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Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag

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ABSTRACT

Suppressor of cytokine signaling 1 (SOCS1) is a recently identified host factor that positively regulates the intracellular trafficking and stability of HIV-1 Gag. We here examine the molecular mechanism by which SOCS1 regulates intercellular Gag trafficking and virus particle production. We find that SOCS1 colocalizes with Gag along the microtubule network and promotes microtubule stability. SOCS1 also increases the amount of Gag associated with microtubules. Both nocodazole treatment and the expression of the microtubule-destabilizing protein, stathmin, inhibit the enhancement of HIV-1 particle production by SOCS1. SOCS1 facilitates Gag ubiquitination and the co-expression of a dominant-negative ubiquitin significantly inhibits the association of Gag with microtubules. We thus propose that the microtubule network plays a role in SOCS1-mediated HIV-1 Gag transport and virus particle formation.

Structured summary:
MINT-7014185: *Gag* (uniprotkb:P05888) and *SOCS1* (uniprotkb:O15524) *colocalize* (MI:0403) by *cosedimentation* (MI:0027)
MINT-7014239: *Cullin 2* (uniprotkb:Q13617) *physically interacts* (MI:0218) with *RelA* (uniprotkb:Q04206), *RBX1* (uniprotkb:P62877), *SOCS1* (uniprotkb:O15524), *elongin B* (uniprotkb:Q15369) and *elongin C* (uniprotkb:Q15370) by *pull-down* (MI:0096)
MINT-7014046: *gag* (uniprotkb:P05888), *SOCS1* (uniprotkb:O15524) and *tubulin alpha* (uniprotkb:Q13748) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7014269: *tubulin alpha* (uniprotkb:Q13748) *physically interacts* (MI:0218) with *Gag* (uniprotkb:P05888) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7014036: *tubulin alpha* (uniprotkb:Q13748) and *SOCS1* (uniprotkb:O15524) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7014201: *Cullin 2* (uniprotkb:Q13617) *physically interacts* (MI:0218) with *RBX1* (uniprotkb:P62877), *SOCS1* (uniprotkb:O15524), *elongin B* (uniprotkb:Q15369) and *elongin C* (uniprotkb:Q15370) by *pull-down* (MI:0096)
MINT-7014257: *Gag* (uniprotkb:P05888) *physically interacts* (MI:0218) with *Ubiquitin* (uniprotkb:P62988) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7014221: *Cullin 2* (uniprotkb:Q13617) *physically interacts* (MI:0218) with *Gag* (uniprotkb:P05888), *elongin C* (uniprotkb:Q15370), *elongin B* (uniprotkb:Q15369), *SOCS1* (uniprotkb:O15524) and *RBX1* (uniprotkb:P62877) by *pull-down* (MI:0096)

1. Introduction

The human immunodeficiency virus 1 (HIV-1) employs multistep and multi-factorial processes for producing progeny viruses during infection [1,2]. Virus must utilize the intrinsic transport machinery of the infected host cells to enable the active transport

Abbreviations: HIV, human immunodeficiency virus; SOCS1, Suppressor of cytokine signaling 1; KIR, kinase inhibitory region; MTOC, microtubule organizing center; Ub, ubiquitin; VLP, virus-like particle

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of viral proteins [3,4]. Several recent studies have identified cellular factors that modulate HIV-1 Gag trafficking and localization. These include AP-3δ, POSH, HP68, GGA and Trim22 [5–9]. Moreover, phosphatidylinositol-(4,5)-bisphosphate (PIP2) has been shown to control the targeting of Gag to the plasma membrane [10]. These findings point to a critical role of host cell factors in Gag assembly and release, but the precise molecular functions of these factors and the specific timing of their roles in this process remain largely unknown.

We recently reported that the suppressor of cytokine signaling 1 (SOCS1) is an inducible host factor during HIV-1 infection and plays an important role in the intracellular trafficking of Gag to the plasma membrane, resulting in the efficient production of HIV-1 particles [11]. Moreover, we have further shown that the function of SOCS1 in Gag trafficking and HIV-1 particle production is not principally due to the suppression of interferon/cytokine signaling, but is mediated via its interaction with the HIV-1 Gag polyprotein [11]. Importantly, the targeted depletion of SOCS1 results in the mistargeting and degradation of Gag in lysosomes, leading to a significant decrease in virus particle production [11].

In our current study, we have utilized SOCS1 as a molecular tool to further reveal the molecular mechanisms underlying the intracellular transport of HIV-1 Gag during viral infection. We reveal from our findings that SOCS1 regulates the Gag trafficking process via the microtubule-dependent cellular machinery. Furthermore, we find that Gag is also regulated by a ubiquitin signaling pathway which is accompanied by Gag ubiquitination. These findings shed new light on the mechanisms involved in the intracellular transport of HIV-1 Gag and provide important clues for the design of future novel therapeutic interventions against AIDS and related disorders.

2. Materials and methods

2.1. Antibodies

Antibodies (Abs) and fluorescent reagents were obtained from the following sources: rabbit polyclonal anti-myc (A-14) and rabbit polyclonal anti-SOCS1 (H-93) Abs (Santa Cruz Biotechnology); rabbit polyclonal anti-SOCS1 (Zymed Laboratories); mouse monoclonal anti-FLAG (M2), anti-α-tubulin, anti-acetylated-α-tubulin and anti-γ-tubulin Abs (Sigma, St. Louis, MO); rabbit polyclonal anti-stathmin antibody (Calbiochem); mouse monoclonal anti-myc antibody (9B11, Cell Signaling Technology); mouse monoclonal anti-cytokeratin 7, cytokeratin 18, vimentin and HIV-p24 Ab (Dako Cytomation). Immunoblotting, immunoprecipitation and immunofluorescent analyses were performed as described previously [11].

2.2. Plasmids and sequences

Expression constructs for SOCS1 have been described previously [12]. HIV-1 Gag constructs have also been described previously [13]. Stathmin cDNA was amplified by RT-PCR from a human kidney cDNA library using the primers 5′-AGCAAG CTTGCCACCATGGCTTCTTCTGATATCCAGG-3′ and 5′-GACGGATCC GTCAGCTTCAGTCTCGTCAG-3′ and then subcloned into the pcDNA3.1 vector. pcDNA3.1-myc-ubiquitin and its mutants were generated by PCR as described previously [14]. The siRNA sequences were as follows: SOCS1-siRNA, GGCCAGAACCTT CCTCCTCTT; control-siRNA, TCGTATGTTGTGTGGAATT. All expression constructs were validated by sequencing.

2.3. Microtubule-associated protein spin-down assays

Microtubule-associated proteins were collected using a microtubule-associated protein spin-down assay kit (Cytoskeleton, BK029) according to the manufacturer's instructions. Briefly, 293T cells were lysed in 0.5 ml of PEM buffer (80 mM PIPES, pH 6.9, 0.3% Triton X-100, 1 mM EGTA, 1 mM GTP, GTP, 1 mM $MgCl_2$) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, and 5 mM NaF. Cell lysates were incubated with taxol-stabilized microtubules, followed by ultracentrifuge at 100000×*g* for 40 min at 25 ℃.

2.4. Cell culture

The 293T, COS-1, COS-7, HeLa and HOS cell lines and mouse embryonic fibroblasts (MEFs) were cultured in DMEM supplemented with 10% FBS. SOCS1−/− MEF cells were cultured as described previously [12].

2.5. In vitro interaction analysis

The in vitro interaction between HIV-1 Gag and the SOCS1-E3 complex was analyzed as follows: ^{14}C-labeled recombinant proteins (SOCS1, elongin B/C, Rbx1, biotin labeled cullin 2, and HIV-1 Gag) were synthesized in a wheat germ cell-free system as described previously [15]. The synthesized proteins were subsequently incubated in 120 μl of reaction buffer (50 mM Tris–HCl, pH 7.6, 50 mM $MgCl_2$, 500 mM CH_3COOK, 0.1 mM DTT and 1 mg/ml BSA) and streptavidin magnetic beads (Promega, Madison, WI) at 23 °C for 1 h. The precipitated proteins were then washed three times with reaction buffer and subjected to autoradiography.

3. Results

3.1. SOCS1 aligns with microtubule forming fibrous structures

SOCS1 has been shown previously to localize at both the perinuclear region and the microtubule organizing center (MTOC) [16]. This finding indicated the possible involvement of the microtubule network in the regulation of Gag by SOCS1. We thus addressed whether the Gag transport system is in fact mediated by microtubule integrity and if SOCS1 enhances this process. Immunofluorescent analysis with α-tubulin antibodies revealed that endogenous SOCS1 forms punctate structures that align with the microtubule network (Fig. 1A). Importantly, these signals were completely abolished when the cells were stained with anti-SOCS1 antibodies that had been pre-absorbed with recombinant SOCS1 protein, confirming the specificity of this antibody (Fig. 1B). Parallel experiments revealed that SOCS1 does not colocalize with other cytoskeletal components such as actin or intermediate filaments (Fig. 1C).

3.2. SOCS1 promotes microtubule stability

Given our finding that SOCS1 can tightly associate with microtubules, we next addressed whether SOCS1 affects microtubule stability. Stabilized microtubules are frequently enriched in tubulin that has undergone post-translational modifications such as acetylation [17]. We found that the high expression of SOCS1 results in higher amounts of acetylated microtubules in three different cell lines when compared with control cells (Fig. 2A) and that this trend is dose-dependent in COS-7 cells (Fig. 2B). On the other hand, SOCS1−/− mouse embryonic fibroblasts (MEFs) exhibited lower levels of acetylated α-tubulin compared with wild-type MEFs (Fig. 2C). These results indicate that SOCS1 does indeed contribute to the stabilization of microtubules.

Mammalian cells usually possess a population of microtubules that are resistant to the depolymerization effects of microtubule disorganizing reagents. We thus addressed whether SOCS1 impacts

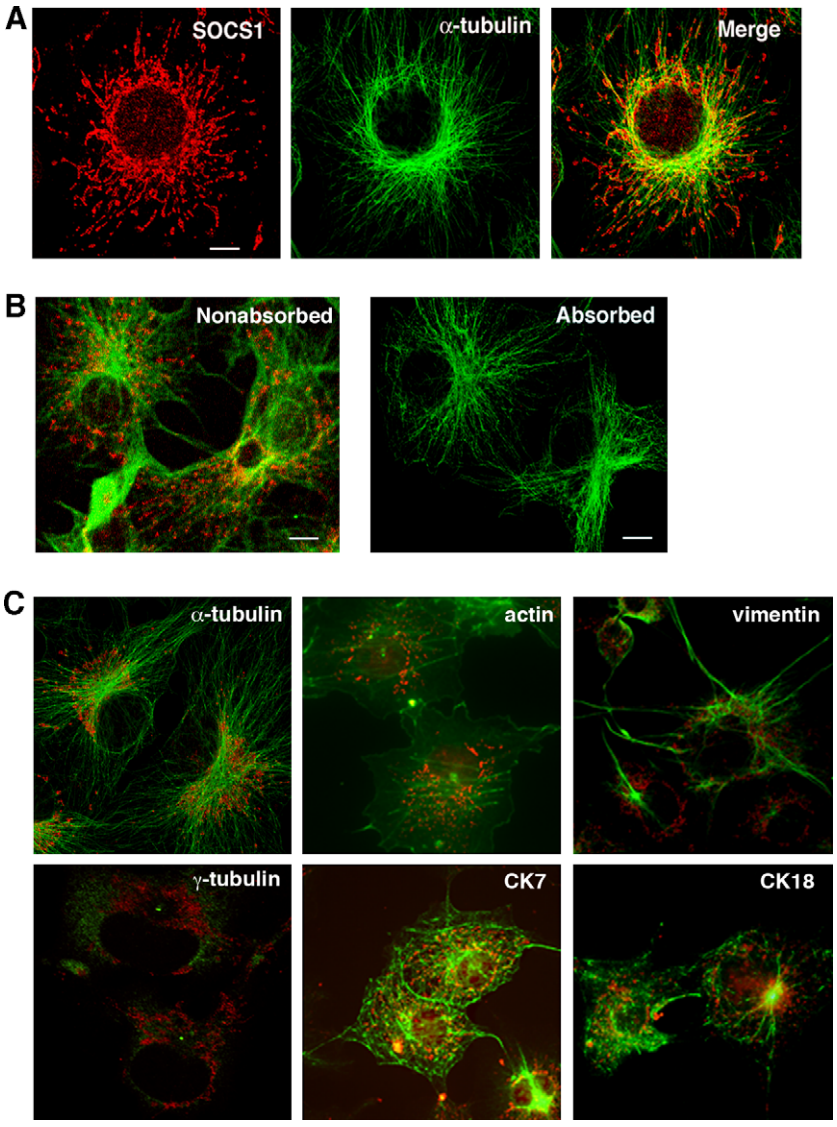

Fig. 1. SOCS1 colocalizes with microtubule forming fibrous structures. (A) COS-1 cells were fixed with 3% formaldehyde followed by 100% cold methanol, and then co-immunostained with polyclonal antibodies targeting SOCS1 (red) and monoclonal antibodies targeting α-tubulin (green). Cells were then analyzed by confocal microscopy. Scale bar, 10 μm. (B) COS-1 cells were immunostained with anti-α-tubulin monoclonal antibodies together with anti-SOCS1 polyclonal antibodies that had either been non-absorbed or pre-absorbed with GST-SOCS1 proteins. This was followed by confocal microscopy. Scale bar, 10 μm. (C) COS-1 cells were fixed with 3% formaldehyde followed by 100% cold methanol, then co-immunostained with polyclonal antibodies targeting SOCS1 (red) and monoclonal antibodies targeting various cytoskeletal components (green). Cells were then analyzed by confocal microscopy.

upon this property in this subpopulation of microtubules in COS-7 cells. The cells were transfected with either SOCS1 or control vector and then treated with 1 μM colchicine for 12 h to fully depolymerize the microtubules. Immunostaining with antibodies against acetylated α-tubulin showed that the SOCS1 expressing cells contained more polymerized microtubules compared with the control cells (Fig. 2D). These results indicate that SOCS1 might contribute to the microtubule stability required for Gag trafficking via this network.

3.3. SOCS1 enhances the association of HIV-1 Gag with microtubules

We next investigated the sub-cellular localization of HIV-1 Gag with SOCS1 and microtubules. COS-1 cells were transfected with Gag-GFP and after 24 h were fixed with 3% formaldehyde, followed by 100% cold methanol. The cells were then immunostained with anti-SOCS1 and anti-α-tubulin antibodies. Consistent with our earlier results, confocal microscopic analysis revealed that SOCS1 can form dotted filamentous structures in the cytoplasm along the microtubules, and that HIV-1 Gag colocalizes with these SOCS1 puncta (Fig. 3A).

To next determine whether cellular SOCS1 and Gag can together mechanically bind microtubules, and thus whether SOCS1 expression has any impact upon the interaction between Gag and microtubules, we performed microtubule pull-down analysis. 293T cells were transfected with either Gag-FLAG, myc-SOCS1, or a combination of these two plasmids, and the lysates from these transfected cells were subsequently incubated with taxol-stabilized microtubules and centrifuged to pellet the microtubule-associated proteins. The pellet fractions were then subjected to immunoblotting using either anti-myc or anti-FLAG antibodies. SOCS1 was found to be co-sedimented with microtubules irrespective of whether Gag had been co-transfected (Fig. 3B). The quantities of microtubule-bound Gag in the pellet fraction, however, were significantly increased when SOCS1 was co-transfected (Fig. 3B). These results together indicate that SOCS1 is itself a microtubule binding protein that may also mediate the interaction between HIV-1 Gag and microtubules.

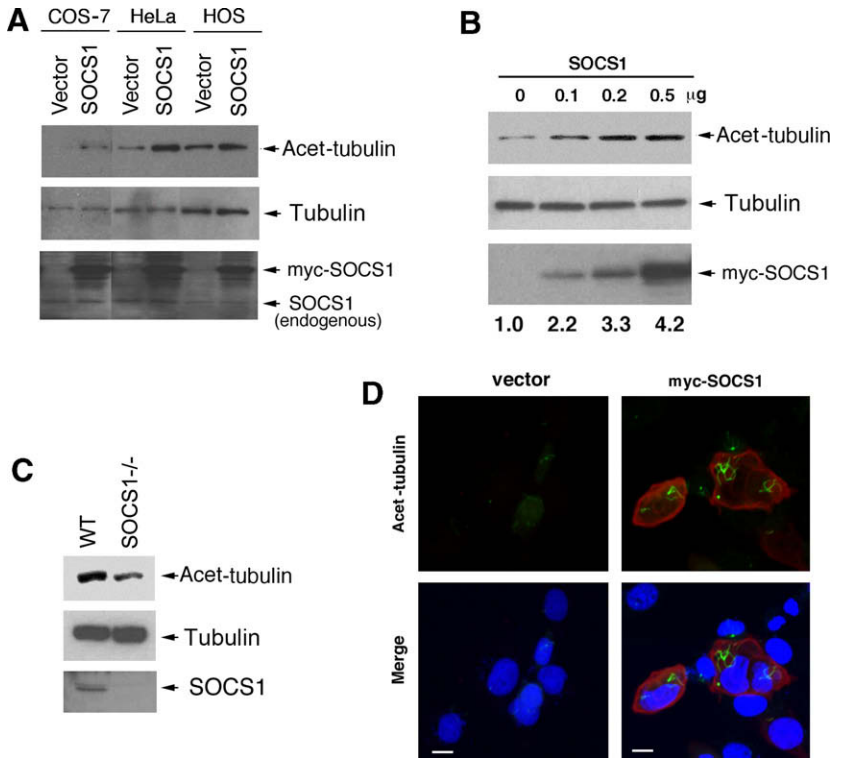

Fig. 2. SOCS1 enhances microtubule stability. (A) COS-7, HeLa or HOS cells were transfected with either empty vector or myc-SOCS1 for 48 h. Cell lysates were then subjected to immunoblotting analysis with antibodies against α-tubulin, acetylated α-tubulin or SOCS1. (B) COS-7 cells were transfected with various amounts of myc-SOCS1 as in (A). Cell lysates were then subjected to immunoblotting analysis with anti-α-tubulin, anti-acetylated-α-tubulin or anti-myc antibodies. Numerical values below the blots indicate acetylated α-tubulin signal intensities normalized by the unmodified α-tubulin intensity derived by densitometry. (C) Exponentially growing wild-type MEFs or SOCS1−/− MEFs were lysed and the cell lysates were immunoblotted with antibodies against α-tubulin, acetylated α -tubulin or SOCS1. (D) COS-7 cells were co-transfected with empty vector or myc-SOCS1, and then treated with colchicine (1 μM) for 12 h to depolymerize the microtubules. Cells were then fixed and immunostained with both anti-acetylated-α-tubulin (green) or anti-myc (red) antibodies and then stained with 4′,6-diamino-2-phenylindole (DAPI, blue), followed by confocal microscopy. Scale bar, 10 μm.

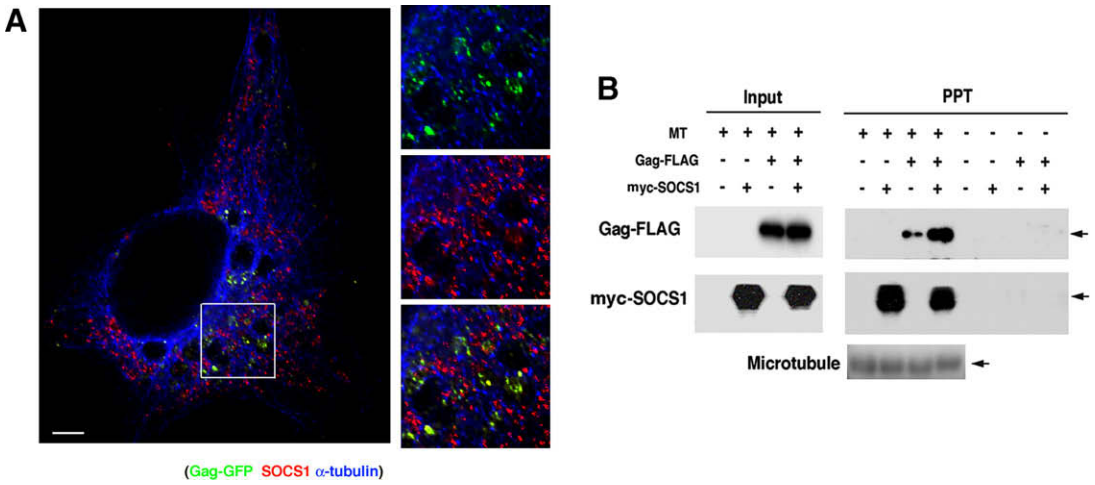

Fig. 3. SOCS1 enhances HIV-1 Gag associated with microtubules. (A) COS-1 cells transiently transfected with HIV-1 Gag-GFP were co-immunostained with antibodies targeting endogenous SOCS1 (red) and microtubules (α-tubulin, blue). The inset indicates the area shown at higher magnification in the right hand panels which reveal the colocalization of Gag-GFP with SOCS1 along the microtubules. Scale bar, 10 μm. (B) Cosedimentation of SOCS1 and HIV-1 Gag with polymerized microtubules. 293T cells were transfected with the indicated plasmids for 36 h. Cell lysates were then incubated with taxol-stabilized microtubules or control buffer and separated into precipitate (PPT) and supernatant fractions. Precipitate fractions were subjected to immunoblotting analysis with anti-myc or anti-FLAG antibodies.

3.4. Microtubule integrity is required for SOCS1 to function in HIV-1 particle formation

Our results shown above indicated that SOCS1 mediates the association of HIV-1 Gag with the microtubule networks. We next examined therefore whether SOCS1-mediated Gag trafficking, and the resultant HIV-1 particle production, are dependent upon an intact microtubule network. 293T cells were transfected with the HIV-1 molecular clone pNL4-3 and co-transfected with either empty vector alone or myc-SOCS1. After 24 h, the cells were washed with PBS and then cultured in the presence or absence of nocodazole for a further 6 h. Subsequent measurement of the p24 antigen levels in the cell supernatant by ELISA revealed nocodazole treatment significantly inhibited the enhancement of HIV-1 particle production in SOCS1-transfected cells more dramatically than in vector control transfected cells, and that this was

dose-dependent (Fig. 4A). Consistent with these results also, SOCS1 localization was observed to be significantly altered by nocodazole treatment, i.e. from a dotted filamentous structure along the microtubules to diffuse and larger aggregations in the cytoplasm (Fig. 4B). The use of trypan blue dye exclusion confirmed that cell viability was not affected by the nocodazole treatment (Fig. 4C).

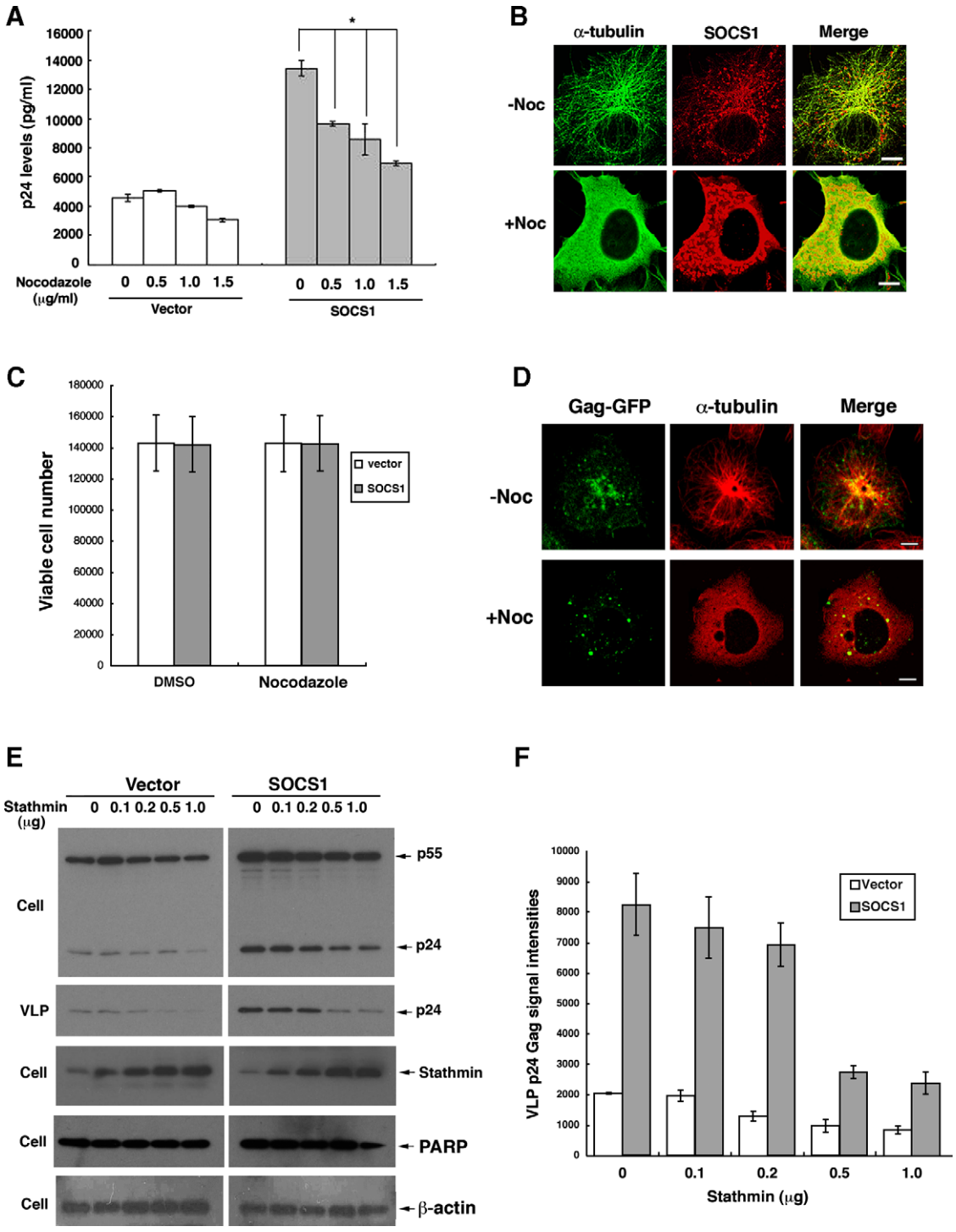

Fig. 4. Microtubule integrity is required for SOCS1 to function in HIV-1 particle formation. (A) 293T cells were transfected with pNL4-3, and co-transfected with either empty vector alone (Vector) or myc-SOCS1. After 24 h, cells were washed with PBS and then cultured with fresh media including the indicated concentrations of nocodazole for 6 h. Supernatant p24 antigen levels were measured by p24 ELISA . The data shown are the average ± S.D. of three independent experiments. $^{*}P \leqslant 0.05$, by the Student's *t*-test. (B and C) Mislocalization of SOCS1 in cells treated with nocodazole. COS-1 cells were treated with vehicle only or with nocodazole (2 μg/ml) for 6 h. Cells were then fixed and immunostained with both anti-SOCS1 (red) and anti-α-tubulin (green) antibodies, followed by confocal microscopy (B). Scale bar, 10 μm. The numbers of viable cells were calculated by trypan blue dye exclusion (C). (D) COS-1 cells transfected with Gag-GFP were treated with vehicle only or with nocodazole (2 μg/ml) for 6 h followed by immunostaining with anti-α-tubulin (red) antibody. Scale bar, 10 μm. (E and F) 293T cells were transfected with pNL4-3 and either vector or SOCS1, and co-transfected with various amounts of stathmin. At 36 h after transfection, cell lysate and supernatant virus-like particle (VLP) were processed for immunoblotting analysis with anti-p24, anti-PARP, anti-β-actin or anti-stathmin antibodies (E). VLP p24 Gag signal intensities, derived by densitometry, are shown in (F).

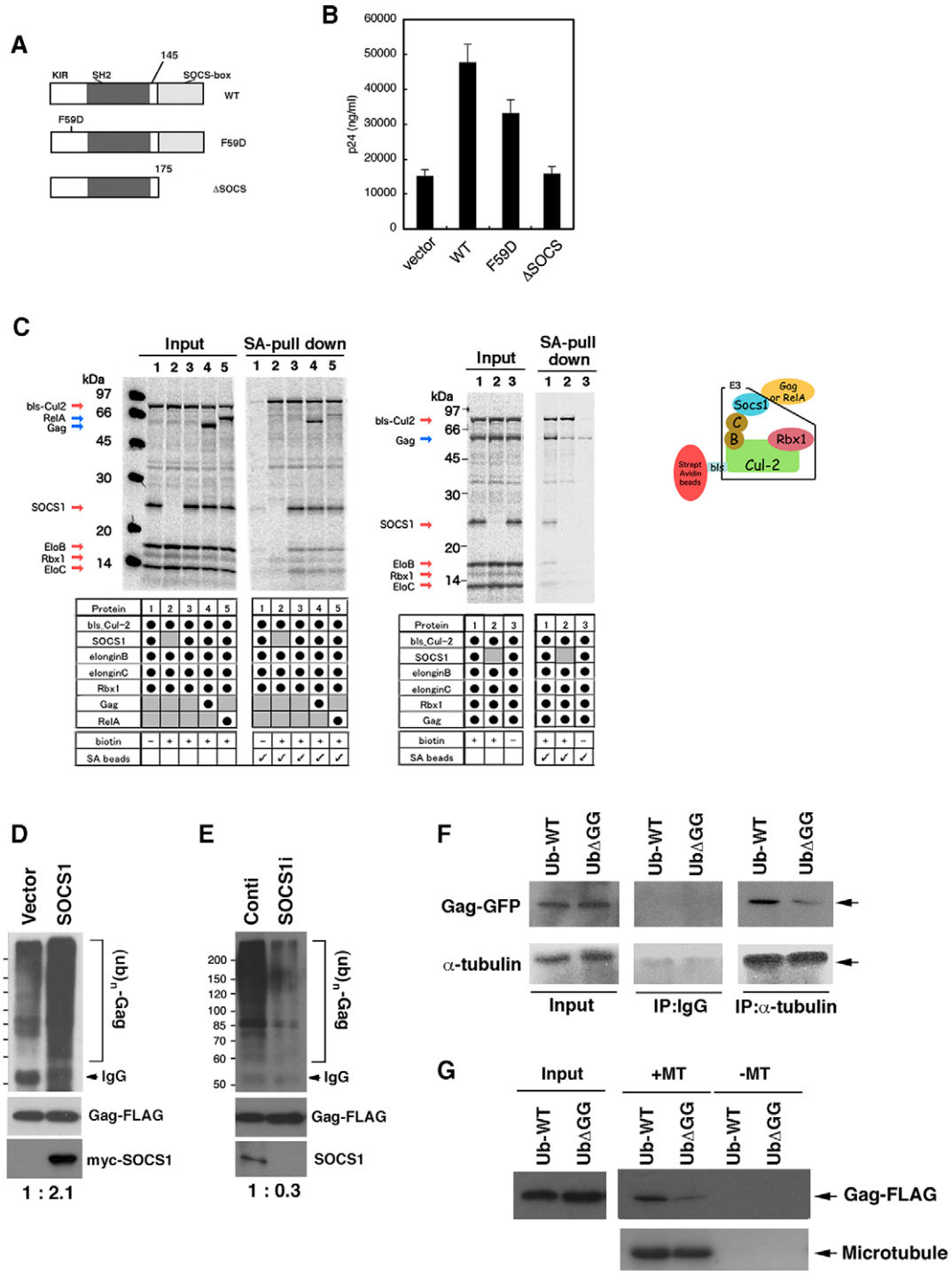

Fig. 5. SOCS1 enhances the ubiquitination of HIV-1 Gag and this affects the association of Gag with the microtubules. (A) Schematic represenation of SOCS1 mutants. (B) 293T cells were transfected with pNL4-3 and co-transfected with control vector, wild-type SOCS1 (WT), SOCS1-F59D mutant or ΔSOCS-box mutant. After 48 h, the p24 levels in the cell supernatants were measured by ELISA. (C) HIV-1 Gag associates with the ubiquitin ligase complex of SOCS1 in vitro. ^{14}C-labeled proteins (SOCS1, biotin labeled Cullin2, elongin B/C, Rbx1, HIV-1 Gag and RelA) were synthesized using a wheat germ cell-free system. Purified proteins were incubated in the indicated combinations for 1 h and subjected to co-purification with streptavidin magnetic beads. Captured proteins were then separated by SDS–PAGE followed by autoradiography. (D) 293T cells were co-transfected with Gag-FLAG, myc-tagged ubiquitin, and either empty vector (Vector) or SOCS1 expression construct. After 48 h, cells were lysed and denatured by boiling them in 1% SDS lysis buffer and diluted to RIPA buffer conditions, and Gag-FLAG proteins were immunoprecipitated (IP) with anti-FLAG antibody and processed for anti-myc immunoblotting to detect ubiquitinated Gag. Ubn, polyubiquitinated. Numerical values below blot indicates the relative levels of ubiquitinated Gag normalized by the amount of total Gag. (E) 293T cells were co-transfected with Gag-FLAG, myc-tagged ubiquitin, and either control-siRNA or SOCS1-siRNA. After 48 h, cell lysates were immunoprecipitated with anti-FLAG antibody and processed for anti-myc immunoblotting to detect ubiquitinated Gag. Ubn, polyubiquitinated. Numerical values below blot indicates the relative levels of ubiquitinated Gag normalized by the amount of total Gag. (F) 293T cells were transfected with Gag-GFP and SOCS1, and co-transfected with either myc-Ub-WT or myc-UbΔGG. After 24 h, cell lysates were harvested and subjected to immunoprecipitation analysis with anti-α-tubulin antibody or non-immunized mouse IgG (IgG) followed by immunoblotting analysis with the indicated antibodies. (G) 293T cells were transfected with Gag-FLAG and SOCS1, and co-transfected with either myc-Ub-WT or myc-UbΔGG. After 24 h, cell lysates were harvested and then incubated with taxol-stabilized microtubules (+MT) or control buffer (−MT), and separated into precipitate and supernatant fractions. Precipitate fractions were collected and then subjected to immunoblotting analysis with either anti-FLAG or anti-α-tubulin (microtubule) antibodies.

Furthermore, a parallel experiment revealed that the Gag-GFP puncta formed larger aggregations in the cytoplasm upon nocodazole treatment (Fig. 4D).

To further delineate the role of microtubule integrity in HIV-1 particle formation, we next performed experiments in which we co-transfected SOCS1 and pNL4-3 with or without the microtubule-destabilizing protein stathmin. Stathmin expression efficiently blocked the effects of SOCS1 upon HIV-1 particle formation in a dose-dependent manner (Fig. 4E and F). Cell viability was not strongly affected as revealed by immunoblotting analysis of either poly (ADP-ribose) polymerase (PARP) or β-actin (Fig. 4E). Our findings together indicate therefore that microtubule integrity may be required for SOCS1 to function in Gag assembly and release.

3.5. SOCS1 facilitates the ubiquitination of HIV-1 Gag

Our previous study has revealed that the SOCS-box of SOCS1 is required for both HIV-1 particle production and the enhancement of Gag association with microtubules [11]. The mechanism by which SOCS1 inhibits cytokine signaling is mediated by the inhibition of kinase activity through its N-terminal kinase inhibitory region (KIR) [18]. We next examined whether SOCS1 mutants lacking the function of either KIR (SOCS1-F59D) or SOCS-box (ΔSOCS) affected virus particle production. Our ELISA results indicate that the SOCS-box deletion mutant (ΔSOCS) of SOCS1 fails to promote virus production, whereas the KIR mutant, F59D, of SOCS1 partially enhances HIV-1 particle production when co-transfected with pNL4-3 in 293T cells (Fig. 5A and B). These data again suggest that the function of SOCS1 in HIV-1 particle production is not principally due to the suppression of interferon/cytokine signaling, but is mediated by its direct interaction with the HIV-1 Gag via the function of the SOCS-box.

The SOCS-box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity [19]. Indeed, several reports have demonstrated that Gag ubiquitination is related to its membrane association and particle release, although the function of HIV-1 Gag ubiquitination remains unclear [20,21]. We thus explored the possibility that SOCS1 modulates the ubiquitination of HIV-1 Gag, leading to enhanced virus particle formation. We initially examined the specific interaction of HIV-1 Gag with the SOCS1-E3 ligase complex. Purified SOCS1 and its E3 component proteins (biotinylated-Cullin 2, elongin B/C and Rbx1) in addition to HIV-1 Gag were synthesized in a wheat germ cell-free system and then subjected to pull-down assays using streptavidin coated magnetic beads. We found that Gag was co-purified with a SOCS1-E3 complex comprising SOCS1-elongin B/C-Rbx1-Cullin2 in a similar manner to RelA, a previously reported SOCS1 binding protein (Fig. 5C, left). Significantly, in the absence of SOCS1, both elongin B and C were not co-purified with Cullin 2 probably due to the unsteady condition of the E3 complex without SOCS1, and the amount of bound Gag was also reduced (Fig. 5C, right).These result indicate that the SOCS1-E3 complex associates with HIV-1 Gag and may promote its ubiquitination.

We next addressed whether SOCS1 affects the ubiquitination of HIV-1 Gag. Immunoprecipitation analysis with cells co-transfected with Gag-FLAG and myc-tagged ubiquitin, with or without SOCS1 co-transfection, revealed that SOCS1 overexpression significantly enhances the ubiquitination of the Gag protein (Fig. 5D). In contrast, the targeted depletion of SOCS1 by siRNA significantly reduced the amount of ubiquitinated Gag (Fig. 5E). These results indicate that SOCS1 could indeed be a potent ubiquitin ligase for HIV-1 Gag.

To clarify the biological significance of Gag ubiquitination via SOCS1, we performed an experiment using a dominant-negative ubiquitin construct lacking two C-terminal glycines (residues 75–76). This mutant ubiquitin (UbΔGG) cannot become conjugated to target substrates, but can bind noncovalently to ubiquitin interacting domains [14]. By immunoprecipitation analysis we revealed that the levels of HIV-1 Gag associated with microtubules were significantly reduced in cells expressing UbΔGG, as compared with those expressing wild-type ubiquitin (Ub-WT) (Fig. 5F). This trend was further revealed by a microtubule sedimentation experiment showing that the expression of UbΔGG reduced the amount of Gag associated with microtubules when compared with the expression of Ub-WT (Fig. 5G). These results together indicate a link between ubiquitin signaling and the microtubule-mediated Gag dynamics involved with HIV-1 particle formation.

4. Discussion

In our current study, we report that microtubule integrity is required for SOCS1 to facilitate Gag trafficking and virus particle production. We demonstrate from our experiments that (1) SOCS1 colocalizes with HIV-1 Gag along microtubules; (2) both SOCS1 and HIV-1 Gag are co-purified with microtubules and SOCS1 can augment the association of Gag with microtubules; (3) an intact microtubule network is required for the function of SOCS1 during Gag trafficking; (4) SOCS1 facilitates Gag ubiquitination; and (5) Gag association with the microtubules is significantly reduced when a dominant-negative Ub mutant is overexpressed. These results together indicate that SOCS1 can regulate the trafficking and stability of HIV-1 Gag via the microtubule-related cellular machinery, which may be in turn enhanced by Gag ubiquitination.

SOCS1 was identified initially as a negative regulator of signaling downstream of cytokines [22–24] and has been shown to localize at both the perinuclear region and the microtubule organizing center (MTOC) in cells [16]. We show from our current data that SOCS1 also forms dotted filamentous structures in the cytoplasm emanating from the perinuclear region, including the MTOC, to the cell periphery. A recent report has also indicated that Gag colocalizes at the MTOC with HIV-1 RNA and is subsequently transported to the cell periphery [25]. These observations together indicate that SOCS1 might facilitate the trafficking of HIV-1 Gag from the MTOC toward the plasma membrane by utilizing the intrinsic transport machinery of infected host cells.

The plus-end directed transport system along the microtubules could provide a means for the targeting of virus capsid proteins to the site of virus assembly and budding in the vicinity of the plasma membrane [26]. This ante-grade transport system is utilized by several viruses, such as herpes simplex virus type 1 (HSV-1), vaccinia virus and African swine fever virus (ASFV) [26–29]. Significantly, we have demonstrated in our present study that HIV-1 can utilize the microtubule-dependent transport mechanism, which may in turn be enhanced by SOCS1. Consistent with this notion, Leblanc et al. have demonstrated previously using a monoclonal antibody raised against unprocessed Gag that intracellular Gag puncta can travel along microtubules [30]. Our current microtubule pull-down analyses also clearly indicate that SOCS1 associates with Gag on microtubules and can enhance this interaction. This in turn might accelerate the intracellular trafficking of Gag to the plasma membrane along these structures, although the topological details are still unknown. Consistent with this observation, a plus-end microtubule motor KIF4 has been shown previously to associate with HIV-1 Gag and to enhance Gag trafficking [31,32]. These results further demonstrate the relevance of microtubule network in the trafficking of the HIV-1 Gag.

The involvement of the microtubule cytoskeleton in Gag assembly and HIV-1 particle egress is somewhat controversial [3,26,33,34]. However, several reports have presented convincing data to indicate the importance of this network in HIV-1 assembly

and propagation [3,35,36]. Our current study further demonstrates that the microtubule depolymerizing reagent, nocodazole, or the expression of microtubule-destabilizing protein stathmin, significantly inhibits the enhancement of HIV-1 particle production by SOCS1, suggesting a possible role of the microtubule network in regular HIV-1 particle production.

Our previous report indicated that the targeted depletion of SOCS1 results in the prominent perinuclear accumulations of HIV-1 Gag in 293T cells [11]. Our current study shows that nocodazole treatment or stathmin expression only slightly affects Gag release in non-SOCS1 overexpressing cells. This difference might be attributable to the following two possibilities. First, SOCS1 may affect Gag at multiple points during trafficking and assembly, and a critical point could be prior to the microtubule-mediated events that can be affected by nocodazole or stathmin. Second, there are multiple pathways to the delivery of exogenously expressed Gag protein from the cytoplasm to the plasma membrane in addition to microtubule-directed transport. Furthermore, we are currently uncertain whether the Gag association with microtubules is mediated by other microtubule binding proteins in cooperation with SOCS1, or whether SOCS1 directly associates with HIV-1 Gag on the microtubules. Further careful analysis must be performed to elucidate these possibilities.

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