitated samples were incubated then with the indicated combinations of 200 mM ubiquitin-E1, 500 mM UbcH5a, ubiquitin (Biomol) and SAE1 or SAE2 IVT ^{35}S-methionine-labeled. The reactions were incubated 2 hours at 30 °C in ATP buffer 10x (500 mM Tris pH 7.4, 50 mM $MgCl_2$, 20 mM ATP). The samples loaded on SDS-PAGE were then stained with Coomassie staining, dried and exposed for autoradiography.

Gel-Filtration - 24 hours after transfection, 4 mg of each total cellular lysates were loaded into Superose 6 HR 10/30 column (Amersham). The running conditions were designed as described in the manufacturer procedures manual. The column was equilibrated with E1A buffer without NP-40. 40 fractions were collected. 1/5 V/V of the indicated fractions were loaded on SDS-PAGE and immunoblotted.

RESULTS

Gam1 recruits Cullin-based ligase complexes (CRLs) - We have demonstrated that the reduction of SAE1 and SAE2 proteins induced by Gam1 expression, is not due to transcription

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regulation and is prevented by the addition of the proteasome inhibitor MG132 (11). Furthermore, the disappearance of SUMO E1 subunits was due to a substantial decrease in their half-lives induced by the viral protein, thus implicating the ubiquitin degradation pathway in Gam1 function (11). We therefore started a bioinformatics analysis to identify any conserved domain in the Gam1 amino acid sequence that might be traced back to known ubiquitin E3 complexes. Using the SMART software (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de) we found a putative SOCS motif (4) in the C-terminus of the Gam1 amino acid sequence. This domain, with its N-terminal shorter motif called a BC-box (16,17), is necessary for the interaction with the specific linker proteins elongin B (EloB) and elongin C (EloC) (3). Examining the multiple-alignment between the amino acid sequence of Gam1 and established SOCS motifs of known proteins, we observed that Gam1 has a conserved pattern of amino acids mainly in the BC-box (Fig. 1).

To determine whether Gam1 could interact with the elongins, we performed an in vitro binding assay using bacterial purified GST fusion version of the Gam1 protein incubated with ^{35}S-methionine labeled in vitro translated (IVT) EloB and/or EloC (Fig. 2A). We demonstrated that Gam1 is able to interact directly with EloC but not with EloB, that is recruited to Gam1 only in the presence of EloC (Fig. 2A). To establish whether Gam1 could physically interact in vivo with the endogenous CRLs and in particular with the elongins, cells were transfected with a myc-tagged Gam1 expression vector followed by immunoprecipitation. In agreement with the architecture of these complexes, Western blot analysis showed that, in vivo, Gam1 is able to immunopecipitate strongly EloB, EloC and the RING protein Roc1, generating non-physiological aggregates (Fig. 2B).

As expected, Gam1 does not interact with Cul1, Cul3 or Cul4A but is able to bind both Cul2 and Cul5 (Fig. 2B). This result is quite peculiar, since the cellular and viral proteins that interact with EloB and EloC, usually form a complex in an exclusive manner with either Cul2 or Cul5. This cullin's specificity seems to be due to the C-terminal P/L-rich region of the SOCS motif (18), that is absent from the Gam1 amino acid sequence. To investigate whether Gam1 recruits Cul2 and Cul5 in a common protein complex, cells were transfected with combinations of Gam1 and differentially tagged Cul2 and Cul5 expression vectors. As shown in Figure 2C, the immunoprecipitated Cul2 or Cul5 does not aggregate with the other overexpressed cullin, even if bound with Gam1. These data confirm the individual interactions between Gam1 and Cul2 or Cul5, and show that the two cullins generate distinct and new complexes with the viral protein.

Subsequently, to validate the in vivo interaction between Gam1 and the Cul2/Cul5-based CRLs, we followed the distribution of Gam1 protein using a gel-filtration technique and observed complexes with a representative range of molecular weights. As shown in Figure 2D, normally EloB and EloC co-elute in the same fractions and are mainly present in aggregates between 44 and 158 KDa (Fig. 2D, upper panel). Significantly, the expression of Gam1 both induces a clear increase in the molecular weight of the elongin complexes and co-elutes with them. The presence of Cul2, Cul5 and Roc1 in the same elution fractions suggests that Gam1 recruiting EloB/C into new complexes (Fig. 2D, lower panel) could reconvert the functionality of these endogenous CRLs.

Gam1 binds Elongins through its SOCS-box motif - To prove the functionality of the Gam1 SOCS-box and its implication in EloC recruitment, few amino acid residues involved in this putative domain were mutated. The resulting Gam1 mutants were expressed and tested for elongins and cullins binding by immunoprecipitation (Fig. 3A). The alignment between Gam1 and the consensus SOCS motif (Fig. 1) reveals that replacement of the highly conserved cysteine with alanine (A256) in Gam1 generates a domain most similar to SOCS domains of other viral proteins (7,19,20). Nevertheless, the mutation of this residue does not cause any effect on its binding property (Fig. 3A). Instead, the Gam1 point mutants L252A, W255A, L258A and L265A exhibited a drastically lower binding with EloB and EloC, consolidating the basic role of the hydrophobic surfaces of the SOCS motif in the functional recruitment of E3 adaptor subunits (Fig. 3A). To exclude the possibility that these mutants have any other structural deficiencies we tested binding with a different and uncorrelated Gam1 interactor, Histone Deacetylase 2 (HDAC2) (14).

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All Gam1 mutants bind HDAC2 in vivo in the same manner (Fig. 3A), demonstrating that these point mutations impair only the hydrophobic pocket necessary for EloC interaction.

The Gam1 L252A, W255A, L258A and L265A mutants show a virtually abolished binding with elongins but a residual interaction with Cul2 and Cul5, while the Gam1 double mutant L258/265A (14) was totally unable to immunoprecipitate all the subunits of the E3 complexes (Fig. 3A). Supposing that Gam1 could interact also directly with Cul2 and Cul5, we evaluated the binding properties of the Gam1 mutant L258/265A (Gam1 LL/AA) in vitro. As shown in Figure 3B, incubating the IVT-EloB and IVT-EloC with bacterial purified GST-Gam1 WT or GST-Gam1 LL/AA, we demonstrated that the Gam1 mutant does not bind the EloB/C heterodimer. Subsequently, we investigated the interaction between bacterial purified GST-Gam1 WT and its mutant incubated with IVT-Cul2 (Fig. 3C) or IVT-Cul5 (Fig. 3D). We demonstrated that Gam1 binds either Cul2 or Cul5 and these direct interactions were not affected by the presence of a functional Gam1 SOCS motif in vitro.

Gam1 recruits SUMO E1 in the CRL complexes - We have shown that the Gam1 LL/AA can no longer bind Cul2/5-EloB/C proteins and cannot induce SUMO E1 disappearance in vivo (11). We therefore reasoned that by recruiting the CRL complexes through its SOCS-box, Gam1 could work as a substrate-receptor allowing SAE1 and SAE2 ubiquitylation and their subsequent proteasomal degradation. Since Gam1 binds directly SAE1 and SAE2 (Figure 4A) (11), we decided to establish whether they could be included into the Gam1-EloB/C-Cul2/5 protein aggregates following their distribution in a gel-filtration assay. As shown in Figure 4B, upper panel, normally EloC does not co-elute with the heterodimer SAE1/SAE2. Instead the presence of Gam1 causes the co-elution of SUMO E1 components and EloC in the same fractions (Fig. 4B, lower panel, dotted box). These in vivo results suggest that Gam1 may recruit SAE1/SAE2 to the Cul2/5-EloB/C complexes, thus promoting their ubiquitylation.

To verify whether the SUMO E1 subunits are actually present in the same protein aggregates together with Gam1, EloB/C and Cul2/Cul5, we immunoprecipitated the overexpressed tagged version of SAE1 and SAE2 with or without Gam1 WT or Gam1 LL/AA (Fig. 4C and 4D). We demonstrated that SAE1 and SAE2 do not bind EloB/C and Cul2/5 normally, but are recruited in the CRL complexes only through the binding to the Gam1 WT protein (Fig. 4C and 4D). As expected, Gam1 LL/AA, defective for SUMO E1 protein degradation, cannot operate as substrate receptor and fails to join SAE1/SAE2 into Cu2/5-based complexes (Fig. 4D).

Gam1 permits SAE1 in vitro ubiquitylation - To tightly correlate the degradation of SUMO E1 and the assembly of the functional ubiquitin E3s induced by Gam1 we followed the enzymatic activity of these non-physiological proteins aggregates. We transfected cells with empty vector, Gam1 WT or Gam1 LL/AA myc-tagged expression vectors and subsequently immunoprecipitated the myc-tagged proteins. Equal amounts of immunoprecipitated materials were used as a source of E3s activities in the in vitro ubiquitylation reactions of ^{35}S-IVT SAE1 or SAE2 (Fig. 4E). Surprisingly, we showed that immunoprecipitated Gam1 could in vitro conjugate ubiquitin only on the SAE1 protein (Fig 4E).

SAE1 and SAE2 are mutually stabilized in vivo - To explain the paradox in which the degradation of SAE1 and SAE2 in vivo mediated by Gam1 contrasts with its inability to post-translationally modify SAE2 in vitro, we investigated whether SUMO E1 subunits are mutually stabilized in vivo. To deplete the protein level of SUMO E1 subunits we set an RNA interference approach (shRNAs) able to reduce successfully SAE1 or SAE2. As shown in Figure 4F, the disappearance of any SUMO E1 subunit caused a strong reduction of the other protein constituent of the enzyme. Therefore, in the presence of Gam1 the disappearance of SAE2 seems to be directly related to the reduction in SAE1 protein level and is not tightly dependent to the effect of Gam1 on endogenous CRLs. Furthermore, we could conclude that the stability of the SUMO E1 heterodimer is tightly related to the presence of its two subunits, SAE1 and SAE2.

Gam1 exploits both Cul2 and Cul5 to degrade SAE1 - The original ability of Gam1 to interact with both Cul2 and Cul5 and the absence of any identifiable cullin selection motif in its amino acid sequence, prompted us to elucidate the

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contribution of each cullin in SAE1 degradation. To understand whether they are both necessary for the function of Gam1 we employed an RNA interference approach to delete specifically Cul2 and/or Cul5. As shown in Figure 4G, the expression of Gam1 in cells in which Cul2 and Cul5 are simultaneously depleted does not induce SAE1 degradation. The single cullin depletion does not restore the disappearance of SAE1 caused by Gam1, suggesting that Cul2 and Cul5 are redundant for Gam1 function (Fig. 4G).

DISCUSSION

A plethora of data has now implicated SUMO in fundamental biological activities, making SUMO as important in regulating cell activity as ubiquitin. We have described a novel mechanism of action adopted by the CELO adenoviral protein Gam1 that induces a total reduction of cellular sumoylated proteins by blocking the formation of an E1-SUMO thioster complex (11). We now reveal the mechanism underlying Gam1 function by showing that it exploits the endogenous ubiquitin pathway to convert the specificity of Cullin2/5-based E3 complexes, triggering a non-physiological ubiquitylation and degradation of SAE1.

Gam1 is the substrate receptor of ubiquitin-E3 complexes - Having previously demonstrated that the function of Gam1 on sumoylation is related to the proteasome activity (11), we then investigated the potential role of the ubiquitin system in the Gam1 phenotype. Similarly to sumoylation, protein ubiquitylation catalyzes the formation of poly-ubiquitin chains onto substrate proteins via isopeptide bonds, through a cascade of enzymes involving activating (E1), conjugating (E2) and ligating (E3) activities (1). Poly-ubiquitylated substrates are then rapidly delivered to and degraded by the 26S proteasome. The substrate specificity of ubiquitin-dependent proteolysis is tightly mediated by hundreds of E3 ubiquitin ligases. Many viral and bacterial pathogens have evolved different proteins that convert the specificity of host multi-subunit ubiquitin ligase complexes (E3) inducing a non-physiological degradation of specific cellular targets (5,21). These considerations led us to identify a putative SOCS domain in the C-terminal region of the Gam1 protein (Fig. 1), implicating an involvement in Cul2/5-based E3 ligase complexes.

Although the amino acid sequences are not fully conserved among the SOCS proteins, they maintain the same helix structure, steric surface and pack similarly with the hydrophobic pocket of EloC. Interestingly, the Gam1 amino acids included between 251-265 are predicted to form an alpha-helix and the L252, W255, L258 and L265 residues could be located on the same face of this generated helix. This probable hydrophobic cluster could support a successful interaction with the hydrophobic pocket of EloC. In agreement with these structural considerations we demonstrated that Gam1 interacts directly with EloC (Fig. 2A and 2B) and this binding is stabilized by the hydrophobic residues L252, W255, L258 and L265 of Gam1 (Fig. 3A). In fact, the double substitutions L258A and L265A (LL/AA) impair totally the Gam1 binding capability both in vitro and in vivo (Fig. 3A and 3B).

Generally, within the SOCS motif, the presence of a C-terminal Proline/Leucine-rich region (P/L-rich) (18), allows to distinguish Cul2 or Cul5-associated proteins (Fig. 1). Even if Gam1 could not be clustered into any of these groups, we demonstrated that this viral protein binds independently both Cul2 and Cul5 (Fig. 2B and 2C). We could assume that other less well conserved residues in the Gam1 amino acid sequence are important for the cullin selection or that Gam1, through its SOCS-box binds specifically elongins but non-specifically either Cul2 and Cul5. Moreover, in despite of the direct binding between Gam1 and Cul2 or Cul5 (Fig. 3C and 3D), we could assume that, in vivo, the correct recruitment of the EloB/C heterodimer is necessary to stabilize the Gam1 entire complex and that its dual interaction with Cul2 and Cul5, could theoretically provide a larger and stronger platform of substrates for Gam1 to ubiquitinylate. We showed that the presence of Gam1 induces, as expected, a strong increase in the molecular weight of elongins complexes (Fig. 2D), supporting the hyphothesis that Gam1 is able to reconvert the functionality of these endogenous proteins aggregates.

Gam1 targets SAE1 into Cul2/5-EloBC-Roc1 complexes – We have shown that the Gam1 LL/AA mutant can no longer bind Cul2/5-EloB/C

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proteins (Fig. 3A and 3B). Interestingly, this double point mutant was initially identified as the inactive version of Gam1 protein (13) and fails to interfere with sumoylation and some SUMO related phenotypes (namely transcription and PML-NBs assembly) (11,22). Consequently, we speculated that Gam1 by recruiting the Cullin E3 ligase complexes through its SOCS motif allowed SAE1 and SAE2 ubiquitylation and their proteasomal degradation. Following this hyphothesis, we demonstrated that SAE1 and SAE2 were stably associated, through the direct link of Gam1 WT, into the Cul2/5-EloB/C-Roc1 complexes (Fig. 3B and 3C). In agreement with the initial assumption, Gam1 LL/AA fails to connect the SUMO E1 subunits to the endogenous CRL complexes (Fig. 3B). Therefore, we could assume that the inability of Gam1 LL/AA to degrade SUMO E1 in vivo is a direct effect of its failure to join, in a stable manner, the CRLs and SAE1/SAE2. Consequently, we followed the ubiquitin conjugating activity of the Gam1-based CRLs in vitro and showed that Gam1 could ubiquitin-modify only the SAE1 protein, suggesting that the in vivo phenotype could be due to overlapping but distinct events (Fig. 4E). Using a RNA interferences approach, we demonstrated that the stability of the SUMO E1 heterodimer is tightly related to the presence of its two subunits, SAE1 and SAE2, and that the effect of Gam1 on SAE2 seems to be a consequence to the induced ubiquitylation and degradation of SAE1 (Fig. 4F).

We have shown that the viral protein Gam1, through a novel dual binding with Cul2- and Cul5-based aggregates (Fig. 2) could theoretically provide two non-physiological larger ubiquitin platforms with overlapping functions. In fact, Cul2 and Cul5 seem to have a redundant role during the degradation of SAE1 induced by Gam1 (Fig. 4G).

The atypical role of Gam1 could also be a viral evolutionary conserved mechanism to assure specific substrate modification and degradation of essential cellular proteins. The flexibility of action of Gam1 supports its pivotal role in viral replication and reveals its potential to study cellular pathways.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. *Gam1 is a putative SOCS-box containing protein.* Sequence alignment of SOCS-box motifs from cellular proteins that bind EloC. Conserved residues are shaded. Proteins that bind differently Cullin2 or Cullin5 are classified in two distinct groups. The alignment and the consensus motif were generated using ClustalW software (PAM matrix). *p*: polar amino acids (a.a.); *l*: aliphatic a.a.; *b*: big a.a.; *s*: small a.a.

Fig. 2. *Gam1 interacts with Cullin2/5-based aggregates.* (A) Gam1 recruits directly EloC and indirectly EloB in vitro. ^{35}S-Methionine labeled in vitro translated (IVT) EloB and/or EloC were incubated with GST or GST-Gam1 as described in Experimental Procedures. The samples were loaded into a 17% SDS-PAGE. GST proteins were detected by staining with Coomassie Blue while the in vitro interactions by autoradiography. (B) Gam1 forms a complex with Cullin2/5-based complexes in vivo. Phoenix cells were transfected with the indicated constructs and lysed in E1A buffer. The samples were then immunoprecipitated (IP), electrophoresed on SDS-PAGE and immunoblotted with indicated antibodies. Input: whole cellular extract (WCE). (C) Gam1 binds Cul2 and Cul5 in distinct complexes. Phoenix cells were transfected with indicated plasmids two days later lysed in E1A buffer, immunoprecipitated (IP) and immunoblotted, as indicated. (D) Gam1 co-elutes with Cul2/Cul5-based complex subunits in vivo. Phoenix cells were transfected with the indicated plasmids, lysed and processed for a gel-filtration analysis (described in Experimental Procedures). 1/5 V/V of the indicated fractions were loaded in 17% SDS-PAGE and immunoblotted. The dotted boxes underline the shift of elongins and cullins complexes induced by the expression of Gam1.

Fig. 3. *Identification of critical residues in the Gam1 SOCS motif required for association with Elongins/Cullins complexes.* (A) Phoenix cells were transfected with indicated plasmids, lysed in E1A buffer, immunoprecipitated (IP) using an anti-myc antibody and immunoblotted, as indicated. (B) ^{35}S-

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Methionine labeled in vitro translated (IVT) EloB and/or EloC were incubated with GST, GST-Gam1 WT or GST-Gam1 LL/AA as described in Experimental Procedures. The samples were loaded onto a 17% SDS-PAGE. GST proteins were detected by staining with Coomassie Blue while the in vitro interactions by autoradiography. (C) and (D) IVT-Cul2-myc tagged (C) or IVT-Cul5-myc tagged (D) were incubated with GST, GST-Gam1 WT or GST-Gam1 LL/AA as described in Experimental Procedures. The samples were loaded onto a 12% SDS-PAGE and immunoblotted with indicated antibodies.

Fig. 4. *Gam1 recruits SAE1/SAE2 in Cullin-based Ubiquitin ligase complexes and ubiquitinates SAE1.* (A) Gam1 binds directly the SUMO E1 subunits, SAE1 and SAE2, in vitro. ^{35}S-Methionine labeled in vitro translated (IVT) SAE1 or SAE2 were incubated with GST or GST-Gam1 WT as described in Experimental Procedures. The samples were loaded onto a 12% SDS-PAGE. GST proteins were detected by staining with Coomassie Blue while the in vitro interactions by autoradiography. (B) SUMO E1 co-elutes with elongin C and Gam1 in vivo. Phoenix cells were transfected with the indicated plasmids, lysed and processed for a gel-filtration analysis (described in Experimental Procedures). 1/5 V/V of the indicated fractions were loaded in 17% SDS-PAGE and immunoblotted. The dotted boxes underline the shift of the proteins that co-elute with Gam1. (C) and (D) Gam1 recruits SAE1 and SAE2 in the Cullin2/5-based complexes in vivo. Phoenix cells were transfected with indicated plasmids, lysed in E1A buffer and immunoprecipitated (IP) using the indicated antibodies. (E) Gam1 induces SAE1 in vitro ubiquitylation. Phoenix cells were transfected with indicated plasmids, lysed in E1A buffer and immunoprecipitated (IP) using the indicated antibodies. The immunoprecipitated samples were incubated as a source of ubiquitin E3 ligase activity in an in vitro ubiquitylation reactions as described in Experimental Procedures. ^{35}S-Methionine labeled IVT-SAE1 or IVT-SAE2 were used as a substrate and detected by autoradiography. (F) SUMO E1 subunits are mutually stabilized. HeLa cells were transfected with plasmids encoding the indicated shRNAs. 5 days later cells were lysed and processed for Western Blot analysis. (G) Gam1 exploits both Cul2 and Cul5 to degrade SAE1. HeLa cells were interfered against the indicated cullins, as described in Experimental Procedures. The interfered cells were transfected then with empty vector or myc-Gam1. After 48 hours cells were lysed and processed for Western Blot analysis.

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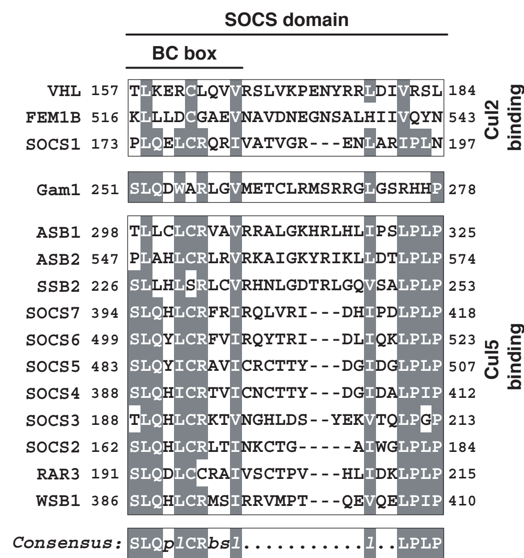

SOCS domain
BC box
VHL 157 TLKERCLQVVRSLVKPENYRRLDIVRSL 184
FEM1B 516 KLLLDCGAEVNAVDNEGNSALHIIVQYN 543
SOCS1 173 PLQELCRQRIVATVGR---ENLARIPLN 197
Cul2 binding
Gam1 251 SLQDWARLGVMETCLRMSRRGLGSRHHP 278
ASB1 298 TLLCLCRVAVRRALGKHRLHLIPSLPLP 325
ASB2 547 PLAHLCRLRVRKAIGKYRIKLLDTLPLP 574
SSB2 226 SLLHLSRLCVRHNLGDTRLGQVSALPLP 253
SOCS7 394 SLQHLCRFRIRQLVRI---DHIPDLPLP 418
SOCS6 499 SLQYLCRFVIRQYTRI---DLIQKLPLP 523
SOCS5 483 SLQYICRAVICRCTTY---DGIDGLPLP 507
SOCS4 388 SLQHICRTVICNCTTY---DGIDALPIP 412
SOCS3 188 TLQHLCRKTVNGHLDS--YEKVTQLPGP 213
SOCS2 162 SLQHLCRLTINKCTG-----AIWGLPLP 184
RAR3 191 SLQDLCCRAIVSCTPV---HLIDKLPLP 215
WSB1 386 SLQHLCRMSIRRVMPT---QEVQELPIP 410
Cul5 binding
Consensus: SLQplCRbsl...........l..LPLP

Boggio et al. Figure 2

A

Input GST GST-Gam1

1 2 3 4 5 6 7 8

	1	2	3	4	5	6	7	8
IVT ^{35}S-EloB	+		+		+	+		+
IVT ^{35}S-EloC		+		+	+		+	+

EloB ▸
EloC ▸

Autoradiography

GST-Gam1 ▸
GST ▸

Coomassie staining

B

Input (WCE) IP α-myc (Gam1)

Gam1-myc - + - +

RING | Roc1

Cullins | Cul1, Cul2, Cul3, Cul4A, Cul5

Adaptors | EloC, EloB

SOCS protein | Gam1

1 2 3 4

C

	1	2	3	4	5	6	7
Cul2-FLAG		+		+	+		+
Cul5-HA			+	+		+	+
Gam1-myc	+				+	+	+

Cul2-FLAG
Cul5-HA
Gam1-myc
Input (WCE)

Cul2-FLAG
Cul5-HA
Gam1-myc
IP α-FLAG (Cul2)

Cul2-FLAG
Cul5-HA
Gam1-myc
IP α-HA (Cul5)

1 2 3 4 5 6 7

D

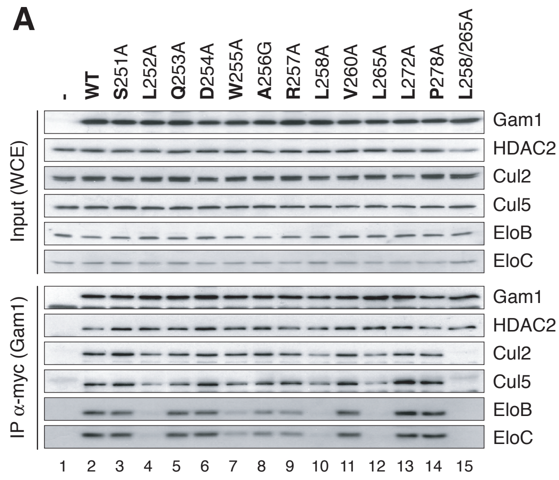
640 158 44 KDa

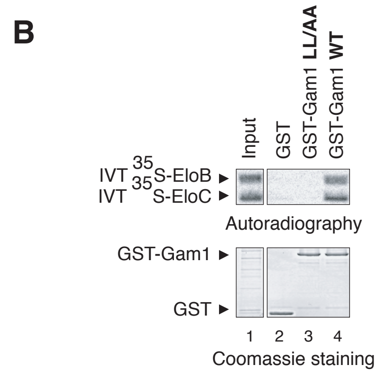
15 16 17 18 19 20 21 22 23 24 25 26 27 Fractions

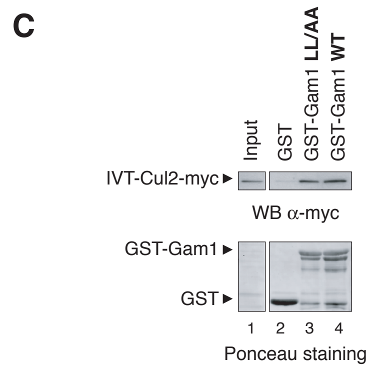
Gam1
EloB
EloC
Cul2
Cul5
Roc1
Empty Vector

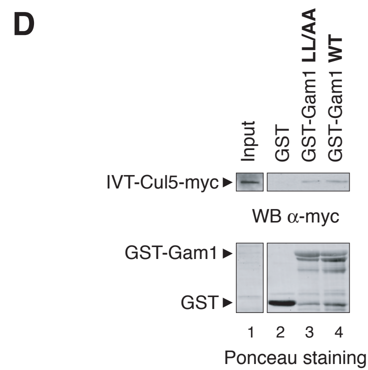
Gam1
EloB
EloC
Cul2
Cul5
Roc1
Gam1

Boggio et al. Figure 3

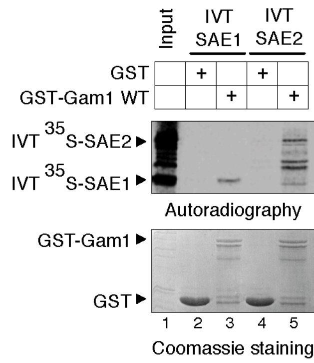


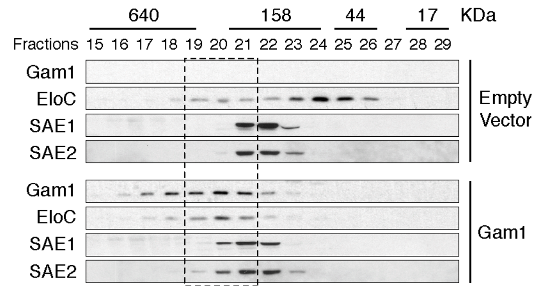


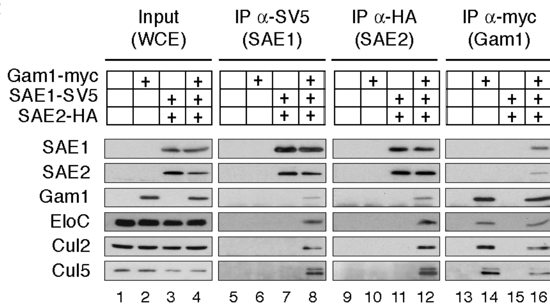


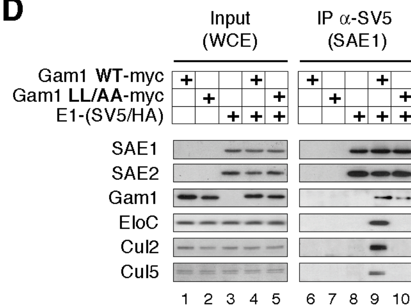


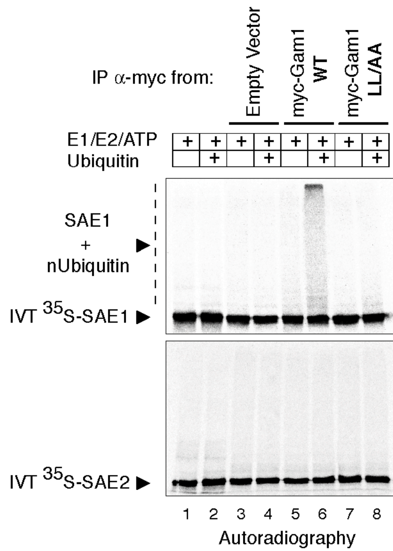
Boggio et al. Figure 4

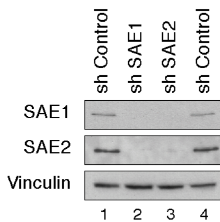


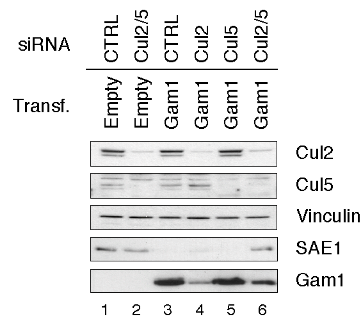












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Targeting sumo E1 to ubiquitin ligases: A viral strategy to counteract sumoylation

Roberto Boggio, Alfonso Passafaro and Susanna Chiocca

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