SUMO is a small ubiquitin-like protein that becomes covalently conjugated to a variety of target proteins, the large majority of which are found in the nucleus. Ulp1 is a member of a family of proteases that control SUMO function positively, by catalyzing the proteolytic processing of SUMO to its mature form, and negatively, by catalyzing SUMO deconjugation. In Drosophila S2 cells, depletion of Ulp1 by RNA interference results in a dramatic change in the overall spectrum of SUMO conjugates, indicating that SUMO deconjugation is substrate-specific and plays a critical role in determining the steady state targets of SUMO conjugation. Ulp1 normally serves to prevent the accumulation of SUMO-conjugated forms of a number of proteins, including the aminoacyl-tRNA synthetase EPRS. In the presence of Ulp1, most SUMO conjugates reside in the nucleus. However, in its absence, SUMO-conjugated EPRS accumulates in the cytoplasm, contributing to an overall shift of SUMO from the nucleus to the cytoplasm. The ability of Ulp1 to restrict SUMO conjugates to the nucleus is independent of its role as a SUMO-processing enzyme because Ulp1-dependent nuclear localization of SUMO is even observed when SUMO is expressed in a preprocessed form. Studies of a Ulp1-GFP fusion protein suggest that Ulp1 localizes to the nucleoplasmic face of the nuclear pore complex. We hypothesize that, as a component of the nuclear pore complex, Ulp1 may prevent proteins from leaving the nucleus with SUMO still attached.

The regulation of protein function by reversible post-translational modification is an effective means for regulating protein stability, activity, subcellular localization, and interaction specificity. One such modification involves the covalent attachment of the small ubiquitin-like modifier protein (SUMO)1

1 The abbreviations used are: SUMO, small ubiquitin-like modifier protein; NPC, nuclear pore complex; EPRS, glutamyl-prolyl-tRNA synthetase; MRS, methionyl-tRNA synthetase; MSC, multiaminoacyl-tRNA synthetase complex; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; ds, double-stranded; GST, glutathione S-transferase; GFP, green fluorescent protein; ORF, open reading frame; HA, hemagglutinin; PBS, phosphate-buffered saline; NEM, N-ethylmaleimide; DAPI, 4',6-diamidino-2-phenylindole; POD, PML oncogenic domains.

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This paper is available on line at http://www.jbc.org
Ulp1 Blocks Accumulation of Cytoplasmic SUMO Conjugates

Ulp1 and Ulp2 (36–38). Yeast Ulp1 is an essential gene product that is responsible for both processing SUMO into its active form and deconjugating SUMO from its target proteins (36). Ulp2 is not required for cell viability and appears to act on a subset of sumoylated substrates that are distinct from the SUMO-conjugated proteins recognized by Ulp1 (37, 38). In vertebrates, there are at least six predicted members of the SUMO protease family (1, 39). These proteases all contain a highly conserved C-terminal catalytic region and a divergent N terminus that appears to be involved in regulating substrate selection or intracellular localization.

Mounting evidence suggests a connection between SUMO conjugation/deconjugation and nucleocytoplasmic trafficking. Yeast Ulp1 and the human SUMO protease SENP2 have both been shown to interact with components of the NPC (38, 40–42). The nuclear pore component RanBP2 has been shown to function as a SUMO ligase in vertebrates (43) and vertebrate nuclear pores, Ulp1 may deconjugate SUMO-conjugated substrates to clear pore complexes. We hypothesize that as a component of the nuclear membrane where it probably associates with nucleoplasmic filaments of the NPC (44). Finally, disruption of the SUMO conjugation machinery in yeast results in impaired nuclear transport and intracellular localization.

While most sumoylation targets are nuclear proteins, in this study we identify two primarily cytoplasmic enzymes, namely the aminocyl-tRNA synthetases glutamyl-prolyl-tRNA synthetase (EPRS) and methionyl-tRNA synthetase (MRS), as substrates for sumoylation. In metazoans, about 9 of the ~20 aminocyl-tRNA synthetases, including EPRS and MRS, associate with one another and additional factors to form an ~1.5 MDa protein complex termed the multiaminoacyl-tRNA synthetase complex (MSC) (46). While the MSC is primarily localized to the cytoplasm (the site of translation), a small fraction of the MSC is also found in the nucleus (47).

In this report, we show that Ulp1 is required to maintain the normal spectrum of SUMO conjugates in Drosophila cells. In untreated S2 cells, the majority of SUMO conjugates reside in the nucleus. However, upon depletion of Ulp1 by RNA interference, a large fraction of SUMO appears in the cytoplasm in the form of high molecular mass SUMO-conjugated proteins, including SUMO-conjugated EPRS. Fluorescence microscopy demonstrates that Ulp1 is found at the nucleoplasmic face of the nuclear membrane where it probably associates with nuclear pore complexes. We hypothesize that as a component of the nuclear pores, Ulp1 may deconjugate SUMO-conjugated proteins as they leave the nucleus thereby preventing the escape of SUMO from the nucleus.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies used for immunofluorescence microscopy and Western blotting are as follows: mouse monoclonal anti-HA 12CA5, mouse monoclonal anti-GFP (Roche Applied Science); mouse monoclonal antibody mAb414 against nuclear pore complex proteins (BAbCO); mouse monoclonal anti-FLAG M2, goat anti-mouse peroxidase conjugate, rabbit anti-rabbit peroxidase conjugate, goat anti-mouse Cy3 conjugate, sheep anti-mouse FITC conjugate, goat anti-rabbit TRITC conjugate (Sigma); rabbit polyclonal anti-Dorsal; rabbit polyclonal anti-EPRS was raised against Echerichia coli expressed EPRS rep domain; rabbit polyclonal anti-SUMO was raised against E. coli expressed HA-SUMO (45)

DNA Constructs and dsRNA Production—In previous publications (18, 48), we used the term Smn3 for the Drosophila member of the SUMO family. We now have switched to using the term SUMO. Accordingly, “Smn3” has been replaced with “SUMO” of all the constructs.

The pRM-Ulp1-GFP vector was created by amplifying the ulp1 cDNA (without the stop codon) from the expressed sequence tag (EST) clone GH303A1 by PCR using primers containing XbaI sites and ligating it into the XbaI site of pRM-GFP (48). A GST-GFP expression construct was created by amplifying the GST open reading frame (ORF) from pRM-GFP using primers containing EcoRI (5′) and Xhol (3′) and inserting it into the pGEX-4T1 vector (Amersham Biosciences). The full-length SUMO-expressing construct pRM-HA-SUMO was processed and SUMO-encoding vector pRM-HA-SUMO was generated by amplifying each ORF from pGEX-HA-SUMO or pGEX-HA-SUMO using primers containing a 5′-BamHI site and 3′-SacI site followed by insertion into the pRM-Ha3 vector. The pRM-HA-SUMO construct was generated by amplifying HA-SUMO from pPAC-HA-SUMO using primers containing EcoRI (5′) and BamHI (3′), and the resulting fragment was inserted into EcoRI/BamHI-digested pRM-ha3. The pPAC-FLAG-SUMO construct was created by amplifying the full-length SUMO open reading frame from pPAC-HA-SUMO followed by insertion into the SacI site in pPAC-FLAG. Construction of the following vectors has been described previously: pPAC-HA-SAE1, pPAC-HA-SAE2, pPAC-HA-SUMO, pPAC-HA-Ubc9, pPAC-HA-MRS, pPAC-HA-EPRS, pPAC-HA-Ulp1, pPAC-Dorsal, pPAC-Twist, pD25-37kluc, p-37luc (18, 48–50).

Double-stranded RNA (dsRNA) was generated by in vitro transcription (Megascript kit; Ambion) using PCR-derived templates containing flanking T7 promoters. The 720-bp control dsRNA template was created by amplifying the GST ORF from pGEX-4T1 using primers that included the T7 promoter. Similarly, the lamin Dm0 dsRNA template was generated by amplifying a 740-bp segment from the EST clone LD46046 and the Ulp1 dsRNA template was created by amplifying a 660-bp segment from pPAC-FLAG-Ulp1. SUMO dsRNA was prepared as described previously (18). All PCR products were generated using Pfu DNA polymerase.

RNA Purification and Enzyme Assays—The GST-tagged expression vectors pGEX-HA-SUMO, pGEX-HA-SUMO-GFP, pGEX-Ulp1, and pGEX-GFP, and pGEX-4T1 were expressed EPRS in E. coli BL21 and purified on glutathione-agarose beads according to the manufacturer’s instructions (Amersham Biosciences). Expression of GST-Ulp1 was induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) at 25 °C for 3 h. GST-HA-SUMO, GST-HA-SUMO-GFP, GST-GFP, and GST were expressed by induction with 1 mM IPTG for 3 h at 37 °C. GST fusions were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). Immobilized GST-GFP was treated with thrombin to yield GFP.

The SUMO-processing assays were performed at 30 °C for 6 h and included 150 ng of GST-HA-SUMO-GFP and 350 ng of GST-Ulp1 (or GST). Finally, 150 ng of purified GST-HA-SUMO and GST-HA-SUMO-GFP were included for comparison. The reaction mixtures contained 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM dithiothreitol. The reactions were terminated by boiling in SDS sample buffer and subjected to 12% SDS-PAGE followed by Western blot (anti-HA and anti-GFP).

For the in vitro deconjugation assay, 52985 U cells (S2 cells stably transfected with Cu2+ inducible expression constructs encoding HA-Ubc9 and FLAG-SUMO) were treated with 500 μM CuSO4. After a 24-h induction, the cells were pelleted, washed once in phosphate-buffered saline (PBS), and lysed by sonication in buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.2% Triton X-100, 2 mM N-ethylmaleimide (NEM), 2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin. The lysate was cleared by centrifugation, and the NEM was consumed by incubation with 2 mM N-tosyl-L-lysine and 2 mM β-mercaptoethanol for 10 min at room temperature. The reactions were incubated at 30 °C for 6 h and contained 20 μg of cleaved 52985 U lysate and 3 μg of crude GST-Ulp1 (or GST) in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, and 0.1% Triton X-100. The reactions were stopped by boiling in SDS sample buffer and subjected to 12% SDS-PAGE. FLAG-SUMO conjugates and free FLAG-SUMO were visualized by Western blot using the anti-FLAG antibody.

Cell Culture, Transfections, and RNAi—Drosophila S2 cells were cultured at 24 °C in Schneider insect medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Intermountain Scientific). Transfections were performed on cells grown in 24-well plates, using the PuGENE6 transfection reagent (Roche Applied Science). Localization of HA-tagged SUMO pathway components was carried out by individually transfecting 250 ng of pPAC-HA-SAE1, pPAC-HA-SAE2, pPAC-HA-Ubc9, or pPAC-HA-SUMO and harvesting cells for immunofluorescence 24 h post-transfection. To visualize the subcellular localization, cells were transfected with Ulp1, cells were fixed with 2% paraformaldehyde, incubated with 500 μM CuSO4, 18-h post-transfection, and harvested for fluorescence microscopy 24-h post-induction. In addition, 1.5 μg of lamin Dm0 dsRNA (or 1.5 μg of control dsRNA) was cotransfected with 200 ng of pRM-Ulp1-GFP. Expression of Ulp1-GFP was induced with 500 μM CuSO4, three days post-transfection, and cells were harvested for fluorescence microscopy 24 h post-induction. The nuclei were stained with DAPI and photographed. For Ulp1 RNAi experiments, cells were left untreated, transfected with 2 μg of Ulp1 dsRNA, 2 μg of SUMO dsRNA, or 2 μg of control dsRNA and harvested 5 days later. These cells were then either left untreated or were heat-shocked at 37 °C for 1 h and then analyzed by
anti-SUMO Western blot. To assess cytoplasmic fractions (Fig. 2C), the
cells were permeabilized in buffer containing 250 mM sucrose, 10 mM
Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM NEM, 0.1% digitonin, and
Complete protease inhibitor mixture (Roche Applied Science). The
samples were incubated on ice for 15 min, centrifuged, and the supernatant
was collected. In addition, cells were left untreated or were transfected
with 1 µg of control dsRNA or 1 µg of Ulp1 dsRNA and harvested 5 days
later. Immunofluorescence using the anti-HA antibody/FITC-conjugated
secondary antibody was also transfected with 500 ng of pRM-HA-SUMO⁵² or
pRM-HA-SUMO⁵² with or without 1 µg of Ulp1 dsRNA. SUMO expression was induced with 500 µM CuSO₄ 3 days post-transfection, and
harvested 24 h later and subjected to immunofluorescence using the anti-HA antibody/FITC-conjugated
secondary antibody.

To evaluate sumoylation of Dorsal, 529SU cells were left untreated or
were transfected with 1 µg of pPAC-Dorsal in addition to 1 µg of GST
dsRNA or 1 µg of Ulp1 dsRNA. The cells were induced with 500 µM
CuSO₄ 24 h post-transfection and harvested 24 h post-induction. The cells
were lysed directly in SDS sample buffer and subjected to Western blot
analysis.

For reporter assays each transfection consisted of 500 ng pDE5-37tkluc, 50 ng p-37luc (internal control), 3 ng pPAC-Twist, 9 ng
pPAC-Dorsal, and the indicated amounts of pRM-HA-Ubc9, pRM-HA-
SUMO⁵², pRM-HA-SUMO⁵², and/or pRM-HA-SUMO⁵². The luciferase
activity was determined using the dual-luciferase reporter assay
system (Promega). For RT-PCR analysis, total RNA was isolated from
cells using the TRIzol reagent according to the manufacturer’s instruc-
tions. Residual genomic DNA was degraded with RNAsese-free DNase and RT-PCR was performed using the Access RT-PCR kit (Promega) using primers specific for ulp1, dorsal, or sumo mRNA.

Identification of FLAG-SUMO Conjugates—S2 cells were transfected
with the pPAC-FLAG-SUMO expression vector. Two days later, cells
were harvested and cell lysates were immunoprecipitated with anti-
FLAG antibodies. The immunoprecipitate was fractionated by 6% SDS-
PAGE and analyzed by Coomassie Blue staining and anti-FLAG immu-
no blotting. Coomassie Blue-stained bands that appeared in the presence of
transfected FLAG-SUMO (see Fig. 2A) were excised, and subjected to in-gel digestion with trypsin. Tryptic peptide masses
determined by MALDI-TOF mass spectrometry (PerSeptive Biosystems
Voyager DE-RI operated in the delayed extraction and Reflector mode)
were used to search the Dro sophila genome database (51). p200 and p120
matched Dro sophila EPRS (CG5394 protein in the Dro sophila genome
data base) and Dro sophila MRS (CG15100 protein in the Dro sophila
genome database), respectively, each with greater than 99% proba-
blility. Unambiguous identifications were not obtained for p140 and p160.

N-terminal Edman degradation sequencing (Applied Biosystems
Procise cLC sequencer) of HPLC-purified tryptic peptides confirmed the
identification of p200 and p120. Four tryptic peptides from the p200
digest were sequenced and found to match predicted EPRS tryptic
fragments, and four tryptic peptides from the p120 digest were se-
quenced and found to match predicted MRS tryptic fragments. Addi-
tional evidence in support of the identifications was provided by the
apparent SDS-PAGE molecular masses of p200 and p120, which agree
within 5–10% with the calculated molecular masses of EPRS and MRS
after correction for the mass of a single SUMO adduct. Furthermore,
the identity of EPRS as a SUMO-conjugated protein was con-
firmed by probing Western blots of Dro sophila cells before and after
heat shock, before and after SUMO-Ubc9 overexpression, and before
and after Ulp1 or SUMO RNAi with anti-EPRS and anti-SUMO anti-
odies (see Fig. 2, C, D, E, and F).

Fluorescence Microscopy and Western Blotting—For fluorescence micro-
scopy, S2 cells were attached to poly-l-lysine-treated coverslips and
fixed in methanol at −20 °C for 10 min. In Fig. 5, C and D, Ulp1-GFP
or GFP-expressing cells were fixed in 2% formaldehyde for 30 min at
room temperature. After washing with PBS, the cells were permeabi-
lized in 0.2% Triton X-100 for 5 min on ice by incubation with 10 µg/ml digitonin for 10 min on ice. The cells were
washed three times in PBS and incubated with the indicated primary
antibody for 1 h at room temperature. After three additional PBS
washes, the cells were incubated with the indicated fluorochrome-conju-
gated secondary antibody for 1 h. DNA staining was performed by
incubating the cells with 500 ng/ml DAPI for 15 min. Following three
additional PBS washes, the coverslips were dried briefly and mounted in
Vectashield (Vector Laboratories). Images were viewed using a Zeiss
Axiostar 2 fluorescence microscope. Images were acquired with an
Optronics cooled CCD camera and captured with Adobe Photoshop. In
addition, Ulp1-GFP was also visualized in unfixed S2 cells using a
Bio-Rad MRC laser scanning confocal microscope and images were
processed using Confocal Assistant software.

For Western blotting, samples were boiled in SDS sample buffer for
10 min and fractionated by SDS-PAGE. Resolved proteins were trans-
ferrered to polyvinylidene difluoride membranes. The blots were blocked
for 1 h using 1% blocking reagent (Roche Applied Science) in TBS, and
subsequent steps were performed according to the manufacturer’s rec-
ommendations. Blots were processed by enhanced chemiluminescence
and exposed to ECL hyperfilm (Amersham Biosciences).

RESULTS

Ulp1 Alters the Spectrum of SUMO-conjugated Proteins in
Drosophila Cells—The genomic sequence of Drosophila mele-
nogaster reveals at least two probable members of the SUMO
protease family: Dro sophila Ulp1 and Dro sophila Ulp2, which,
based on sequence homology, may be the orthologues of yeast
Ulp1 and yeast Ulp2, respectively. To determine if Drosophila
Ulp1 can catalyze both the deconjugation and maturation re-
actions, we assayed recombinant Ulp1 in vitro. In Dro sophila,
the C-terminal extension that is removed during SUMO
maturation is just two amino acids long, and thus processing
does not result in a size change that can be easily detected by
SDS-PAGE. However, fusion of additional amino acid residues
to the C terminus of the unprocessed SUMO family proteins
does not generally interfere with SUMO processing (36). Thus,
to determine if Dro sophila Ulp1 could catalyze the processing
reaction, we incubated GST-Ulp1 with GST-HA-SUMO-GFP.
This resulted in the production of mature GST-HA-SUMO as well as
free GFP (Fig. 1A), confirming that Dro sophila Ulp1 can
catalyze SUMO maturation. The ability of Dro sophila Ulp1 to
catalyze deconjugation is illustrated by an experiment in which
FLAG-SUMO and Ubc9 were overexpressed in S2 cells gener-
ating an array of SUMO-conjugated proteins, as detected by an
anti-FLAG immunoblot (Fig. 1B, lane 1). Treatment of an
extract of these cells with GST-Ulp1 (lanes 2), but not with GST
alone (lane 3) results in the disappearance of these SUMO-
conjugated species, but not free FLAG-SUMO. The free FLAG-
SUMO is present in large excess in this experiment thus ac-
counting for the lack of a discernable increase in free FLAG-
SUMO upon treatment with GST-Ulp1.

The above experiments show that Dro sophila Ulp1 is able to
mediate both SUMO maturation and deconjugation in vitro. To
determine how Ulp1 functions in cells, we treated cells with
double-stranded RNA (dsRNA) to deplete Ulp1 by RNA inter-
ference (RNAi). RT-PCR assays (Fig. 1C) demonstrate the spec-
ificity of the RNAi treatment.

When whole cell lysates of untreated cells were probed by
immunoblot with anti-SUMO antibody, we observed free
SUMO plus a complex array of SUMO-conjugated proteins
(Fig. 1D, lane 1). Treatment with SUMO dsRNA eliminated
almost all the anti-SUMO reactive bands (lanes 3 and 7), while
control GST dsRNA had no effect (lanes 2 and 6), thus demon-
strating the specificity of the antibodies and confirming that
the observed bands are SUMO conjugates. Depletion of
Ulp1 by RNAi results in a distinct change in the spectrum of
SUMO-conjugated proteins. In particular, SUMO conjugates
in the 80–120 kDa range decrease in abundance, while a group
of high molecular mass SUMO conjugates in the 120–200 kDa
range appear (lane 4). High molecular mass SUMO conjugates
also appear when cells are subjected to heat shock (lanes 5 and
6) or other forms of stress (e.g. oxidative stress, data not
shown). In other systems, it has been shown that SUMO con-
jugation is stimulated in response to cellular stress (52–54), and
these results demonstrate that the same is true in Dro-
sophila cells. The similar appearance in the high molecular
mass regions of the banding patterns in lanes 4 and 5 suggests
that some of the same conjugates that are induced by heat
shock are also induced by Ulp1 depletion. We provide further
evidence below that at least one of the high molecular mass conjugates induced by heat stress is identical to a conjugate induced by Ulp1 depletion. We note, however, that while Ulp1 depletion results in the specific accumulation of the high molecular mass conjugates, heat shock results in increased levels of a broader array of SUMO-conjugated proteins (compare lanes 1, 4, and 5). We also observed that disruption of Ulp1 prevents the elevation in SUMO conjugates seen in heat shock (compare lanes 5 and 8). This suggests that Ulp1 may be required for the elevation of sumoylation that occurs in response to heat shock. For example, perhaps this aspect of the heat shock response requires the ability to recycle SUMO from one conjugate to another.

The finding that new SUMO conjugates appear upon Ulp1 depletion suggests that Ulp1 may not be essential for SUMO maturation in vivo. Indeed, overall levels of SUMO conjugation may be slightly higher in the Ulp1-depleted cells as indicated by the decrease in the level of free SUMO (Fig. 1D, lane 4). This is more clearly shown in Fig. 2C, below. Thus, other SUMO specific proteases (perhaps Ulp2) must contribute to SUMO maturation. These results are consistent with an in vivo role for Ulp1 in SUMO deconjugation and with the notion that the efficiency of deconjugation by Ulp1 is substrate specific. In the absence of Ulp1, some SUMO-conjugated proteins (presumably those that are normally efficiently deconjugated by Ulp1) accumulate in the SUMO-conjugated form apparently resulting in decreased availability of SUMO for other conjugation targets. However, we cannot completely rule out the possibility that Ulp1 depletion alters the spectrum of SUMO-conjugated proteins by altering conjugation specificity.
Identification and characterization of high molecular weight SUMO conjugates. A, S2 cells were transfected with a vector encoding FLAG-SUMO. Two days later, cells were harvested and cell lysates were immunoprecipitated with anti-FLAG antibodies. The immunoprecipitate was fractionated by 6% SDS-PAGE and analyzed by Coomassie Blue staining and anti-FLAG immunoblotting. The bands indicated by the arrows were excised and p120 was identified as MRS, whereas p200 was identified as EPRS.

B, S2 cells were fixed and stained for immunofluorescence using anti-EPRS antibody and anti-rabbit TRITC-conjugated IgG. Cells were treated with DAPI to stain DNA.

C, S2 cells were left untransfected (lane 1) or were transfected with GST dsRNA (lane 2) or with Ulp1 dsRNA (lane 3). The cells were harvested 5-days post-transfection, permeabilized with digitonin, and the extract was cleared by centrifugation. Samples of the cytoplasmic extract (15 μg of total protein) were subjected to 6% (top) or 12% (bottom) SDS-PAGE and analyzed by immunoblotting with the anti-SUMO antibody. The 6% gel was reprobed with an anti-EPRS antibody (top, right).

D, S2 cells were left untransfected (lanes 1 and 2) or were transfected with SUMO dsRNA (lanes 3 and 4) and heat-shocked by incubation at 37 °C for 1 h prior to harvesting (lanes 2 and 4). The cells were boiled in SDS sample buffer, subjected to 5% SDS-PAGE, and analyzed by anti-EPRS Western blot.

E, 529SU cells were left untreated, induced with CuSO_4 for 36 h (lanes 2 and 3), and heat-shocked by incubation at 37 °C for 1 h prior to harvesting (lane 3). The cells were boiled in SDS sample buffer, subjected to 5% SDS-PAGE, and analyzed by anti-EPRS Western blot.

F, 529SU cells were transfected with Ulp1 dsRNA (lanes 3 and 4) and induced with CuSO_4 for 3 days (lanes 2 and 5). The cells were boiled in SDS sample buffer, subjected to 5% SDS-PAGE, and analyzed by anti-EPRS Western blot.

G, EPRS contains an N-terminal glutamyl-tRNA synthetase domain (glu) and a C-terminal prolyl-tRNA synthetase domain (pro). The two catalytic domains are connected by the rep domain, which consists of 6 imperfect repeats of an ~46 amino acid sequence (green ovals). The sequence of the rep domain is shown. Matches for the consensus sumoylation site are highlighted in red.

H, 529SU cells were transfected with a vector encoding the wild-type HA-tagged EPRS rep domain (WT) or one in which all five lysines within consensus SUMO conjugation sequences were mutated to arginine (m5). Cells were either left untreated (−) or treated with CuSO_4 to induce expression of FLAG-tagged SUMO (+). Whole cell lysates were analyzed by immunoblotting with an anti-HA antibody.
Dorsal is a known SUMO conjugation target (18). To provide further evidence that Ulp1 is a deconjugating enzyme in vivo, we evaluated the effect of Ulp1 RNAi on formation of the SUMO-Dorsal conjugate. Dorsal was transiently expressed in a stably transformed S2 cell line (529SU cells) bearing Ubc9 and full-length SUMO encoding transgenes under the control of the Cu" inducible metallothionein promoter. An anti-Dorsal immunoblot showed that depletion of Ulp1 resulted in a substantial increase in the level of the SUMO-Dorsal conjugate (Fig. 1E, compare lanes 3 and 5). This result strongly suggests that Ulp1 is not required for SUMO processing in vivo and supports the conclusion that Ulp1 is capable of deconjugating SUMO from known target proteins in living cells.

Identification of High Molecular Weight SUMO Conjugates—In an effort to identify new SUMO conjugation targets, cells were transfected with an expression vector encoding FLAG-tagged SUMO. Whole cell lysates of the transfected cells were subjected to anti-FLAG immunoprecipitation, and the purified proteins were resolved by SDS-PAGE. The Coomassie Blue-stained gel revealed four bands, termed p120, p140, p160, and p200, that were specific for the FLAG-SUMO-transfected cells (Fig. 2A). Since these are similar to the high molecular weight SUMO conjugates that are induced in response to Ulp1 depletion (compare Figs. 1D and 2A), we speculate that overexpression of the FLAG-SUMO chimera may have overwhelmed the endogenous Ulp1 causing the cells to behave as if they were Ulp1-deficient.

We attempted to identify the high molecular weight FLAG-SUMO conjugates by mass spectroscopy and microsequencing and obtained unambiguous identification (see “Experimental Procedures”) of p120, which is MRS, and p200, which is EPRS. The apparent SDS-PAGE molecular masses of these two proteins (120 kDa for MRS and 200 kDa for EPRS) are in excellent agreement with the calculated masses (109 kDa for MRS and 189 kDa for EPRS), with the discrepancy being accounted for by the mass of a SUMO adduct.

The finding that aminoacyl-tRNA synthetases might be major targets of sumoylation in Ulp1-depleted cells was somewhat surprising, since almost all previously known sumoylation targets are nuclear proteins and since the sumoylation machinery is primarily found in the nucleus (Ref. 55 and see Fig. 3A). tRNA aminoclylation is a cytoplasmic process and previous studies of aminoacyl-tRNA synthetases confirm that they are primarily cytoplasmic enzymes. When cells were examined by immunofluorescence with an anti-EPRS antibody (Fig. 2B), EPRS was, as expected, found to reside predominantly in the cytoplasm. Furthermore, treatments that alter the level of SUMO-conjugated EPRS including Ulp1 depletion or Ubc9/SUMO overexpression (see below) have no influence on EPRS subcellular localization (Supplemental Fig. B).

We employed several approaches to confirm that EPRS is sumoylated in vivo. First, we probed Western blots containing digitonin extracts of untreated and Ulp1-depleted cells with anti-EPRS and anti-SUMO antibodies (Fig. 2C). The anti-EPRS immunoblot (Fig. 2C, right panel) shows that untreated cells (lane 1) or cells treated with control GST dsRNA (lane 2) exhibit a band of the expected molecular weight. Upon Ulp1 depletion, we observe a near quantitative shift of the immunoreactive species to an apparently higher molecular weight (lane 3). This shifted species almost certainly represents SUMO-conjugated EPRS as is confirmed by probing the same blot with the anti-SUMO antibody (Fig. 2C, left panel). The difference between the appearance of the anti-SUMO blot in this experiment and its appearance in the previous Ulp1-depletion experiment (Fig. 1D) is due to the different procedures used to generate the proteins samples. In the previous experiment, we generated whole cell lysates, while in this experiment, cytoplasmic proteins were extracted with digitonin, a procedure that results in significant enrichment of EPRS relative to other SUMO-conjugated proteins. The anti-SUMO blot (Fig. 2C, lower panel) shows that the cytoplasmic-free SUMO completely disappears after Ulp1 depletion. This supports the conclusion that the cytoplasmic SUMO immunoreactivity detected after Ulp1 depletion (see Fig. 3B, below) is because of SUMO-conjugated cytoplasmic proteins including EPRS and not to cytoplasmic free SUMO.

Further confirmation that EPRS is a SUMO conjugation target came from an experiment in which we assessed the effect of heat shock on untreated cells or cells in which SUMO was depleted using RNAi (Fig. 2D). In untreated cells (lanes 1 and 2) heat shock results in a substantial increase in sumoylated
EPRS (lane 2). When cells are treated with SUMO dsRNA (lanes 3 and 4) heat shock fails to induce sumoylation of EPRS. In addition we probed a Western blot of cells before and after induction of Ub9 and SUMO overexpression and/or before and after subjecting the cells to heat shock. Upon expression of Ub9 and SUMO, we observe the appearance of EPRS-SUMO conjugates (Fig. 2E, compare lanes 1 and 2) and the abundance of the conjugates increases upon heat shock (lane 3). Continued overexpression of Ub9 and SUMO results in a nearly complete shift of EPRS from the unsumoylated to sumoylated form (Fig. 2F, compare lanes 1 and 2). We determined that disruption of Ulp1 by RNAi is as effective as Ub9/SUMO overexpression in generating the EPRS-SUMO conjugate (compare lanes 2 and 3). In conclusion, we strongly suspect that Ulp1 normally suppresses the formation of SUMO-EPRS by specifically deconjugating this target.

EPRS is an unusual member of the aminoaocyl-tRNA synthetase family in that it consists of two independent catalytic domains, a glutamyl-tRNA synthetase domain and a prolyl-tRNA synthetase domain, held together by a linker domain termed the rep domain. Inspection of the rep domain revealed 5 lysine residues that lie in the context of a consensus SUMO conjugation site (Fig. 2G). When the rep domain was expressed in 529SU cells, induction of SUMO expression resulted in the appearance of a rep-SUMO conjugate (Fig. 2H, compare lanes 1 and 2). Mutagenesis of the 5 lysines prevented formation of the rep-SUMO conjugate (lanes 3 and 4). In contrast, the isolated glutamyl-tRNA synthetase and prolyl-tRNA synthetase domains were not substrates for SUMO conjugation in this assay (data not shown).

Nucleocytoplasmic Distribution of SUMO Conjugates Is Dependent upon Ulp1—Previous studies have suggested that sumoylation is primarily a nuclear process. The vast majority of known SUMO-conjugated proteins are found in the nucleus. Furthermore, studies of mammalian cells show that the enzymes catalyzing SUMO conjugation are predominantly located in the nucleus (55). To determine if enzymes required for SUMO conjugation are also nuclear in Drosophila cells, we expressed HA-tagged forms of the SUMO-conjugating enzyme (Ub9), the two subunits of the SUMO-activating enzyme (SAE1 and SAE2), and SUMO itself in S2 cells. Anti-HA immunofluorescence reveals that all four polypeptides are predominantly nuclear (Fig. 3A).

Given the evidence that SUMO-conjugated proteins are primarily nuclear, while major targets of SUMO conjugation that appear both in response to heat shock and Ulp1 depletion are primarily cytoplasmic, we investigated the effect of Ulp1 on the overall distribution of endogenous SUMO in S2 cells. In control S2 cells, endogenous SUMO was largely restricted to the nucleus (Fig. 3B, top row). Transfecting a control GST dsRNA had no effect on the subcellular localization of SUMO (Fig. 3B, middle row). In cells transfected with Ulp1 dsRNA, we observed a significant increase in cytoplasmic SUMO yielding essentially uniform staining throughout the cell (Fig. 3B, bottom row). This change in SUMO localization does not reflect a general defect in transport of proteins into the nucleus since the other components of the conjugation machinery (SAE1, SAE2, and Ub9) and other nuclear proteins (e.g. Dip3 and Groucho) show normal nuclear localization (Supplemental Fig. A and data not shown).

Predominant Nuclear Localization of SUMO Depends on SUMO Conjugation but Not SUMO Processing.—The above findings suggest that Ulp1 may prevent the accumulation of cytoplasmic SUMO conjugates by specifically catalyzing the deconjugation of SUMO from cytoplasmic proteins including certain aminoaocyl-tRNA synthetases. However, although unlikely, it is also possible that the specific targeting of sumoylation by Ulp1 is somehow coupled to the function of Ulp1 in SUMO processing. To test this possibility, we carried out experiments in which full-length HA-tagged SUMO (HA-SUMOFL) and two truncated forms of SUMO (HA-SUMO GG and HA-SUMO AG, Fig. 4A) were expressed in S2 cells. HA-SUMO AG is missing the final two amino acids (Ala-Pro) found in the full-length SUMO translation product and therefore ends with the diglycine residue normally found at the end of the mature protein after processing. Thus, HA-SUMO AG is ready for conjugation immediately after translation without the need for an intermediate processing step. HA-SUMO GG is missing the two glycines in addition to the Ala-Pro. It has been previously shown that these C-terminal glycine residues are essential for recognition by the SUMO activating enzyme and HA-SUMO GG is therefore unable to undergo conjugation (31). Further evidence that HA-SUMO FL and HA-SUMO GG are functional, while HA-SUMO AG is non-functional comes from an analysis of the effects of these three forms of SUMO on Dorsal-mediated transcriptional activation. Previous studies have shown that Dorsal-mediated activation is stimulated by the attachment of SUMO to Lys-382 (18). Transient transfection assays using a luciferase reporter containing a promoter that is synergistically activated by Dorsal and Twist show that HA-SUMO FL and HA-SUMO GG can potentiate Dorsal-mediated activation while HA-SUMO AG cannot (Fig. 4B). Cells expressing HA-SUMO FL and cells expressing HA-SUMO GG both exhibited predominantly nuclear anti-HA immunofluorescence (Fig. 4, C and D, top row). In addition to diffuse staining throughout the nucleus, we also observed punctate dots of nuclear staining. These dots may represent the Drosophila counterpart of the nuclear bodies to which many mammalian SUMO-conjugated proteins are found to localize. Both HA-SUMO FL and HA-SUMO GG largely relocated to the cytoplasm upon depletion of Ulp1 by RNAi (Fig. 4, C and D, bottom two rows). Interestingly, the nuclear dots also appeared to relocalize to the cytoplasm upon Ulp1 depletion. The number of nuclear and cytoplasmic dots was higher in SUMO GG-expressing cells than in SUMO FL-expressing cells, although the significance of this difference is unclear. The finding that SUMO is still predominantly nuclear even when expressed in a preprocessed form rules out the possibility that Ulp1 regulates the localization or spectrum of SUMO conjugates by controlling the processing step.

In contrast to functional forms of SUMO, when the non-functional form of SUMO, HA-SUMO AG, was expressed in S2 cells, we observed uniform localization throughout the cell (Fig. 4E, bottom two rows). This finding suggests that nuclear accumulation of SUMO requires SUMO conjugation. Perhaps conjugation of SUMO to nuclear proteins is required for the restriction of SUMO to the nucleus in wild-type cells.

Drosophila Ulp1 Localizes to the Nucleoplasmic Face of the Nuclear Pores—The results presented thus far suggest that Ulp1 normally acts to prevent the accumulation of a specific subset of SUMO conjugates, including EPRS, that appear to have a different overall subcellular distribution than the majority of SUMO-conjugated proteins. To determine if this ability to target select proteins for deconjugation could be due to the restricted subcellular localization of Ulp1, we expressed a Ulp1-GFP fusion protein in S2 cells and determined its localization by fluorescence microscopy. We found that Ulp1-GFP was primarily localized to the nuclear envelope (Fig. 5A). Using confocal microscopy, we observed a small number of speckles within the nucleus, in addition to predominant localization at the nuclear periphery (Fig. 5B). An antibody (mAb 414) that
recognizes a group of FG repeat-containing NPC proteins was used in immunofluorescent staining of cells expressing Ulp1-GFP. A considerable overlap between Ulp1-GFP and mAb414 staining was seen (Fig. 5C).

Further evidence that the Ulp1-GFP fusion protein is localized to the nuclear pores is provided by an experiment in which the distribution of nuclear pores was perturbed. Experiments employing a hypomorphic lamin allele in the adult central nervous system have shown that when lamin expression is reduced, nuclear pores tend to cluster (56). Furthermore, reduction of lamin expression in Caenorhabditis elegans by RNA interference also results in clustering of nuclear pores (57). When we used dsRNA interference to block lamin expression in S2 cells, the nuclear pores, as observed by staining with mAb414, clustered in a fraction of the cells in a manner very similar to that previously observed in C. elegans. Anti-lamin Western blots reveal that lamin protein levels were greatly reduced in the dsRNA-treated cells (data not shown). The clustered nuclear pores in these cells always colocalized with the

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Ulp1-GFP fluorescence (Fig. 5C, bottom row, and Supplemental Fig. C).

To determine if Ulp1 localizes to the cytoplasmic or the nuclear face of the nuclear membrane, we permeabilized GFP or Ulp1-GFP expressing cells with either digitonin or Triton X-100. The GFP or Ulp1-GFP in the detergent treated cells was then visualized by direct GFP fluorescence or by anti-GFP immunofluorescence. Digitonin renders the plasma membrane, but not the nuclear membrane, permeable to antibodies, while Triton X-100 permeabilizes both the cytoplasmic and nuclear membranes. This is verified by examination of the GFP-expressing cells (Fig. 5D, bottom two rows). Direct fluorescence shows that the GFP is localized throughout the cell although it is more concentrated in the nucleus than in the cytoplasm. The anti-GFP antibody detects only the cytoplasmic GFP in digitonin-treated cells, while it detects both the cytoplasmic and nuclear GFP in Triton X-100-treated cells.

In the Ulp1-GFP expressing cells, we once again observed nuclear membrane localization by direct fluorescence. However, the antibody only detected the nuclear membrane bound protein after Triton X-100 treatment and not after digitonin treatment (Fig. 5D, top two rows). Thus, Ulp1 localizes to the nucleoplasmic face of the nuclear pores.

DISCUSSION

SUMO Deconjugation as a Means to Control SUMO Targeting—Depletion of Ulp1 from S2 cells by RNAi results in increased levels of SUMO conjugation indicating that deconjugation rather than SUMO maturation is the dominant role of Ulp1. Furthermore, Ulp1 depletion results in a change in the spectrum of SUMO-conjugated proteins indicating that the specificity with which proteins are selected for deconjugation may play an important role in the specificity of SUMO targeting. Substrate-specific deconjugating enzymes may prevent limiting amounts of SUMO from becoming irreversibly conjugated to inappropriate targets that encounter the conjugation machinery. In this way, specific SUMO conjugation could be achieved even if the specificity of the enzymes responsible for conjugation was relatively low. This contrasts with ubiquitin conjugation in which the specificity of conjugation is ensured by a myriad of ubiquitin ligases, which select specific ubiquitin conjugation targets in response to a huge variety of extrinsic and intrinsic cues (58, 59). While analogous SUMO ligases do exist, they appear to be fewer in number than ubiquitin ligases, and may not always be required for SUMO conjugation (34, 35).

Restricted Localization of SUMO Proteases to Nuclear Subcompartments—There are multiple ways in which SUMO deconjugation could be rendered substrate specific. For example, the deconjugating enzymes could recognize specific sequence or structural motifs in the deconjugation targets. However, it is difficult to see how a limited number of deconjugating enzymes could specifically recognize all the proteins (probably the majority of cellular proteins) that should not be conjugated to SUMO. An alternative strategy might be to control deconjugation specificity by targeting the deconjugating enzymes to specific subcellular locales.

In support of this latter possibility, we have found that Drosophila Ulp1 localizes to the nucleoplasmic face of the NPC where it is apparently required to prevent the accumulation of SUMO-conjugated cytoplasmic proteins. Upon depletion of Ulp1 from S2 cells, there is a dramatic shift in the localization of SUMO from the nucleus to the cytoplasm, which at least partly reflects the attachment of SUMO to high molecular mass cytoplasmic proteins such as EPRS and MRS. While these aminoacyl-tRNA synthetases are predominantly located in the cytoplasm, a growing body of evidence suggests that they also spend time in the nucleus, where they may catalyze an initial round of tRNA aminoacylation. These enzymes may help to channel aminoacyl-tRNA directly to the cytoplasmic translational elongation factor EF1 (60, 61), and thus it is possible that they shuttle in and out of the nucleus. Since the SUMO conjugation machinery is concentrated inside the nucleus, enzymes like EPRS that may transiently enter the nucleus could become inappropriately conjugated to SUMO during the time spent inside the nucleus. The localization of Ulp1 to the nucleoplasmic face of the NPC might then assure that SUMO deconjugation occurs during re-export. If this was the case, then we might expect inhibition of EPRS export to result in the accumulation of SUMO-conjugated forms of EPRS. However, the mechanism of EPRS re-export is not understood and our attempts to block EPRS export by the specific inhibition or RNAi-mediated depletion of Crm1, the best characterized of the exportins (62), were not successful (data not shown).

With one possible exception (63), previous studies of other SUMO proteases have also demonstrated localization to specific nuclear subcompartments. Yeast Ulp1 and human SENP2 are found at the nuclear pores (38, 40–42), and furthermore, the targeting of yeast Ulp1 to the nuclear membrane is required to maintain the normal spectrum of SUMO-conjugated proteins (64). Yeast Ulp2 is found in the nucleoplasm (38). Murine SUMO protease-1 localizes to the nuclear bodies (65). Finally, human SENP3 (SMT3P1) localizes to the nucleolus (66). Localization may be a means to limit the access of these proteases to proteins in particular subcellular compartments thereby targeting them to a particular subset of SUMO-conjugated proteins. In addition to the evidence presented here, further evidence for this hypothesis comes from experiments in which removal of the NPC-targeting sequence from the human SUMO protease SENP2 was found to result in a significant change in the intracellular spectrum of sumoylated proteins (42).

Speculation on the Functional Significance of tRNA Synthetase Sumoylation—Our findings suggest that one purpose of Ulp1 is to prevent the conjugation of SUMO to cytoplasmic proteins such as EPRS, which spend most of their life in the cytoplasm, but which may encounter the SUMO conjugation machinery during transient passage through the nucleus. Thus, it is possible that EPRS sumoylation plays no beneficial cellular role. However, we have found that EPRS also becomes sumoylated upon cellular stress such as heat shock, raising the possibility that aminoacyl-tRNA synthetase sumoylation plays a role in the stress response. Given the variety of functions that have been associated with aminoacyl-tRNA synthetases (46), sumoylation of these enzymes could help mediate the stress response in any number of ways. For example, both EPRS and MRS are components of the MSC, and the rep domain, which is the region of EPRS targeted for sumoylation, is required for the formation of this complex (67). The functional significance of the MSC is unclear, although it may play a role in the trafficking of tRNA from the nucleus to the ribosome (47). Celluar stress and the resulting protein damage may lead to a need for increased protein synthesis, which in turn, would require increased levels of aminoacyl-tRNA at the ribosome. By regulating the function of the MSC, sumoylation could therefore help cells up-regulate protein synthesis to replace proteins damaged during cellular stress.

Sumoylation as a Nuclear Process—Whereas a few SUMO target proteins may be exclusively extranuclear (68), the vast majority of such proteins including NPC-associated factors and numerous transcription factors spend some or most of their life in the nucleoplasm or at the nuclear periphery. For example, in vertebrates, SUMO seems to play a role in the structure and/or function of the intranuclear PODs (10, 11, 69). Anti-SUMO
staining of Drosophila cells reveals punctate nuclear dots above a diffuse background of SUMO throughout the nucleus. These dots may represent the Drosophila counterpart of the POIs (70). Interestingly, we find that when SUMO is excluded from the nucleus by interference with Ulp1 expression, the dots relocalize to the cytoplasm. While we cannot be sure that these dots are truly equivalent to the nuclear dots seen in normal cells, this finding suggests that dot formation may be an intrinsic property of SUMO itself that is independent of SUMO nuclear localization.

Restriction of SUMO conjugation to the nucleus is largely achieved by the localization of the SUMO conjugation machinery to the nucleus. However, the findings presented here indicate that Ulp1 plays an important role in enforcing this nuclear restriction through substrate-specific deconjugation.

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