CRM1-mediated Nuclear Export Is Required for 26 S Proteasome-dependent Degradation of the TRIP-Br2 Proto-oncoprotein*

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Overexpression of the proto-oncogene TRIP-Br2 (SERTAD2) has been shown to induce E2F activity and promote tumorigenesis, whereas ablation of TRIP-Br2 arrests cell proliferation. Timely degradation of many cell cycle regulators is fundamental to the maintenance of proper cell cycle progression. Here we report novel mechanism(s) that govern the tight regulation of TRIP-Br2 levels during cell cycle progression. TRIP-Br2 was observed to be a short-lived protein in which the expression level peaks at the G1/S boundary. TRIP-Br2 accumulated in cells treated with 26 S proteasome inhibitors. Co-immunoprecipitation studies revealed that TRIP-Br2 forms ubiquitin conjugates. In silico analysis identified a putative leucine-rich nuclear export signal (NES) motif that overlaps with the PHD-Bromo interaction domain in the acidic C-terminal transactivation domain (TAD) of TRIP-Br2. This NES motif is highly conserved in widely divergent species and in all TRIP-Br family members. TRIP-Br2 was shown to be stabilized in G2/M phase cells through nuclear entrapment, either by deletion of the acidic C-terminal TAD, which includes the NES motif, or by leptomycin B-mediated inhibition of the CRM1-dependent nuclear export machinery. Mutation of leucine residue 238 of this NES motif abolished the interaction between CRM1 and TRIP-Br2, as well as the nuclear export of TRIP-Br2 and its subsequent 26 S proteasome-dependent degradation. These data suggest that CRM1-mediated nuclear export may be required for the proper execution of ubiquitin-proteasome-dependent degradation of TRIP-Br2.

The TRIP-Br/SERTAD (henceforth referred to as TRIP-Br (transcriptional regulator interacting with the PHD-bromodomain)) family of mammalian transcriptional coregulators has been shown to play important roles in governing cell cycle progression, at least in part, by regulating the expression of E2F-responsive genes that are implicated in or directly linked to the regulation of cell proliferation (1, 2). The mammalian TRIP-Br family comprises four members: TRIP-Br1/p34SER-TAD1/SEI-1 (henceforth referred to as TRIP-Br1); TRIP-Br2/SERTAD2/SEI-2 (henceforth referred to as TRIP-Br2); TRIP-Br3/HEPP/CDC4/SEI-3 (henceforth referred to as TRIP-Br3); and RBT1§ (replication protein A-binding transactivator 1)/SERTAD3 (henceforth referred to as RBT1) (3). In addition, the TRIP-Br homolog in Drosophila, Taranis (TARA), was identified in a screen for functional partners of the homeotic loci and was shown to represent a novel member of the trithorax group of regulatory proteins (4). TRIP-Br proteins are recruited to the retinoblastoma (Rb) protein-disassociates E2F-1/DP-1 complexes on E2F-responsive promoters through physical association with DP-1. PHD zinc finger- and/or bromodomain-containing proteins such as p300/CBP, PCAF, and KRP-1, present in differing amounts and possessing different binding affinities, have been proposed to compete for binding to TRIP-Br proteins and confer positive or negative regulatory signals to E2F-1/DP-1 transcription complexes assembled on E2F-responsive promoters (1, 5, 6). Antagonism of the TRIP-Br integrator function has been shown recently to elicit a proliferative block and caspase-3-independent cellular subdiploidization, associated with the transcriptional down-regulation of a subset of E2F-responsive genes in vivo (7).

Members of the TRIP-Br family possess four evolutionarily conserved regions including a putative cyclin A-binding motif [including a conserved nuclear localization signal (NLS), KRK] at the amino terminus, a novel SERTA (S.E.I-1, RBT1 and TARA) domain and an acidic TAD at the C terminus, which also includes a PHD zinc finger and/or bromodomains (PHD-Bromo) interaction domain (4). Although the function of the SERTA domain remains to be elucidated, studies have shown that this motif of TRIP-Br1 binds to cyclin-dependent kinase 4 (CDK4) and facilitates the assembly and activation of cyclin D-CDK4 complexes (8). Notably, the C terminus of TRIP-Br proteins is required not only for the stimulation of E2F-medi-

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§ These abbreviations used are: RBT1, replication protein A-binding transactivator 1; Rb, retinoblastoma; TAD, transactivation domain; CDK, cyclin-dependent kinase; NES, nuclear export signal; HA, hemagglutinin; aa, amino acid(s); HU, hydroxyurea; CHX, cycloheximide; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ALLN, N-acetyl-Leu-Leu-leucinal; LMB, leptomycin B; WB, Western blot; RT, reverse transcription; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ub, ubiquitin.
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TRIP-Br2, TRIP-Br, and RBT1 (1, 5, 9) are the first report of TRIP-Br-mediated suppression of E2F-dependent transcription (6).

Members of the TRIP-Br protein family share a similar but unusual genomic structure. All members have intronless coding regions but possess one intron in their 5′-untranslated region. In addition, the splice acceptor site is always found within 7 bp of the ATG start codon. TRIP-Br, the Drosophila homolog of mammalian TRIP-Br genes, contains an intron in its 5′-untranslated region (4). It remains unknown whether TRIP-Br proteins are regulated by post-transcriptional modifications such as alternative splicing.

We recently reported that TRIP-Br2 is a novel proto-oncogene that is overexpressed in multiple human tumors such as prostate carcinoma, squamous cell lung carcinoma, lung adenocarcinoma, ovarian cystadenocarcinoma, colorectal carcinoma, renal cell carcinoma, osteosarcoma, and hepatocellular carcinoma. Its overexpression was associated with up-regulation of E2F-mediated transcription, transformation of NIH3T3 fibroblasts, and promotion of tumor growth in athymic nude mice. In contrast, knockdown or global knock-out strategies resulted in cell proliferation arrest (2).

Because the loss and gain of function of TRIP-Br2 leads to cell proliferation arrest and tumor progression, respectively, we investigated whether transcriptional and/or translational regulation of TRIP-Br2 may serve to tightly control the precise execution of its function during cell cycle progression. We demonstrate that TRIP-Br2 protein (not transcript) expression peaks at the G1/S boundary and progressively decreases through S and G2/M phases of the cell cycle. The oscillatory nature of TRIP-Br2 expression in cell cycle progression is shown to be tightly regulated by the closely related ubiquitin-proteasome-dependent degradation and CRM1-mediated nuclear export pathways. We provide the first evidence that a TRIP-Br protein may form ubiquitin conjugates and may possess an intrinsic NES motif that directly regulates its CRM1-mediated nuclear export pathways. We provide the first report of TRIP-Br proteins regulated by post-transcriptional modifications such as alternative splicing.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Transfection, and Other Reagents—The human osteosarcoma cell line U2OS and the African green monkey SV40-transfected kidney fibroblast cell line COS-7 were purchased from American Type Culture Collection (Manassas, VA). NIH3T3 mouse fibroblasts stably overexpressing C-terminal hemagglutinin (HA)-tagged TRIP-Br1 or TRIP-Br2 were generated as described previously. All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and maintained at 37°C in a 5% CO2 environment. COS-7 cells were transfected with expression plasmids using Lipofectamine™ transfection reagent (Invitrogen) in accordance with the manufacturer’s instructions. Polymerase chain reactions (PCR) were performed on 1-μl cDNA samples in the presence of 10 μM dNTPs and specific primer pairs (at 10 μM) in a total reaction volume of 20 μl. PCR was performed as follows: 20 cycles of denaturation (94°C, 30 s), annealing (51°C, 30 s), and extension (72°C, 1 min) with a 2-min initial denaturation step at 94°C and a 3-min terminal polishing step at 72°C. The primer sequences used for RT-PCR are available upon request.

Protein Degradation and 26 S Proteasome Inhibition Assays—A CHX chase assay for the analysis of protein degradation was performed as described previously (12). Briefly, cells (untransfected and transfected) were exposed to CHX (20 μg/ml) over a given time course. To achieve 26S proteasome inhibition, cells were exposed to ALLN (50 μM), MG132 (25 μM), or lactacystin (5 μM) over a given time course. Whole cell lysates were prepared from the drug-treated cells at the indicated time points for semi-quantitative RT-PCR and WB analyses.

Semiquantitative RT-PCR—Total RNA was isolated from cells using the TRIzol® reagent (Invitrogen). Total RNA (3 μg) was reverse transcribed using the ABI high capacity cDNA archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Polymerase chain reactions (PCR) were performed on 1-μl cDNA samples in the presence of 10 μM dNTPs and specific primer pairs (at 10 μM) in a total reaction volume of 20 μl. PCR was performed as follows: 20 cycles of denaturation (94°C, 30 s), annealing (51°C, 30 s), and extension (72°C, 1 min) with a 2-min initial denaturation step at 94°C and a 3-min terminal polishing step at 72°C. The primer sequences used for RT-PCR are available upon request.

Immunoprecipitation and Immunocytochemistry—Transfected cells were washed twice with PBS, and cell lysates were prepared by resuspending cells with gentle rocking in ice-cold
modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and aprotinin, leupeptin, and pepstatin, 1 µg/ml each). The cell lysates were precleared with protein A/G plus-agarose beads (sc-2003, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 30 min prior to centrifugation at 14,000 × g (4 °C, 15 min). The supernatants were then incubated overnight with preconjugated anti-HA-protein A/G plus-agarose beads (sc-7392AC, Santa Cruz Biotechnology) or anti-GAL4DBD-protein A/G plus-agarose beads (sc-510AC, Santa Cruz Biotechnology). These agarose beads were precipitated with centrifugation at 1000 × g (4 °C, 5 min) and washed five times with ice-cold modified radioimmune precipitation assay buffer. They were then resuspended in 60 μl of 2× WB sample loading buffer and boiled for 5 min prior to denaturing SDS-PAGE and WB analysis.

To analyze the subcellular localization of endogenous TRIP-Br2, cells were grown to 80% confluence on coverslips, washed three times with PBS, and permeabilized in 0.1% Triton-X for 10 min. Primary immunostaining with rabbit anti-TRIP-Br2 antibody (G4195, 1:4000) was performed at room temperature for 1 h. Secondary immunostaining with goat anti-rabbit-Cy3 or goat anti-mouse-Cy3 antibody was performed at room temperature for 1 h following three washes with PBS. Cellular DNA was subsequently counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Staining was visualized and photographed using a Nikon Eclipse E1000 fluorescence microscope.

**NES Motif Prediction Analysis and Nuclear Export Inhibition Assay**—NES motif prediction was achieved by *in silico* interrogation of the human TRIP-Br2 primary amino acid sequence (GenBank™ accession number NP_055570) using a Web-
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FIGURE 2. Rapid turnover of C-terminal HA-tagged TRIP-Br2. A, following de novo protein synthesis inhibition by treatment with CHX (20 μg/ml) in COS-7 cells, the endogenously expressed N-terminal GAL4DBD-tagged TRIP-Br2 steadily degraded over time. B, C-terminal HA-tagged TRIP-Br2 (TRIP-Br2-HA) degraded more rapidly over time. (Cyclin D1 (CCND1) and CDK2 were included as a positive and a negative control, respectively, upon CHX treatment.) C, the half-life of TRIP-Br2-HA was as short as 20 min. D, TRIP-Br2-HA mRNA levels remained constant over the time course of CHX treatment. β-Tubulin was used as a loading control in WB analyses; β-actin was used as a loading control in RT-PCR analyses. All data were obtained from three independent experiments performed in triplicates.

RESULTS

TRIP-Br2 Is Differentially Expressed during Cell Cycle Progression—Members of the TRIP-Br family of mammalian transcription coregulators have been shown to play key roles in regulating cell cycle progression. Our group and others have previously demonstrated that some members of this novel family of proto-oncogenes and/or tumor suppressors, such as TRIP-Br1, RBT1, and TRIP-Br3, are themselves tightly controlled during cell cycle progression by regulatory processes that remain elusive (1, 5, 6). We have also recently shown that the loss and gain of function of the poorly studied TRIP-Br2 protein leads to cell proliferation arrest and tumor progression, respectively (2).

We envisaged that the tight regulation of TRIP-Br2 might serve to control the precise execution of its function(s) during cell cycle progression.

To study whether TRIP-Br2 is regulated spatially and/or temporally during cell cycle progression, we synchronized human osteosarcoma U2OS cells at the G1/S boundary by exposure to 10 mM HU for 24 h before reversing the drug treatment. We found that the transcript levels of both TRIP-Br1 and TRIP-Br2 remained constant in all phases of the cell cycle (Fig. 1A). In contrast, TRIP-Br1 and TRIP-Br2 protein levels were differentially regulated during cell cycle progression (Fig. 1B). Both TRIP-Br1 and TRIP-Br2 levels accumulate at the G1/S boundary and decrease progressively through S phase until the G2/M phase of the cell cycle is reached. Proper cell cycle progression after the release from HU arrest was confirmed by the presence of phosphorylated retinoblastoma protein, Rb (P-Ser-780Rb), in the S phase and its subsequent dephosphorylation in the G2/M phase. Unlike non-oscillatory cell cycle-related proteins such as CDK2, TRIP-Br2 was also differentially expressed during cell cycle progression in COS-7 cells (Fig. 1C). In contrast to cyclin A, a known proliferation marker that is rapidly degraded when cells progress out of S phase, residual expression of TRIP-Br2 was observed in the G2/M and early G1 phases of the cell cycle in HU-synchronized cells. This suggests that TRIP-Br2 may play novel roles during these phases of the cell cycle, which may include cell division in the G2/M phase and mitogen sensing in the early G1 phase to facilitate its subsequent accumulation at the G1/S boundary in a positive autoregulatory loop. The higher molecular weight endogenous species observed in Fig. 1B are specific bands that we have observed in only a subset of human cancer cell lines, as detected by the rabbit polyclonal anti-TRIP-Br2 antibody used for immunoblot analyses.

C-terminal HA-tagged TRIP-Br2 Is Susceptible to Rapid Turnover—Given that the transcript level of TRIP-Br2 did not vary across the different phases of the cell cycle, a post-translational regulatory mechanism may serve to limit the function of...
FIGURE 3. Inhibition of 26 S proteasome activity enhances TRIP-Br2 stability. A, treatment of pcDNA3.1-TRIP-Br2-HA-transfected COS-7 cells with the proteasome inhibitor MG132 (25 \( \mu \)M) or ALLN (50 \( \mu \)M) led to the accumulation of TRIP-Br2-HA. Upon exposure of transfected cells to CHX (20 \( \mu \)g/ml), TRIP-Br2-HA expression was undetectable within 2 h. Co-incubation of transfected COS-7 cells with CHX (20 \( \mu \)g/ml) and MG132 (25 \( \mu \)M) was associated with reduced accumulation of TRIP-Br2-HA relative to untreated cells. B, treatment of pcDNA3.1-TRIP-Br2-HA-transfected COS-7 cells with another specific proteasome inhibitor, lactacystin (5 \( \mu \)M), also led to the accumulation of TRIP-Br2-HA. C, the TRIP-Br2-HA protein stability increased over time in lactacystin (5 \( \mu \)M)-treated COS-7 cells. D, treatment of NIH3T3TRIP-Br1-HA and NIH3T3TRIP-Br2-HA fibroblasts with MG132 (25 \( \mu \)M) led to the accumulation of TRIP-Br1-HA and TRIP-Br2-HA respectively. V, vector-only clones; R1, TRIP-Br1-HA-overexpressing clones; R2, TRIP-Br2-HA-overexpressing clones. E, treatment with MG132 (25 \( \mu \)M) led to the accumulation of TRIP-Br2-HA in COS-7 cells over time. p27 was used as a positive control. F, treatment with MG132 (25 \( \mu \)M, up to 6 h) or ALLN (50 \( \mu \)M up to 10 \( \mu \)M for 16 h) led to an increase in the accumulation of endogenous TRIP-Br2 in U2OS cells. Higher molecular weight (MW) isoforms of TRIP-Br2 were observed after treatment with these proteasome inhibitors. G, treatment with MG132 (25 \( \mu \)M), endogenous TRIP-Br2 accumulated to a greater degree in asynchronously growing and G2/M phase COS-7 cells than in G1/S phase COS-7 cells. H, immunochemical analysis revealed that treatment with MG132 (25 \( \mu \)M) was associated with an increase in the intensity of immunostaining in G2/M phase COS-7 cells (right panel) but not in G1/S phase COS-7 cells (left panel). Cellular DNA was counterstained with DAPI (blue). I, subcellular fractionation analysis revealed that endogenous TRIP-Br2 accumulates in G2/M phase COS-7 cells upon treatment with either LMB (10 ng/ml) or MG132 (25 \( \mu \)M). LMB treatment of G2/M phase COS-7 cells was associated with TRIP-Br2 levels comparable with that of G1/S phase COS-7 cells. Cytoplasmic TRIP-Br2 was observed only in the MG132-treated COS-7 cells. Lamin B and GAPDH were used as loading controls for nuclear (N) and cytoplasmic (C) fractions, respectively. β-Tubulin was used as a loading control in WB analyses. All data were obtained from three independent experiments performed in triplicates.
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A

[Cartoon of gel electrophoresis with lanes labeled 1, 2, 3, 4. Lanes contain bands for POLYUB, GAL4DBD-tagged proteins, IgG heavy chain, and GAL4DBD only.]

B

[Cartoon of gel electrophoresis with lanes labeled 1, 2, 3, 4. Lanes contain bands for POLYUB, GAL4DBD only, IgG heavy chain, and GAL4DBD-hTRIP-Bt2(1-314).]

C

[Cartoon of gel electrophoresis for COS-7 cells with lanes labeled 1, 2, 3, 4. Lanes contain bands for pCDNA3.1, TRIP-Bt2-HA, and MG132.]

D

[Cartoon of gel electrophoresis for COS-7 cells with lanes labeled 1, 2, 3, 4. Lanes contain bands for GAL4DBD only, GAL4DBD-hTRIP-Bt2, and MG132.]

E

[Cartoon of gel electrophoresis for COS-7 cells with lanes labeled 1, 2, 3, 4. Lanes contain bands for GAL4DBD only, GAL4DBD-hTRIP-Bt1, and MG132.]

[Additional annotations and labels for each gel lane, indicating experimental conditions and results.]
TRIP-Br2 in the G1/M and early G2 phases of the cell cycle by controlling the turnover of TRIP-Br2 proteins. We first performed cycloheximide chase analysis to examine the half-lives of endogenously expressed TRIP-Br2 and exogenously expressed N-terminal GAL4DBD-tagged TRIP-Br2 in COS-7 cells. Both endogenously expressed TRIP-Br2 and exogenously expressed N-terminal GAL4DBD-tagged TRIP-Br2 exhibited similar half-lives of approximately 2 h (Fig. 2A). However, when we performed cycloheximide chase analysis on C-terminal HA-tagged TRIP-Br2 in COS-7 cells, we observed that its half-life was significantly reduced to less than 30 min (Fig. 2B). Compared with known short-lived cell cycle regulatory proteins like cyclin D, which has a half-life of ~1 h, we found that the C-terminal HA-tagged TRIP-Br2 was rapidly turned over with its half-life estimated to be about 20 min (Fig. 2C). It is unlikely that such control over the protein turnover of C-terminal HA-tagged TRIP-Br2 reflects a transcriptional mechanism, as its exogenous expression in transiently transfected COS-7 cells was driven by a strong and constitutively active cytomegalovirus promoter. As shown in Fig. 2D, we demonstrated that the transcript level of C-terminal HA-tagged TRIP-Br2 did not vary across the time intervals of the cycloheximide chase analysis. These data suggest that the introduction of epitope-tagged sequences to TRIP-Br2 at the C terminus may have exposed encrypted degradation signals of TRIP-Br2 through changes in its native protein conformation.

Degradation of TRIP-Br2 through the 26 S-dependent Proteasome Pathway—Protein turnover in eukaryotic cells is governed by multiple proteolytic systems, including the lysosomal proteases, calpains, the ATP-ubiquitin-proteasome-dependent pathway, and an ATP-independent non-lysosomal process (15). The ubiquitin-proteasome-dependent pathway is known to be involved in regulating the turnover of key regulators of the cell cycle machinery such as cyclins and cyclin-dependent kinase inhibitors (16, 17). To determine whether the 26 S proteasome complex is involved in the regulation of TRIP-Br2 protein turnover, we treated TRIP-Br2-HA-transfected COS-7 cells with known 26 S proteasome inhibitors MG132 (25 µM, 2 h) and ALLN (50 µM, 2 h). As shown in Fig. 3A, either MG132 or ALLN was sufficient to cause accumulation of TRIP-Br2-HA in COS-7 cells (compare treatment lanes 4 and 5). To investigate whether the 26 S proteasome complex may play a role in regulating TRIP-Br2 levels, we treated TRIP-Br2-HA-transfected COS-7 cells with a combination of cycloheximide (20 µg/ml) and MG132 (3A, lane 1). We have earlier shown that TRIP-Br2-HA is a short-lived protein and has a half-life of ~20 min in a cycloheximide chase analysis. The addition of MG132 prevented the 26 S proteasome-dependent depletion of the existing pool of TRIP-Br2-HA in these cycloheximide-treated cells (Fig. 3A, compare lanes 1 and 6). In the absence of de novo synthesis of TRIP-Br2-HA and inhibition of the degradation of the existing pool of TRIP-Br2-HA, the TRIP-Br2-HA level was comparable with that observed under normal physiologic conditions (Fig. 3A, compare lanes 1 and 5) in which TRIP-Br2-HA is robustly synthesized and actively degraded. We observed a similar increase in TRIP-Br2-HA protein stability when COS-7 cells were treated with lactacystin, another specific inhibitor of 26 S proteasome activity (Fig. 3B). Notably, the protein stability of TRIP-Br2-HA increased over time in lactacystin (5 µM)-treated COS-7 cells (Fig. 3C).

In addition, MG132 (25 µM, 2 h) was sufficient to prevent the degradation of both TRIP-Br1-HA and TRIP-Br2-HA in NIH3T3 murine fibroblasts stably overexpressing these TRIP-Br proteins (Fig. 3D). The accumulation of TRIP-Br2-HA in these cells was clearly observed within 1 h of MG132 treatment (Fig. 3E). A similar phenomenon was observed when U2OS cells, which abundantly express endogenous TRIP-Br2, were treated with MG132 or ALLN. WB analysis of endogenous TRIP-Br2 in U2OS cells following a MG132- or ALLN-prolonged treatment time course (6 h) revealed a ladder of higher molecular weight TRIP-Br2 species, which is a characteristic of polyubiquitinated proteins (Fig. 3F). These data suggest that the 26 S proteasome may regulate the turnover of the two TRIP-Br family members, TRIP-Br1 and TRIP-Br2.

As the protein level of TRIP-Br2 has been shown to be higher at the G1/S boundary and throughout most of the S phase, we sought to determine whether TRIP-Br2 is more susceptible to the 26 S proteasome-dependent proteolysis in other phases of the cell cycle, such as the G2/M phase, by treating asynchronously cycling COS-7 cells as well as G1/S phase-synchronized and G2/M phase-synchronized COS-7 cells with MG132. The synchronized cell populations were derived from treatment of asynchronously cycling COS-7 cells with 10 mM HU for 24 h; the drug effect was reversed to allow cycling of the synchronized cell populations. The G1/S phase-synchronized cells and G2/M phase-synchronized cells were collected at 1 and 20 h following the release from HU arrest, respectively, prior to a 2-h MG132 treatment (25 µM). The asynchronously cycling COS-7 cells that were subjected to MG132 treatment were used as a positive control. WB analysis of endogenously expressed TRIP-Br2 following MG132 treatment of asynchronous or synchronously cycling COS-7 cells with 10 mM HU for 24 h; the drug effect was reversed to allow cycling of the synchronized cell populations. The G1/S phase-synchronized cells and G2/M phase-synchronized cells were collected at 1 and 20 h following the release from HU arrest, respectively, prior to a 2-h MG132 treatment (25 µM). The asynchronously cycling COS-7 cells that were subjected to MG132 treatment were used as a positive control. WB analysis of endogenously expressed TRIP-Br2 following MG132 treatment of asynchronous or synchronously cycling COS-7 cells with 10 mM HU for 24 h; the drug effect was reversed to allow cycling of the synchronized cell populations. The G1/S phase-synchronized cells and G2/M phase-synchronized cells were collected at 1 and 20 h following the release from HU arrest, respectively, prior to a 2-h MG132 treatment (25 µM). The asynchronously cycling COS-7 cells that were subjected to MG132 treatment were used as a positive control. WB analysis of endogenously expressed TRIP-Br2 following MG132 treatment of asynchronous or synchronously cycling COS-7 cells with 10 mM HU for 24 h; the drug effect was reversed to allow cycling of the synchronized cell populations. The G1/S phase-synchronized cells and G2/M phase-synchronized cells were collected at 1 and 20 h following the release from HU arrest, respectively, prior to a 2-h MG132 treatment (25 µM).
nized COS-7 cells showed that MG132 alone was sufficient to inhibit the degradation of TRIP-Br2 and to enable its accumulation in cells (Fig. 3G). TRIP-Br2 expression was markedly increased following MG132 treatment of the cells in G2/M phase, whereas the increase of TRIP-Br2 in the MG132 treated-cells in the G1/S phase remained modest. This observation was
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It has been reported that nuclear proteins such as p53 are polyubiquitinated in the nucleus and exported to the cytoplasm for degradation (18). To investigate whether TRIP-Br2 follows a similar fate, we treated cells with a nuclear export inhibitor, LMB. LMB is a drug that inhibits the formation of complexes consisting of CRM1, RanGTP, and NES-containing proteins, thus non-specifically blocking nuclear export (19–22). TRIP-Br2 levels accumulated upon exposure to MG132, suggesting that the ubiquitin-26 S proteasome pathway may regulate the protein turnover of at least two members of the TRIP-Br family (TRIP-Br1 and TRIP-Br2).

Deletion of the TRIP-Br2 C Terminus, Which Includes a Putative Nuclear Export Signal Motif, Inhibits TRIP-Br2 Degradation Independent of Its Ubiquitination Status—An analysis of GAL4DBD-hTRIP-Br2 truncation mutants (Fig. 5A, left panel) revealed that the C terminus of TRIP-Br2, which includes a PHD-Bromo interaction domain and an acidic TAD, is required for its protein turnover. As shown in Fig. 5A (right panel), increased protein stability of GAL4DBD-hTRIP-Br2 was achieved only when its C terminus was deleted (GAL4DBD-hTRIP-Br2-(1–179)). Conversely, fusion of the TRIP-Br2 C-terminal TAD to GAL4DBD (GAL4DBD-hTRIP-Br2-(235–311)) resulted in its destabilization compared with GAL4DBD alone.

Next, we performed cycloheximide chase analysis to examine the half-lives of GAL4DBD-hTRIP-Br2 truncation mutants in COS-7 cells. GAL4DBD-hTRIP-Br2-(235–314) exhibited the shortest half-life (<30 min) followed by the N-terminal truncation mutant GAL4DBD-hTRIP-Br2-(72–314) and the full-length GAL4DBD-hTRIP-Br2-(1–314) with half-lives of ~30 and 60 min, respectively. Notably, the C-terminal truncation mutant, GAL4DBD-hTRIP-Br2-(1–179), exhibited resistance to protein degradation, with levels remaining constant for longer than 2 h after cycloheximide treatment (Fig. 5B). The level of GAL4DBD-hTRIP-Br2-(1–314) did not decrease when the transfected cells were treated with DMSO control.

Earlier we showed that GAL4DBD-hTRIP-Br2 forms ubiquitin conjugates and is regulated by the 26S proteasome-dependent proteolytic pathway. We next investigated whether the GAL4DBD-hTRIP-Br2 truncation mutants differ in their ability to form ubiquitin conjugates that earmark them for recognition by the 26S proteasome complex. COS-7 cells were co-transfected with GAL4DBD-hTRIP-Br2 truncation mutant expression vectors and HA-Ub expression vectors. These cells were subsequently treated either with or without MG132 (25 μM, 2 h) followed by immunoprecipitation with anti-GAL4DBD antibody (Fig. 4B, lower panel). Furthermore, we demonstrated that either TRIP-Br2-HA or GAL4DBD-hTRIP-Br2 was capable of associating with endogenous ubiquitin in vivo, as evident in the immunoprecipitation analyses (Fig. 4, C and D). We also showed that GAL4DBD-TRIP-Br1 immunoprecipitated with endogenous ubiquitin (Fig. 4E), suggesting that the ubiquitin-26 S proteasome pathway may regulate the protein turnover of at least two members of the TRIP-Br family (TRIP-Br1 and TRIP-Br2).

A deletion of the TRIP-Br2 C terminus, which includes a putative nuclear export signal motif, inhibits TRIP-Br2 degradation independent of its ubiquitination status. A, left panel, schematic representation of GAL4DBD and GAL4DBD-hTRIP-Br2 (wild type and its derivatives). Right panel, COS-7 cells were transfected with pBXG1N, pBXG1N-hTRIP-Br2, or its derivatives for 48 h. Cell lysates were prepared and analyzed by SDS-PAGE and WB. β-Tubulin was used as a loading control. B, degradation of GAL4DBD-hTRIP-Br2 or its derivatives was assayed by treatment with CHX (20 μg/ml), and cell lysates were harvested at the indicated time points. GAL4DBD-hTRIP-Br2-(1–314) remained constant when the transfected cells were treated with DMSO control. B, left panel, schematic representation of GAL4DBD and GAL4DBD-hTRIP-Br2 (wild type and its derivatives). Right panel, COS-7 cells were co-transfected with pMT123-TRIP-Br2 or its derivatives for 48 h. Cell lysates were prepared and analyzed by SDS-PAGE and WB. β-Tubulin was used as a loading control. C, COS-7 cells were co-transfected with pMT123-TRIP-Br2 or its derivatives for 48 h, following which they were subjected to either no exposure or exposure to MG132 (25 μM) for 2 h. Top panel, extracts from MG132-treated or untreated cells were prepared and immunoprecipitated with anti-GAL4DBD antibody followed by immunoblotting with anti-HA antibody. Middle panel, expression of HA-Ub in the cell extracts from MG132-treated or untreated co-transfectants was confirmed by immunoblotting with anti-HA antibody. Bottom panel, expression of GAL4DBD-fused proteins in the cell extracts from MG132-treated or untreated co-transfectants was confirmed by immunoblotting with anti-GAL4DBD antibody. β-Tubulin was used as a loading control in WB analyses of the whole cell extracts (WCE). D, deletion of the C-terminal region (aa 180–314) of TRIP-Br2 inhibits its degradation independent of its ubiquitination status. IP, immunoprecipitation; WCE, Western blot (with the appropriate antibody). All data were obtained from three independent experiments performed in triplicates. D, a conserved leucine-rich NES motif is found in the C terminus of TRIP-Br2. At residues 238–243, the TRIP-Br2 NES motif overlapped with the PHD-Bromo interaction domain (aa 242–254) in the C terminus. The TRIP-Br2 NES motif is aligned with homologous sequences of TRIP-Br family members TRIP-Br1, RB1T, and TRIP-Br3 and the NES motifs of p53, p73, and MDM2 (27). The putative NES motifs of other TRIP-Br family members are found to be either localized to the SERTA domain or overlapping the PHD-Bromo interaction domain. The NetNES1.1 Web-based program was used to predict putative NES motifs in TRIP-Br proteins. Hs, Homo sapiens; n, amino terminus; c, carboxyl terminus.
GAL4DBD antibody-conjugated protein A/G plus-agarose beads prior to HA immunoblot analysis. A ladder of polyubiquitinated GAL4DBD-hTRIP-Br2-(1–179) proteins was observed in lysates from GAL4DBD-hTRIP-Br2-(1–179)/HA-Ub co-transfected cells in the absence of MG132, whereas the polyubiquitinated forms of full-length GAL4DBD-hTRIP-Br2 and all of its derivatives were observed only upon inhibition of 26 S proteasome activity by MG132 treatment (Fig. 5C, top).
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We confirmed that HA-Ub and full-length GAL4DBD-hTRIP-Br2 or its truncation mutants were transfected and properly expressed in cells used for these immunoprecipitation studies (Fig. 5C, middle and bottom panels). Collectively, these data suggest that ubiquitination most likely takes place on multiple sites of TRIP-Br2, as we have demonstrated that full-length TRIP-Br2 and all its derivatives form ubiquitin conjugates. We have also demonstrated that other regulatory mechanisms may be involved in regulating the turnover of TRIP-Br2, as deletion of the acidic C-terminal TAD of TRIP-Br2 resulted in a mutant that was highly stable yet heavily ubiquitinated. It remains unknown whether key ubiquitin-conjugating sites, which are critical in the regulation of TRIP-Br2 turnover, are preferentially localized to the C terminus and whether the loss of such ubiquitination sites in the C terminus may result in an increase in TRIP-Br2 protein stability.

As our data indicated that CRM1-mediated nuclear export may be involved in maintaining a low steady-state level of TRIP-Br2 in G2/M phase cells and that LMB stabilizes TRIP-Br2 in these cells (Fig. 3I), we investigated whether the increased stability of the C-terminal TRIP-Br2-(1–179) truncated protein may be due to a loss of key NES motifs. NES motifs have been shown to be important regulators of the subcellular localization of proteins that function in diverse cellular processes ranging from cell proliferation to programmed cell death. Proteins of >40 kDa must use a nuclear export receptor complex to pass through the nuclear pore (23, 24). We interrogated the primary amino acid sequence of TRIP-Br2, using a Web-based NES motif predictor, NetNES 1.1, and examined this sequence manually to determine whether it contains a leucine-rich sequence of conserved spacing and hydrophobicity that fits the criteria established for an NES motif (25, 26). We observed that the C-terminal residues between aa 238 and 243 conform to this motif, as indicated by their similarity to other known NES sequences on p53, p73, and MDM2 (27). This putative NES LXXLX motif overlaps with the PHD-Bromo interaction domain (aa 242–254) and is highly conserved in widely divergent species and in the other TRIP-Br family members such as TRIP-Br1, RBT1, and TRIP-Br3 (Fig. 5D).

CRM1-mediated Export of TRIP-Br2 Is Required for Its Degradation by the 26 S Proteasome in G2/M—To determine whether the putative C-terminal NES LXXLX motif of TRIP-Br2 is functional, we transiently transfected COS-7 cells with GAL4DBD-hTRIP-Br2-(1–314), GAL4DBD-hTRIP-Br2-(72–314), GAL4DBD-hTRIP-Br2-(1–179), or GAL4DBD-hTRIP-Br2-(235–311) and analyzed the subcellular localization of these TRIP-Br2 wild type and truncation mutants by immunofluorescence and subcellular fractionation. As shown in Fig. 6A, the exogenously expressed GAL4DBD-hTRIP-Br2 wild type and truncation mutants were predominantly localized to the nucleus, with only scant cytoplasmic immunostaining of GAL4DBD-hTRIP-Br2-(1–314) and GAL4DBD-hTRIP-Br2-(72–314) observed. Subcellular fractionation analysis of TRIP-Br2 wild type and truncation mutants demonstrated exclusive nuclear expression of wild type and mutant proteins (Fig. 6B). GAL4DBD-hTRIP-Br2-(235–311), which consisted of only the putative NES LXXLXL motif and the PHD-Bromo interaction domain in the acidic C-terminal TAD, was found to be the least stable form of TRIP-Br2. In contrast, GAL4DBD-hTRIP-Br2-(1–179), which lacks the C-terminal TAD (including the putative NES motif and the PHD-Bromo interaction domain), was found to be the most stable form of TRIP-Br2.

We further examined a potential role for the putative NES motif in mediating nuclear export of TRIP-Br2 for proteolytic degradation. GAL4DBD-hTRIP-Br2 wild type and truncation mutant-transfected COS-7 cells were treated with the proteasome inhibitor MG132 (25 μM) and subjected to subcellular fractionation analysis. We found that inhibition of the 26 S proteasome activity led to the accumulation of GAL4DBD-hTRIP-Br2-(1–314), GAL4DBD-hTRIP-Br2-(72–314), and GAL4DBD-hTRIP-Br2-(235–311) in both the cytosol and nucleus (Fig. 6C). In sharp contrast, GAL4DBD-hTRIP-Br2-(1–179) remained exclusively localized to the nucleus, suggesting that the putative C-terminal NES motif of TRIP-Br2 may play a critical role in mediating TRIP-Br2 nuclear export for proteolytic degradation. Further supporting our hypothesis, we observed that wild type GAL4DBD-hTRIP-Br2 and its truncated mutants were accumulated exclusively in the nuclear fraction, when the 26 S proteasome-dependent degradation and the CRM1-mediated nuclear export pathways of COS-7 cells were inhibited by MG132 (25 μM) and LMB (10 ng/ml) (Fig. 6D). These data suggest that CRM1-mediated nuclear export of TRIP-Br2 precedes its 26 S proteasome-dependent degradation.

Because TRIP-Br2 protein expression levels are lowest during the G2/M phase of the cell cycle (Fig. 1, B and C), we investigated whether loss of the C-terminal TAD alone may abrogate the proteolytic degradation of TRIP-Br2 during G2/M. COS-7 cells were transfected with pBXG1N-hTRIP-Br2 or its derivatives for 48 h followed by immunocytochemical analysis. GAL4DBD-hTRIP-Br2 and its derivatives were localized predominantly in the nucleus. Red channel, Cy3 staining; blue channel, DAPI staining. B, similar results were observed when asynchronously growing COS-7 cells were transfected with pBXG1N-hTRIP-Br2 or its derivatives for 48 h and analyzed by subcellular fractionation and immunoblotting with anti-GAL4DBD antibody. Lamin B and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively. C, asynchronously growing COS-7 cells were transfected with pBXG1N-hTRIP-Br2 or its derivatives for 48 h and analyzed by subcellular fractionation and immunoblotting with anti-GAL4DBD antibody. The absence of GAL4DBD-hTRIP-Br2-(1–179) in the cytosol, despite inhibition of 26 S proteasome activity by MG132, is depicted by an asterisk. Lamin B and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively. D, asynchronously growing COS-7 cells were transfected with pBXG1N-hTRIP-Br2 or its derivatives for 48 h followed by MG132 (25 μM) and LMB (10 ng/ml) for 2 h. These cells were then analyzed by subcellular fractionation and immunoblotting with anti-GAL4DBD antibody. GAL4DBD-hTRIP-Br2-(1–179) remained exclusively localized to the nucleus, despite inhibition of 26 S proteasome activity by MG132, as depicted by an asterisk. Lamin B and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively. E, COS-7 cells were transfected with pBXG1N-hTRIP-Br2 or its derivatives for 24 h followed by 10 mM HU treatment for an additional 24 h. 20 h after removal of HU, these G2/M phase cells were subjected to immunocytochemical analysis with anti-GAL4DBD antibody. Only GAL4DBD-hTRIP-Br2-(1–179) was detectable by immunostaining during the G2/M phase of the cell cycle. Red channel, Cy3 staining; blue channel, DAPI staining. F, when COS-7 cells transfected with pBXG1N-hTRIP-Br2 or its derivatives were treated with LMB (10 ng/ml), positive immunostaining was readily observed in a nuclear pattern for GAL4DBD-hTRIP-Br2-(1–314) and all its derivatives during G2/M. Red channel, Cy3 staining; blue channel, DAPI staining. All data were obtained from three independent experiments performed in triplicates.
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cells transiently transfected with GAL4DBD-hTRIP-Br2 wild type or its derivatives were arrested by HU and subsequently released after 20 h. These cells synchronized at the G2/M phase of the cell cycle were subjected to immunocytochemical analysis. Although GAL4DBD-hTRIP-Br2 wild type and N-terminal truncation mutants were completely undetectable by immunostaining, the C-terminal GAL4DBD-hTRIP-Br2-(1-179) truncation mutant, was readily detectable in an exclusively nuclear pattern (Fig. 6E). When these G2/M phase cells were treated with a potent nuclear export inhibitor, leptomycin B, we observed nuclear accumulation of GAL4DBD-hTRIP-Br2 wild type and its derivatives (Fig. 6F). These data provide strong evidence that nuclear export may play a critical role in the tight regulation of TRIP-Br2 protein levels in the nucleus during the G2/M phase of the cell cycle.

Mutation of Leucine Residue 238 of the NES Motif of TRIP-Br2 Abolished the CRM1/TRIP-Br2 Interaction—To further elucidate the mechanism by which the putative NES motif of TRIP-Br2 mediates the CRM1-dependent nuclear export pathway of GAL4DBD-hTRIP-Br2, we mutated the three highly conserved and hydrophobic leucine residues of this NES motif, Leu-238, Leu-241, and Leu-243, to alanine residues by site-directed mutagenesis. The precise mutation of Leu-238, Leu-241, and Leu-243 in the respective NES mutant was confirmed by automated sequencing (Fig. 7A, upper panel). In comparison with the GAL4DBD-hTRIP-Br2 wild type and other NES mutants, L238A exhibited an increase in protein stability (Fig. 7A, lower panel). These data are consistent with our subsequent observation that L238A is resistant to protein degradation, as shown previously for GAL4DBD-hTRIP-Br2-(1-179) (Fig. 5B), with levels remaining constant for over 2 h after cycloheximide treatment (Fig. 6B). Although L241A and L243A also exhibited a slight increase in resistance to protein degradation (Fig. 6B), mutations of these leucine residues was not associated with as dramatic an effect as the mutation of Leu-238 in hindering the proteolytic degradation of GAL4DBD-hTRIP-Br2.

As TRIP-Br2 has been shown previously to associate with CRM1 (13), and short segments containing only the hydrophobic residues of an NES motif can mediate export of a reporter protein (27), we examined whether Leu-238, Leu-241, and Leu-243 dictate the interaction of TRIP-Br2 with CRM1. We observed that the mutation of leucine residue 238 completely abolished the interaction of GAL4DBD-hTRIP-Br2 with endogenous CRM1 and rendered this NES mutant insensitive to MG132 treatment (Fig. 7C, lanes 1 and 2 of the first and fourth panels). It appeared that the mutation of leucine residue 243 also slightly weakened the CRM1/GAL4DBD-hTRIP-Br2 interaction (Fig. 7C, lanes 5 and 6 of the first panel), but this NES mutant remained sensitive to MG132 treatment (Fig. 7C, lanes 5 and 6 in the fourth panel from top). Mutagenesis of leucine residue 241 led to no observable effect on the CRM1/GAL4DBD-hTRIP-Br2 interaction; this NES mutant remained sensitive to MG132 treatment (Fig. 7C, lanes 3 and 4 in the first and fourth panels). Notably, L238A was found to be polyubiquitinatated even in the presence of 26 S proteasome activity (Fig. 7C, lane 1 in the second panel), reminiscent of the unique property exhibited by GAL4DBD-hTRIP-Br2-(1-179), in which the NES motif was removed entirely (Fig. 5C).

Next, we examined the subcellular localization of these NES mutants following the inhibition of 26 S proteasome by MG132. Although the wild type GAL4DBD-hTRIP-Br2 and all of its NES mutants exhibited predominant nuclear localization under normal condition (data not shown), we found that inhibition of the 26 S proteasome activity led to the accumulation of wild type GAL4DBD-hTRIP-Br2, L241A, and L243A in both the cytosol and nucleus (Fig. 7D). In sharp contrast, L238A remained exclusively localized to the nucleus. Collectively, these data suggest that leucine residue 238 of the NES motif of TRIP-Br2 may play a critical role in mediating the interaction of TRIP-Br2 with CRM1 and regulating the nuclear export of TRIP-Br2 for proteolytic degradation.

DISCUSSION

The timely degradation of many transiently induced and/or oscillatory cell cycle regulators is fundamental to the maintenance of proper cell cycle progression. Deregulation of such regulatory mechanisms may lead to oncogenic transformation or apoptosis. Key cell cycle regulators such as cyclins, CDK inhibitors, and E2F transcription factors have been found to be short-lived proteins that are degraded through the ubiquitin-proteasome-dependent pathway (16, 17, 28). The ubiquitin-proteasome-dependent degradation pathway is known to be characterized uniquely by conjugation of ubiquitin to target substrates, resulting in the formation of a polyubiquitin chain on these substrates that earmarks them for recognition by the 26 S proteasome complex (29, 30). As the process from recognition to degradation of polyubiquitinated substrates by the 26 S proteasome complex occurs rapidly, these species are usually transient and are rarely detected. In the present study, we provide evidence that the cell cycle regulatory protein TRIP-Br2 is also a short-lived protein. Expression of TRIP-Br2 is tightly regulated throughout the cell cycle by precise spatial and temporal control mechanisms involving the closely related ubiquitin-proteasome-dependent degradation and nuclear export pathways. We demonstrate that the stability of TRIP-Br2 proteins increased when cells were treated with known 26 S proteasome inhibitors such as ALLN, MG132 or lactacystin and inhibitors of CRM1-dependent nuclear export such as LMB. We provide first evidence that the association of a TRIP-Br protein with ubiquitin may result in the differential regulation of its turnover and function during cell cycle progression. As truncation mutagenesis of TRIP-Br2 revealed that TRIP-Br2 may likely be targeted for ubiquitination at multiple sites, we postulated that the nuclear export of TRIP-Br2 is a rate-limiting step in the regulation of TRIP-Br2 turnover.

First, we report the identification of a novel putative NES LXXLXL motif in the C-terminal region of TRIP-Br2. This leucine-rich NES motif is highly conserved in widely divergent species and in all other TRIP-Br family members. Notably, the TRIP-Br2 NES motif overlaps with the PHD-Bromo interaction domain in the acidic C-terminal TAD, suggesting that the binding of TRIP-Br2 to PHD zinc finger- and/or bromodomain-containing transcription factors during the G2/M phase of the cell cycle may mask the NES motif from recognition by and
FIGURE 7. Mutation of leucine residue 238 of the NES motif of TRIP-Br2 abolished the CRM1/TRIP-Br2 interaction. A, upper panel, all three highly conserved and hydrophobic leucine residues of the NES motif of p8XG1N-hTRIP-Br2 were mutated to alanine residues by site-directed mutagenesis and labeled as L238A, L241A, and L243A, respectively. The precise mutation of each leucine residue (in bold and underlined) in the respective NES mutant was confirmed by automated sequencing. Lower panel, WB analyses revealed that L238A exhibits an increase in protein stability as compared with the wild type GAL4DBD-hTRIP-Br2 (WT) and other NES mutants. β-Tubulin was used as a loading control. NT, not transfected. B, COS-7 cells were transfected with p8XG1N-hTRIP-Br2 or its derivatives (p8XG1N-hTRIP-Br2-(1–179) or NES mutants) for 48 h. Degradation of GAL4DBD-hTRIP-Br2 or its derivatives were assayed by treatment with CHX (20 μg/ml), and cell lysates were harvested at the indicated time points. GAL4DBD-hTRIP-Br2-(1–179) and L238A exhibited resistance to proteolytic degradation following CHX treatment. GAL4DBD-hTRIP-Br2-(1–314) remained constant when the transfected cells were treated with DMSO control. β-Tubulin was used as a loading control. C, COS-7 cells were transfected with p8XG1N-hTRIP-Br2-(1–179) or p8XG1N-hTRIP-Br2-(1–314) for 48 h, following which they were subjected to either no exposure or exposure to MG132 (25 μM) for 2 h. Extracts from MG132-treated or untreated cells were prepared and immunoprecipitated with anti-GAL4DBD antibody followed by immunoblotting with anti-CRM1 antibody (first panel), anti-Ub antibody (second panel), or anti-GAL4DBD antibody (third panel). Fourth panel, expression of GAL4DBD-fused proteins in the cell extracts from MG132-treated or untreated co-transfectants was confirmed by immunoblotting with anti-GAL4DBD antibody. β-Tubulin was used as a loading control in WB analyses of the whole cell extracts (WCE). Mutation of leucine residue 238 of the NES motif of GAL4DBD-hTRIP-Br2 abolished the CRM1/GAL4DBD-hTRIP-Br2 interaction and hindered the proteolytic degradation of GAL4DBD-hTRIP-Br2. IP, immunoprecipitation; WB, Western blot (with the appropriate antibody). D, asynchronously growing COS-7 cells were transfected with p8XG1N-hTRIP-Br2 or its NES mutants for 48 h followed by MG132 (25 μM) for 2 h. MG132-treated cells were then analyzed by subcellular fractionation and immunoblotting with anti-GAL4DBD antibody. The absence of L238A in the cytosol, despite inhibition of 26 S proteasome activity by MG132, is depicted by an asterisk. Lamin B and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively. All data were obtained from three independent experiments performed in triplicates.
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FIGURE 8. Proposed model of the regulation of TRIP-Br2 during cell cycle progression. Nuclear translocation of TRIP-Br2 is induced by mitogenic stimulation (step 1). TRIP-Br2 is recruited to the Rb-dissociated-E2F1-DBD-1 transcriptional complexes on E2F-responsive promoters and integrate signals from PHD zinc finger- and/or bromodomain-containing transcription factors or viral oncoproteins to induce E2F-mediated transcription for proper G1/S transition (step 2). The binding of these PHD zinc finger- and/or bromodomain-containing transcription factors at the PHD-Bromo interaction domain of TRIP-Br2 at the G1/S phase transition acts to mask the TRIP-Br2 NES motif and thereby maintain TRIP-Br2 in the nucleus to carry out its function of transactivating E2F-responsive genes, including cyclin A. Cyclin A binds to and activates CDK2 from the G1/S boundary to late S phase. Phosphorylation of the E2F1-DBD-1-TRIP-Br2 transcriptional complexes by cyclin A/CDK2 may lead to their release from a subset of E2F-responsive promoters in which transcriptional activity is no longer required in late S phase, while further priming these transcription complexes for ubiquitin ligase recognition (step 3). Following the dissociation of TRIP-Br2 from its interacting PHD zinc finger- and/or bromodomain-containing transcription factors, it may be targeted for ubiquitination by a yet-to-be elucidated E3 ubiquitin ligase (step 4). Ubiquitination near the TRIP-Br2 NES motif at the C terminus may enhance its unmasking for recognition by the CRM1-mediated nuclear export machinery and thus mediate the eventual nuclear export of polyubiquitinated TRIP-Br2 (step 5). As most E3 ubiquitin ligases, such as MD2, are known to possess NES motifs, the TRIP-Br2-associated E3 ubiquitin ligase may enhance the nuclear export of TRIP-Br2. The polyubiquitinated TRIP-Br2 and E3 ubiquitin ligase presumably dissociate in the perinuclear region of the cell (step 6), whereupon the polyubiquitinated TRIP-Br2 may be targeted to the 26 S proteasome for proteolysis (step 7). The E3 ubiquitin ligase may be recycled back into the nucleus to facilitate further ubiquitination and nuclear export of other TRIP-Br2 proteins (step 8). Solid line, proposed path of TRIP-Br2 nuclear entry; Dashed line, proposed path of TRIP-Br2 nuclear exit/proteolysis.

binding to the CRM1-mediated nuclear export machinery. The result would be to inhibit proteolytic degradation to maintain high levels of TRIP-Br2 in the nucleus for G1/S phase progression. Our observation that treatment of G1/S cells with MG132 did not affect the TRIP-Br2 protein level by WB analyses (Fig. 3G) or the intensity of TRIP-Br2 immunostaining (Fig. 3H) is consistent with the model we have proposed. Several examples of NES activity regulated by NES masking have been reported (27, 31–35). As highly conserved NES motifs are also found in TRIP-Br1, RBT1, and TRIP-Br3 (Fig. 5D), we postulate that the CRM1-mediated nuclear export pathway may have evolved to provide a mechanism for regulating the turnover of the TRIP-Br family of mammalian transcriptional coregulators. Indeed, CRM1-mediated nuclear export has been shown to be required for the degradation of a number of proteins, including p53 (36). In addition, ubiquitination of the C terminus of p53, in a region close to both the nuclear export and oligomerization sequences, has been shown to unmask the NES and to allow interaction of p53 with the CRM1-mediated nuclear export machinery (18).

Second, we demonstrate that the regulation of TRIP-Br2 turnover during the G2/M phase of the cell cycle is mediated by the putative NES motif in an LMB-sensitive manner. We speculate that deletion of the C-terminal region (aa 180–314), as in the case of the C-terminal truncation mutant GAL4DBD-hTRIP-Br2-(1–179), not only results in the loss of the key protein proteolysis-inducing ubiquitination sites that may reside within the acidic TAD but, more importantly, leads to the loss of the NES motif that is required for the shuttling of polyubiquitinated TRIP-Br2 to the perinuclear 26 S proteasome complex for degradation. Notably, LMB alone is sufficient to induce nuclear entrapment of wild type TRIP-Br2 and its truncated mutants and hinder their 26 S proteasome-dependent degradation in asynchronous or G2/M phase cells (Fig. 6, D and F). Accumulation of TRIP-Br2 in the nucleus upon LMB exposure suggests that TRIP-Br2 may not be a legitimate target of nuclear proteasomes. Nuclear proteasomes have been shown to possess different subtype patterns compared with cytoplasmic proteasomes, such that these differences may contribute to their non-overlapping substrate specificities (37).

Third, we show that mutation of the highly conserved and hydrophobic leucine residue 238 of this NES motif is sufficient to abolish the CRM1/Trp2 interaction, leading to the nuclear entrapment and stabilization of TRIP-Br2 proteins (Fig. 7, C and D). We envisage that TRIP-Br2 may likely be ubiquitinated prior to its nuclear exit. Notably, two mutant forms of TRIP-Br2 that are incapable of nuclear exit (GAL4DBD-hTRIP-Br2-(1–179) and GAL4DBD-hTRIP-Br2-(L238A)) have been shown to be polyubiquitinated in the presence of 26 S proteasome activity (Figs. 5C and 6C). Take together, CRM1-mediated nuclear export of TRIP-Br2 may be the rate-limiting process that is involved in the maintenance of low levels of TRIP-Br2, particularly in G2/M.

We observed a consistently low protein level of GAL4DBD-hTRIP-Br2-(235–311) when we exogenously expressed this truncation mutant in COS-7 cells. It is unlikely that the GAL4DBD-hTRIP-Br2 wild type expression construct and its derivatives differed in their gene expression levels, because they
were driven by a common SV40 promoter. The low level expression of GAL4DBD-hTRIP-Br2-(235–311) is more likely due to the loss of positive regulatory elements that are present in TRIP-Br2 protein residues between amino acids 1 and 234 or the loss of a native TRIP-Br2 folding scaffold that usually masks its NES motif and potential ubiquitination sites in the acidic C-terminal TAD. The barely detectable level of GAL4DBD-hTRIP-Br2-(235–311) expression in the cytoplasmic fraction suggests that its proteolytic degradation may not be mediated solely by the 26 S proteasome degradation pathway (Fig. 6C). We envisage that fusion of the TRIP-Br2 C-terminal TAD (aa 235–311), in which the NES is fully exposed, to GAL4DBD may prime the latter for promiscuous degradation by multiple proteolytic pathways.

We have previously shown that the highly unstable GAL4DBD-hTRIP-Br2-(235–311) protein retains transcriptional activity, whereas the highly stable C-terminal truncation mutants of GAL4DBD-hTRIP-Br2 are transcriptionally inactive (1). We hypothesize not only that ubiquitination of the acidic C-terminal TAD of TRIP-Br2 (and TRIP-Br1, to a lesser extend) serves to activate the acidic C-terminal TAD for gene transactivation but also that extensive ubiquitination of the same TAD could lead to the eventual proteolysis of TRIP-Br2. Indeed, ubiquitination recently has been shown to regulate the TAD function of the VP16 transcription factor by serving as a dual signal for activation and activator destruction (38). Ongoing studies will establish whether unmasking of the TRIP-Br2 NES motif is dependent on extensive ubiquitination of the acidic C-terminal TAD in G2/M. As we have recently reported the overexpression of TRIP-Br2 in many human cancers, it would be of interest to determine whether point mutations of key leucine residues in the NES motif or key ubiquitinated lysine residues in the acidic C-terminal TAD contribute to TRIP-Br2-mediated tumorigenesis. To this end, we have shown that a single mutation in the NES motif of TRIP-Br2-(L238A) greatly increased its protein stability. Establishing the role of such mutant forms of TRIP-Br2 in cell cycle progression and cancer pathogenesis is an important goal for our future investigations.

In summary, we propose a model in which the closely related ubiquitin-proteasome-dependent degradation and CRM1-mediated nuclear export pathways are involved in the regulation of TRIP-Br2 levels during cell cycle progression (Fig. 8). In response to mitogen stimulation, TRIP-Br2 is induced and its translocation to the nucleus may be mediated by a putative NLS motif at its N terminus or through its association with NLS-containing proteins (step 1). Upon nuclear entry, TRIP-Br2 is recruited to the Rb-dissociated-E2F1-DP-1 transcriptional complexes on E2F-responsive promoters through physical association with DP-1. At the G1/S transition, signals from a variety of PHD zinc finger- and/or bromodomain-containing transcription factors and viral oncoproteins may be integrated by their interactions with TRIP-Br2 to drive cell cycle progression through the induction of E2F-mediated transcription (step 2). The binding of these PHD zinc finger- and/or bromodomain-containing transcription factors to the PHD-Bromo interaction domain of TRIP-Br2 at the G1/S phase transition acts to mask the TRIP-Br2 NES motif and thereby maintain TRIP-Br2 in the nucleus to carry out its function of transactivating E2F-responsive genes, including cyclin A. Cyclin A binds to and activates CDK2 from the G1/S boundary to late S phase. The cyclin A-CDK2 complex has been shown to phosphorylate the E2F1-DP-1 heterodimer (39–42). TRIP-Br2 has been hypothesized to be a substrate of the cyclin A-CDK2 complex through its direct interaction with cyclin A.5 Phosphorylation of the E2F1-DP-1/TRIP-Br2 transcriptional complexes may lead to their release from a subset of E2F-responsive promoters in which transcriptional activity is no longer required in late S phase, while further priming these transcription complexes for ubiquitin ligase recognition (step 3). Following the dissociation of TRIP-Br2 from its interacting PHD zinc finger- and/or bromodomain-containing transcription factors, it may be targeted for ubiquitination by a yet-to-be elucidated E3 ubiquitin ligase (step 4). Ubiquitination near the TRIP-Br2 NES motif at the C terminus may enhance its unmasking for recognition by the CRM1-mediated nuclear export machinery and thus mediate the eventual nuclear export of polyubiquitinated TRIP-Br2 (step 5). As most E3 ubiquitin ligases, such as MDM2, are known to possess NES motifs, the TRIP-Br2-associated E3 ubiquitin ligase may enhance the nuclear export of TRIP-Br2. The polyubiquitinated TRIP-Br2 and E3 ubiquitin ligase presumably dissociate in the perinuclear region of the cell (step 6), whereupon the polyubiquitinated TRIP-Br2 may be targeted to the 26 S proteasome for proteolysis (step 7). The E3 ubiquitin ligase may be recycled back into the nucleus to facilitate further ubiquitination and nuclear export of other TRIP-Br2 proteins (step 8).

This model is supported by several lines of new evidence. First, treatment of cells with MG132 inhibited the proteolysis of TRIP-Br2 and led to its accumulation. Second, a highly conserved leucine-rich putative NES motif that overlaps with the PHD-Bromo interaction domain was found in the acidic C-terminal TAD of TRIP-Br2. Third, the stabilization of TRIP-Br2 in G2/M phase cells was achieved through “nuclear entrapment” of TRIP-Br2 either by removal of the NES motif that resides in the acidic C-terminal TAD of TRIP-Br2 (via truncation or site-directed mutagenesis) or by inhibition of the CRM1-mediated nuclear export machinery by LMB. Deregulation of any of these important regulatory pathways that govern the protein stability of TRIP-Br2 during cell cycle progression may ultimately lead to tumorigenesis.

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