MOLECULAR AND CELLULAR BIOLOGY, Oct. 2008, p. 6056–6065 Vol. 28, No. 19
0270-7306/08/$08.00+0 doi:10.1128/MCB.00583-08

Dual Modification of BMAL1 by SUMO2/3 and Ubiquitin Promotes Circadian Activation of the CLOCK/BMAL1 Complex▽†

Jiwon Lee, Yool Lee, Min Joo Lee, Eonyoung Park, Sung Hwan Kang, Chin Ha Chung, Kun Ho Lee,* and Kyungjin Kim*

School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

Received 10 April 2008/Returned for modification 7 May 2008/Accepted 11 July 2008

Heterodimers of BMAL1 and CLOCK drive rhythmic expression of clock-controlled genes, thereby generating circadian physiology and behavior. Posttranslational modifications of BMAL1 play a key role in modulating the transcriptional activity of the CLOCK/BMAL1 complex during the circadian cycle. Recently, we demonstrated that circadian activation of the heterodimeric transcription factor is accompanied by ubiquitin-dependent proteolysis of BMAL1. Here we show that modification by SUMO localizes BMAL1 exclusively to the promyelocytic leukemia nuclear body (NB) and simultaneously promotes its transactivation and ubiquitin-dependent degradation. Under physiological conditions, BMAL1 was predominantly conjugated to poly-SUMO2/3 rather than SUMO1, and the level of these conjugates underwent rhythmic variation, peaking at times of maximum E-box-mediated circadian transcription. Interestingly, mutation of the sumoylation site (Lys^{259}) of BMAL1 markedly inhibited both its ubiquitination and its proteasome-mediated proteolysis, and these effects were reversed by covalent attachment of SUMO3 to the C terminus of the mutant BMAL1. Consistent with this, SUSP1, a SUMO protease highly specific for SUMO2/3, abolished ubiquitination, as well as sumoylation of BMAL1, while the ubiquitin protease UBP41 blocked BMAL1 ubiquitination but induced accumulation of polysumoylated BMAL1 and its localization to the NB. Furthermore, inhibition of proteasome with MG132 elicited robust nuclear accumulation of SUMO2/3- and ubiquitin-modified BMAL1 that was restricted to the transcriptionally active stage of the circadian cycle. These results indicate that dual modification of BMAL1 by SUMO2/3 and ubiquitin is essential for circadian activation and degradation of the CLOCK/BMAL1 complex.

Circadian rhythms are fundamental mechanisms that optimize the physiology and behavior of most organisms. Extensive genetic and molecular studies have shown that these biological rhythms are driven by molecular clockwork composed of autoregulatory transcription-translation feedback loops involving several clock gene products (27, 36). In mammals, the heterodimeric transcription factor CLOCK/BMAL1 activates transcription of its own negative regulators, including cryptochromes (CRYs) and periods (PERs), and the products of the newly synthesized transcripts form complexes that block their own transcription by binding to the heterodimeric transcription activator. Of the negative regulators, the CRYs play a central role in this feedback inhibition by interacting directly with the C terminus of BMAL1 (15, 26, 29).

There is, however, some evidence that this canonical feedback mechanism is insufficient to account for the robust oscillation of the circadian clock. During the circadian cycle, the mRNA profiles of the key negative regulators, the CRYs, exhibit weak oscillations compared to those of other clock genes controlled by the CLOCK/BMAL1 complex (14, 31, 35). Moreover, the abundance of BMAL1 and CLOCK proteins in the nucleus reaches a minimum at the time that expression of their target genes reaches a peak (28, 30, 31).

Recently, work has emphasized the role of posttranslational modifications of the clock components in regulating the molecular time-keeping system (12, 16). In particular, BMAL1 and CLOCK are subject to diverse posttranslational modifications that are essential for the circadian control of expression of their target genes. For instance, protein kinase C-mediated phosphorylation of CLOCK triggered rhythmic gene expression in cultured fibroblasts (42). In addition, ubiquitination of BMAL1 was found to be tightly coupled with transactivation of the CLOCK/BMAL1 heterodimer, as well as its proteolysis (30), whereas acetylation of BMAL1 repressed its transcriptional activity by facilitating recruitment of CRY1 to the CLOCK/BMAL1 complex (18). Another important posttranslational modification is that due to the small ubiquitin-related modifier (SUMO). Sassone-Corsi and coworkers have shown that BMAL1 can serve as a substrate for sumoylation and that this modification is essential for circadian clock oscillation (6). However, the role of BMAL1 sumoylation in the molecular clock machinery is unclear.

SUMO modification has been implicated in diverse processes, including protein stability, subcellular localization, and protein-protein interaction (17, 23, 47). In mammals, there are at least three SUMO paralogues (13, 25, 48). Two of these, SUMO2 and SUMO3, are 95% identical to each other (and are therefore referred to as SUMO2/3 when it is not possible to distinguish between them) but have only 45% homology to

* Corresponding author. Mailing address for Kun H. Lee: School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea. Phone: 82-2-873-6690. Fax: 82-2-872-1993. E-mail: leekho@snu.ac.kr. Mailing address for Kyungjin Kim: Neuroendocrine Research Laboratory, School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea. Phone: 82-2-880-6694. Fax: 82-2-884-6560. E-mail: kyungjin@snu.ac.kr.

† Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 21 July 2008.

SUMO1. Like ubiquitin, SUMO2/3 can form polysumoylation chains due to the intrinsic SUMO consensus sequence close to their N termini, whereas SUMO1 lacks this sequence and can apparently not form such chains (44). In addition, the in vivo dynamics of SUMO1 and SUMO2/3 during the cell cycle are different (1). Consistent with this, a large number of SUMO substrates are modified in a paralogue-specific fashion under physiological conditions (2, 39, 48). Thus, the mammalian SUMO paralogues appear to be functionally distinct, although the physiological relevance of the individual SUMO paralogues remains largely unclear.

In the present study, we investigated in vivo BMAL1 sumoylation and its functional relevance to the molecular mechanism generating circadian gene expression. Our data demonstrate that in vivo BMAL1 is predominantly modified by SUMO2/3 and that this polysumoylation leads to ubiquitination of BMAL1 in discrete nuclear foci and potentiates its transcriptional activity.

MATERIALS AND METHODS

Cell culture and transfection. NIH 3T3 and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) at 37°C under 9% CO_2. For luciferase reporter assays, NIH 3T3 cells were transfected by using Lipofectamine Plus reagents (Invitrogen). At 24 h after transfection, cells were lysed and subjected to luciferase assay, normalized with total protein.

Immunoprecipitation and immunoblotting. Cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, and 1× protease inhibitor cocktail [Roche]) and centrifuged at maximum speed for 20 min at 4°C. Equal amounts of total protein were incubated with 2 μg of anti-BMAL1 (30), anti-Myc (9E10; Santa Cruz Biotechnology) and anti-Flag M2 (Sigma) antibodies for 1.5 h at 4°C and then added to a protein A/G-Sepharose bead slurry. The final immune complexes were analyzed by immunoblotting.

For tissue immunoprecipitation, tissues were homogenized in 1 volume of RIPA buffer 1 (50 mM Tris-HCl [pH 8.0], 450 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na_3VO_4, and 1× protease inhibitor cocktail [Roche]). Homogenates were cleared by dilution with 2 volumes of RIPA buffer 2 (RIPA buffer 1 without NaCl). Further procedures were as described above.

Immunoblot analyses were performed on 6 or 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). Target proteins were detected with anti-BMAL1 generated in rabbit (30), anti-SUMO2/3 (Sentrin2), anti-SUMO1 (GMP-1; Zymed Laboratory), anti-Myc (9E10), anti-HA probe (F-7; Santa Cruz Biotechnology), and anti-Flag M2 (Sigma) antibodies. The immune complexes were visualized with an ECL detection kit (Pierce).

BiFC and immunofluorescence assays. For the bimolecular fluorescence complementation (BiFC) assay, YN-SUMO1 was kindly provided by T. Kerppola (University of Michigan Medical School) (20). Partial sequences encoding amino acid residues 1 to 172 of yellow fluorescent protein (YFP) (YN) and cyan fluorescent protein (CFP) (CN) were fused to the 5′ ends of the coding regions for SUMO3 and ubiquitin to produce plasmids encoding YN-SUMO3, YN-ubiquitin, and CN-SUMO3. The sequences encoding amino acid residues 173 to 238 of YFP and CFP (YC and CC, respectively) were fused upstream of those encoding Bmal1 using linker sequences for GGGGSGGGGS, and the chimeric coding regions were cloned into pcDNA3.1 (Invitrogen) to produce YC-Bmal1 and CC-Bmal1, respectively. COS-7 cells were transfected with the indicated constructs, incubated at 37°C for 24 h, transferred to 30°C for 12 h to promote fluorophore maturation, and visualized after fixation using an LSM 510 laser scanning confocal microscope (Carl Zeiss).

For immunofluorescence analysis, cells were fixed with 3.75% paraformaldehyde in phosphate-buffered saline and incubated with anti-BMAL1, anti-SUMO1, anti-SUMO2/3 (Zymed Laboratory), anti-PML (PG-M3), and anti-ubiquitin (P4D1; Santa Cruz Biotechnology) antibodies. Cells ware visualized with secondary antibodies conjugated with fluorescein isothiocyanate or TRITC (tetramethyl rhodamine isothiocyanate).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's instructions (Upstate Biotechnology). Each cross-linked sample was immunoprecipitated with BMAL1, Myc (9E10), acetyl-histone H3, and histone H3 (Upstate Biotechnology) antibodies, and the DNA was purified by phenol-chloroform extraction and ethanol precipitation. For PCR, the primers were as follows: for the first and second E-box of *Dbp*, 5′-CGACCCCAATCAAGACAAG-3′ and 5′-CGAGGACACCAGTTCAG G-3′; for the first *Per1* E-box, 5′-CCCTCACTTCCCTTTCATTATTGAC G-3′ and 5′-TGCATAATGCCAGGCCCTGCCCCTCATTGG-3′; and for the first exon of *Per1*, 5′-CAGATGCCAGGAAGAGATCCTTAGCCAAC C-3′ and 5′-GACTAACCCTAGGATTGCAGCAGGGATCC-3′.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were obtained as described previously (28). Oligonucleotides containing the E-box motif of *Per1* (5′-CGCGCAAGTCCACGTGCAGGGAT-3′ and 5′-CGCGATCCCTGCACG TGGACTTG-3′) were annealed and end labeled with [α-^{32}P]dCTP using Klenow polymerase. Binding reactions were performed by incubating the nuclear extract with reaction buffer (40 mM HEPES [pH 7.4], 0.2 mM KCl, 2 mM dithiothreitol, 0.2% NP-40, and 20% glycerol) and 2 μg of poly(dI-dC) · poly(dI-dC) in the presence or absence of a cold competitor for 20 min and then with the end-labeled probes (100,000 cpm) for 25 min at room temperature. The reaction mixtures were resolved on 5% nondenaturing polyacrylamide gel. The gel was dried and visualized by autoradiography.

Real-time bioluminescence monitoring. NIH 3T3 cells were plated in 35-mm dish and transfected with the *Per2* promoter fused with destabilized firefly luciferase (*Per2*-dsLuc). The cells were preincubated in serum-free DMEM and synchronized with 1 μM dexamethasone (Dex; Sigma) for 2 h, and their medium was replaced with culture medium (DMEM supplemented with 10% fetal bovine serum and 0.1 mM luciferin [Promega]) as previously described (30). Bioluminescence was measured with a luminometer (AB-2550 Kronos-Dio; ATTO).

RESULTS

BMAL1 is predominantly conjugated to SUMO2/3 under physiological conditions. BMAL1 has been shown to be a target molecule of SUMO modification (6). To characterize the SUMO modification of BMAL1 in vivo, we coexpressed Myc-tagged BMAL1 with each SUMO paralogue (SUMO1, SUMO2, or SUMO3 tagged with Flag) in NIH 3T3 cells and assessed SUMO modification in immunoprecipitates with anti-Myc antibody (Fig. 1A). Consistent with the results of in vitro sumoylation assays (see Fig. S1 in the supplemental material), BMAL1 formed conjugates with all three SUMO paralogues in this culture model. Western blot analysis with anti-Myc antibody revealed more slowly migrating proteins, ~15 kDa larger than Myc-BMAL1, in the lysates of cells cotransfected with any of the SUMO paralogues, and anti-Flag antibody detected only the slowly migrating bands in the same blot. SUMO1 conjugated to BMAL1 more efficiently than the other paralogues. However, SUMO3-conjugated BMAL1 was greatly enhanced and displayed a polysumoylated pattern when Ubc9, a SUMO conjugating enzyme, was coexpressed. Next, we identified the putative in vitro sumoylation site of BMAL1 by using both deletion and site-directed mutagenesis (data not shown). Sumoylation mainly occurred at lysine 259 of BMAL1 in vivo, regardless of the SUMO species (Fig. 1B), a finding consistent with previous observations (6).

To examine the specificity of BMAL1 sumoylation under normal circumstances, we analyzed the abundance of endogenous SUMO-conjugated BMAL1 in several tissues and cell lines using specific antibodies directed against SUMO1 and SUMO2/3. As shown in Fig. 1C, immunoprecipitation assays with anti-BMAL1 antibodies revealed ladders of slowly migrating BMAL1 in all tissues and cell lines tested. These bands precisely overlapped with the doublet bands detected by anti-SUMO2/3 antibodies in the same blot but not with that of

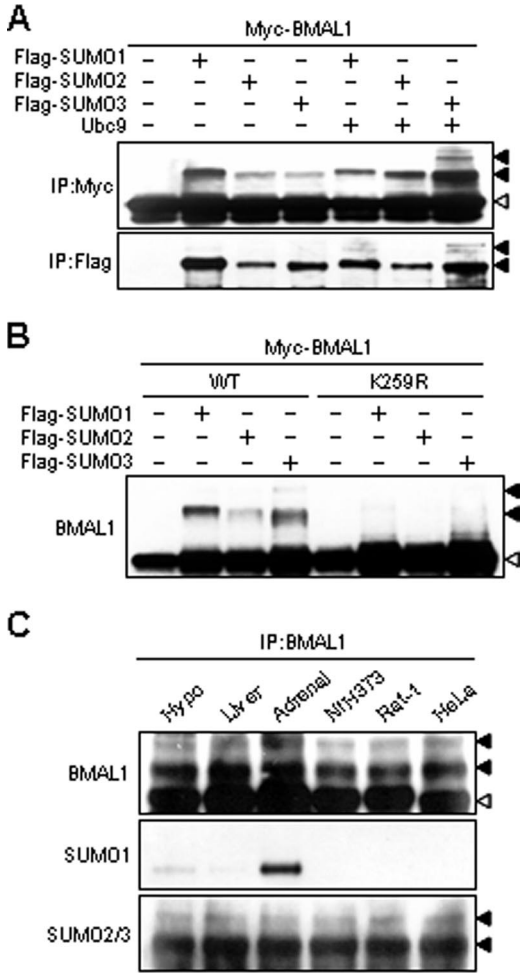

FIG. 1. BMAL1 is mainly sumoylated by SUMO2/3 in vivo. (A) Transient expression of SUMO paralogues induces BMAL1 sumoylation. NIH 3T3 cells were cotransfected with Myc-BMAL1 and Flag-SUMO paralogues (SUMO1, SUMO2, and SUMO3) in the presence or absence of Ubc9, and cell extracts were immunoprecipitated with anti-Myc or anti-Flag antibodies. Samples were then analyzed by immunoblotting with anti-Myc antibodies. Arrowheads indicate the SUMO-conjugated BMAL1 (black) and naked BMAL1 (white) bands. (B) Localization of the BMAL1 sumoylation site. Cells were transfected with Myc-tagged BMAL1 constructs encoding wild-type BMAL1 or the K259R mutant, together with Flag-SUMO paralogues, and analyzed by immunoblotting with anti-Myc. (C) Sumoylation of endogenous BMAL1 by SUMO2/3. Whole-cell lysates from mouse tissues (hypothalamus, liver, and adrenal gland) at the time of highly expressed BMAL1 (ZT 03) and various cell lines such as NIH 3T3 mouse fibroblasts, Rat-1 fibroblasts and HeLa human epithelial cells were immunoprecipitated with BMAL1 antibodies, and the immune complexes were analyzed by immunoblotting with the indicated antibodies. Hypo, hypothalamus; Adrenal, adrenal gland.

anti-SUMO1 blotting. Moreover, we failed to detect any SUMO1-conjugated band in the cell lines, and the SUMO1 signals from the tissue samples consisted of a single band in contrast to SUMO2/3. These observations indicate that SUMO2/3 is the major modifier of BMAL1 sumoylation in vivo.

Circadian oscillations of BMAL1 sumoylation coincide with target gene transcription. To explore the functional relevance of the SUMO2/3 modification of BMAL1 in the circadian clock machinery, we examined temporal changes in BMAL1 sumoylation in mouse liver extracts over the circadian cycle. For this experiment, mice were entrained to a light/dark cycle (12 h/12 h) for 3 weeks and then killed at 3-h intervals in constant darkness. BMAL1 sumoylation in the liver extracts was visualized with anti-SUMO2/3 antibodies in immunoprecipitates with anti-BMAL1 antibodies (Fig. 2A). Polysumoylated BMAL1, the upper band of the anti-SUMO2/3 blot, exhibited a robust oscillation peaking at circadian time 9 to 12 when naked BMAL1 levels reached a minimum. In the same period, the transcript levels of the clock-controlled gene *Dbp* and *Rev-erbα* reached the maxima of their circadian profiles parallel with BMAL1 binding to E-box elements in the intronic region of the *Dbp* gene (Fig. 2B and C). These observations indicate that polysumoylation of BMAL1 may be involved in transcriptional activation of the clock-controlled genes.

To further examine the relationship between BMAL1 sumoylation and its transcriptional activity, we compared the effects of wild-type BMAL1 and a sumoylation-defective mutant (K259R) on clock gene activation. A transcriptional reporter assay using the *Per1* promoter fused with luciferase showed that the site-directed mutation of the sumoylation site (K259) significantly reduced BMAL1-dependent promoter activity, although its inhibitory effect was moderate compared to that caused by CRY1 overexpression (Fig. 2D). These results were substantiated by ChIP assays (Fig. 2E) and EMSAs (Fig. 2F), demonstrating that the K259R mutation attenuated the E-box binding abilities of BMAL1 in vivo and in vitro, respectively. Thus, SUMO2/3 conjugation appears to potentiate the transcriptional activity of the CLOCK/BMAL1 complex by stimulating its E-box binding activity.

Nuclear speckles of BMAL1 colocalize with SUMO2/3 and PML in vivo. Nuclear translocation of the CLOCK/BMAL1 heterodimer is essential to activate E-box-dependent clock gene transcription (30, 42). Sumoylation often affects the subcellular localization of target proteins (32, 37). To investigate the relationship between the subcellular localization of BMAL1 and its sumoylation, we first examined the distribution of BMAL1 tagged with green fluorescent protein at its N terminus (GFP-BMAL1) and endogenous SUMO paralogues in COS-7 cells. A few of the GFP-BMAL1-positive cells ($\leq$7%) contained speckles of the GFP signal in the nucleus (Fig. 3A and C), although large portion of the cells exhibited strong diffuse signals over their nuclei (data not shown). The nuclear speckles overlapped perfectly with the nuclear body (NB) structures stained with anti-SUMO2/3 antibodies and anti-promyelocytic leukemia protein (PML) antibodies, but only partially with immunostaining for SUMO1 (Fig. 3A), suggesting that BMAL1 may be recruited to the PML NB enriched with SUMO2/3. Next, we assessed the effects of exogenous SUMO3 fused with red fluorescent protein (RFP-SUMO3) and Ubc9 on the subnuclear localization of BMAL1. As shown in Fig. 3B and C, overexpression of RFP-SUMO3 increased the number of cells displaying BMAL1 nuclear speckles colocalized with RFP signals. These effects were intensified when cells were additionally transfected with Ubc9. In contrast, when BMAL1 was replaced with the K259R mutant, the BMAL1 signals localized to NB structures fell drastically despite the presence of RFP-SUMO3 signals.

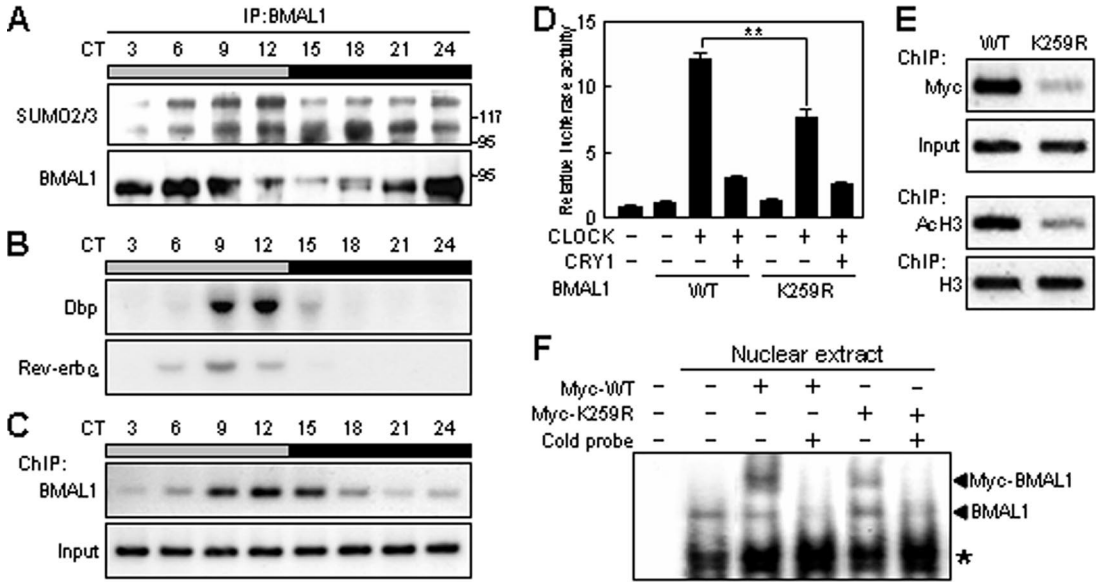

FIG. 2. Sumoylation of BMAL1 regulates CLOCK/BMAL1-mediated transcriptional activation. (A) Circadian cycle of BMAL1 sumoylation by SUMO2/3. Liver extracts were immunoprecipitated with anti-BMAL1 and visualized with SUMO2/3 antibodies. Day is gray, and night is black. (B) mRNA levels of clock-controlled genes, including *Dbp* and *Rev-erbα* in the liver, were determined by Northern blotting. (C) ChIP assays were performed with chromatin extracts from mice liver and immunoprecipitated with anti-BMAL1 antibodies. Chromatin complexes were analyzed by PCR using a primer set for the first and second E-box regions of the *Dbp* gene. The results are representative of three independent experiments. (D) Role of SUMO in transactivation by the CLOCK/BMAL1 heterodimer. NIH 3T3 cells were transfected with the *Per1*-luciferase reporter and the indicated transcriptional factors. At 24 h after transfection, the luciferase activities were determined. Each value is the mean ± the standard error of the mean (SEM) of three independent experiments (**, $P < 0.001$). (E) Chromatin was extracted from NIH 3T3 cells transfected with Myc-tagged BMAL1 constructs encoding wild-type or the K259R mutant. ChIP assays were performed with the indicated antibodies. The association of each protein with the *Per1* promoter was analyzed by PCR using a primer set flanking the proximal E-box and first exon. ChIP data for H3 and input are shown as controls. (F) Binding of BMAL1 to the E-box region of *Per1* promoter in vitro. Nuclear extracts were isolated from Myc-tagged wild-type BMAL1 or K259R mutant expressing cells and then examined by EMSA using radiolabeled E-box probe in the presence or absence of 100-fold molar excess cold probe. An asterisk (*) indicates specific complexes of unidentified factor. AcH3, acetyl-histone H3; H3, histone H3.

SUMO3 conjugation compartmentalizes BMAL1 in the NB. The data presented above indicate that there is strong colocalization of BMAL1 and SUMO2/3 in vivo. Nonetheless, they do not directly demonstrate that SUMO-conjugated BMAL1 is localized in the NB since PML NB contains diverse proteins that are susceptible to SUMO conjugation (22, 52). To clarify the role of sumoylation in the subcellular distribution of BMAL1, we used the BiFC assay, which generates a fluores-

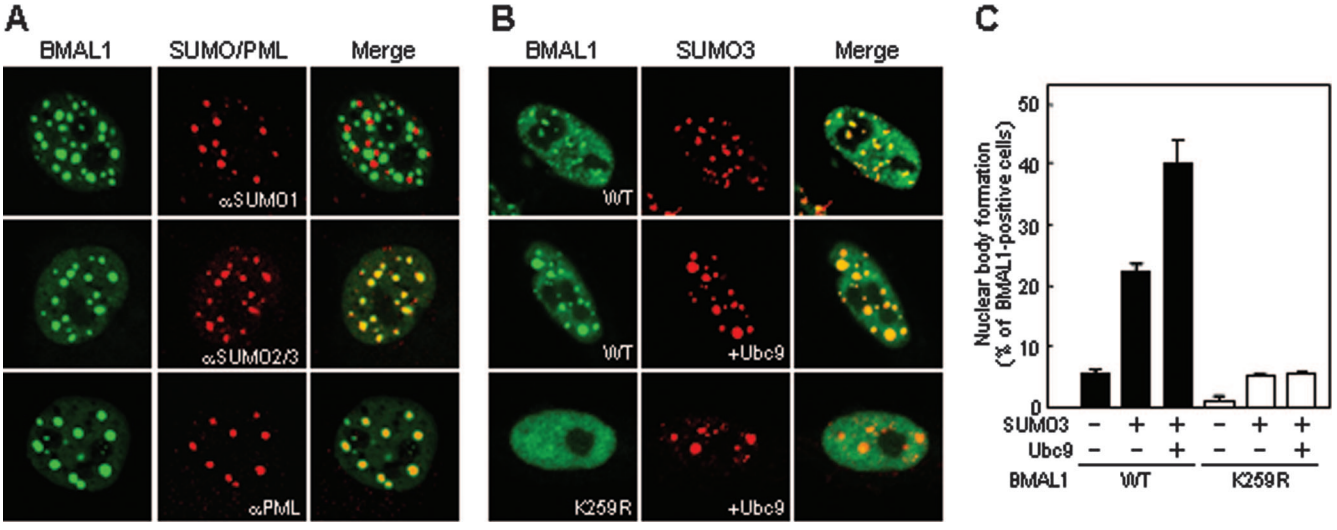

FIG. 3. BMAL1 colocalizes with SUMO2/3 in NB structures. (A) Representative fluorescence images demonstrate that nuclear speckles of BMAL1 overlap with SUMO2/3 and PML NBs. COS-7 cells were transfected with GFP-fused BMAL1 and immunostained with anti-SUMO1, anti-SUMO2/3, or anti-PML antibodies. (B) The cells were transiently cotransfected with constructs encoding either GFP-fused wild-type BMAL1 or K259R mutant, together with RFP-fused SUMO3. Ubc9 was cotransfected to enhance the sumoylation signals. (C) Quantitation of NB formation in BMAL1-positive cells. The data are expressed as mean ± the SEM ($n = 10$).

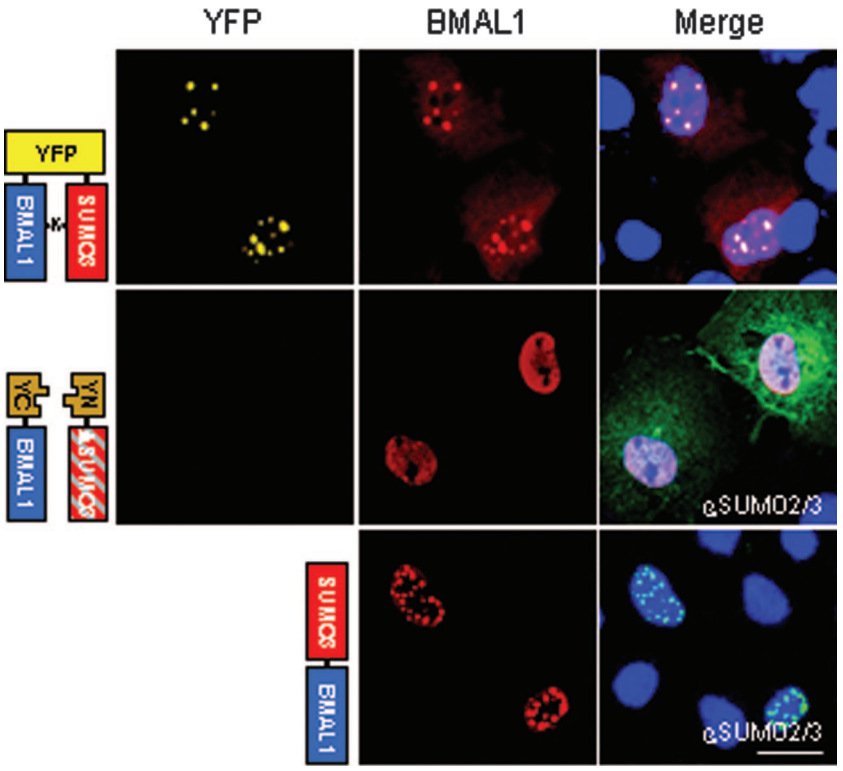

FIG. 4. BMAL1 directly interacts with SUMO3 in the NB. The BiFC technique was used to visualize the direct interaction between BMAL1 and SUMO3. YC-BMAL1 was transiently expressed with YN-SUMO3, or with YN-ΔSUMO3 lacking the C-terminal glycine residue, in COS-7 cells and then immunostained with anti-BMAL1 and anti-SUMO2/3 antibodies. SUMO3-fused BMAL1 was used as a control. Scale bar, 10 μm.

cence signal only when two molecules interact directly in vivo (20). For this experiment we constructed two plasmids, one encoding SUMO3 fused with the N-terminal half of YFP (YN-SUMO3) and the other encoding BMAL1 fused with the remaining C-terminal half of YFP (YC-BMAL1), and introduced them into COS-7 cells. Intriguingly, the YFP signal produced by complexes of the two fusion proteins was detected only at the nuclear foci overlapped with anti-PML immunostaining, even though there was diffuse anti-BMAL1 staining throughout the cells (Fig. 4, upper panels; see Fig. S2 in the supplemental material). No BiFC signal was detected when the mutant SUMO3 (YN-ΔSUMO3) was used instead of YN-SUMO3 (Fig. 4, middle panels). To further confirm whether sumoylation causes the compartmentalization of BMAL1 in the NB, we determined the subcellular distribution of BMAL1 fused with a SUMO3 moiety at its C terminus (BMAL1-SUMO3). Consistent with the results from the BiFC assay, the fusion protein was exclusively localized in the NB (Fig. 4, lower panels), suggesting that sumoylation is responsible for accumulating BMAL1 in the NB.

Sumoylation accelerates ubiquitin-dependent proteolysis of BMAL1. Most unstable transcription factors are known to activate target genes concomitant with their own degradation through the ubiquitin-proteasome system (34). We previously have shown that ubiquitin-dependent BMAL1 degradation occurs concomitantly with transactivation of the CLOCK/BMAL1 heterodimer (30). The present observations indicate that BMAL1 sumoylation may be implicated in circadian activation of E-box-dependent clock gene transcription (Fig. 2). Furthermore, the fact that the abundance of the exogenous mutant K259R protein was higher than that of the wild-type (Fig. 1B) raised the possibility that sumoylation affects the stability of the BMAL1 protein. This notion prompted us to compare the stability of wild-type BMAL1 and the sumoylation-defective mutant in NIH 3T3 cells in the presence or absence of MG132, a specific inhibitor of the 26S proteasome (Fig. 5A). After treatment with cycloheximide, the amount of the wild-type protein rapidly diminished, with an estimated half-life of ~3 h, whereas the half-life of the K259R mutant extended to ~8 h. Both forms of BMAL1 were strongly stabilized in cells treated with MG132, implying that sumoylation of BMAL1 plays a role in the ubiquitin-proteasome pathway leading to its turnover.

To clarify the relation between sumoylation and ubiquitination of BMAL1, we introduced type-specific SUMO proteases into cells by transient transfection (Fig. 5B). Treatment with MG132 caused dramatic accumulation of ubiquitinated BMAL1 (Ub-BMAL1) in cells ectopically expressing SENP1, a SUMO1 specific protease (7), whereas exogenous SUSP1, a SUMO2/3 specific protease (24, 33), completely blocked the Ub-BMAL1 accumulation induced by MG132. To further confirm the role of SUMO2/3 in BMAL1 ubiquitination, we assessed the effect of exogenous SUMO3 on ubiquitination of both wild-type BMAL1 and the K259R mutant. As shown in Fig. 5C, ectopic expression of SUMO3 markedly enhanced ubiquitination of the wild-type but not of the K259R mutant. Moreover, artificial fusion with SUMO3 reversed the inhibitory effect of the K259R mutation on BMAL1 ubiquitination (Fig. 5D).

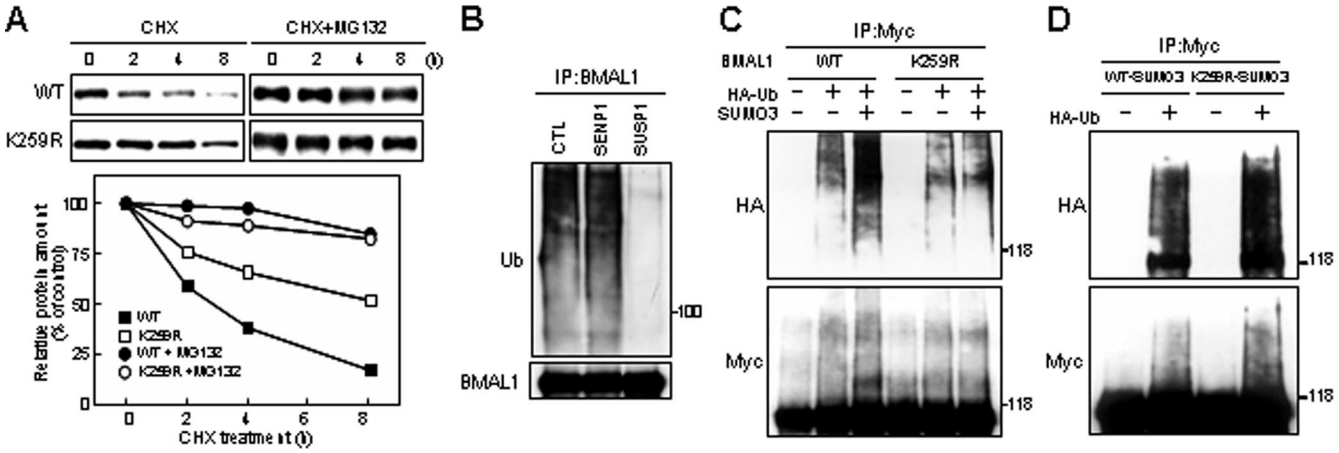

FIG. 5. SUMO3 facilitates ubiquitin-dependent BMAL1 degradation. (A) Stability of wild-type BMAL1 and the K259R mutant. NIH 3T3 cells expressing Myc-tagged wild-type BMAL1 or the K259R mutant were treated with CHX (30 μg/ml) alone or together with MG132 (50 μM) for the indicated times. The level of BMAL1 was analyzed by immunoblotting with anti-Myc and then quantitated by densitometer. (B) Effect of SUMO on BMAL1 ubiquitination. Cells were transfected with SENP1 or SUSP1 and incubated with 50 μM MG132 for 5 h. Cell extracts were subjected to immunoprecipitation with anti-BMAL1 and analyzed with anti-Ub antibodies. (C) Ubiquitination of BMAL1 is enhanced by SUMO3. Cells were cotransfected with Myc-tagged wild-type BMAL1 or K259R mutant, Flag-SUMO3, and HA-tagged ubiquitin, as indicated. At 36 h after transfection, cells were incubated for 5 h with 50 μM MG132, and then BMAL1 was immunoprecipitated with anti-Myc, and precipitates were analyzed by immunoblotting with anti-HA and anti-Myc antibodies. (D) Ubiquitination status of SUMO3-fused wild-type BMAL1 or K259R mutant was measured by immunoprecipitation followed by immunoblotting. CHX, cycloheximide; SENP1, SUMO1-specific protease 1; SUSP1, SUMO2/3-specific protease 1.

SUMO3 promotes ubiquitination of BMAL1 in the NB. SUMO3 conjugation localizes BMAL1 to the NB and also facilitated its ubiquitination. To explore the possibility that ubiquitination of BMAL1 occurs in the NB, we performed BiFC assays using ubiquitin fused with the N-terminal half of YFP (YN-Ub) and BMAL1 fused with the C-terminal half of YFP (YC-BMAL1) (Fig. 6). The joint expression of these proteins in COS-7 cells resulted in a BiFC signal in NB-like structures, as well as in the cytoplasm (Fig. 6, upper panels). Surprisingly, coexpression of SUMO3 led to concentration of the BiFC signal generated by YN-Ub and YC-BMAL1 into the NB-like structures (Fig. 6, middle panels), whereas coexpression of ΔSUMO3 led to disappearance of the complemented signal from most cells despite expression of YN-Ub and YC-BMAL1 (Fig. 6, lower panels and data not shown). It is therefore likely that sumoylation is crucial not only for nuclear compartmentalization of BMAL1 but also for its ubiquitination.

Sumoylation is a prerequisite for ubiquitination of BMAL1. To further see whether sumoylation of BMAL1 leads to its subnuclear compartmentalization and ubiquitination at the same position in the nucleus, we used a dual-color BiFC assay enabling us to discriminate between the forms of BMAL1 modified by SUMO and by ubiquitin in the same cell. To detect sumoylated BMAL1, we fused the N-terminal half of CFP to SUMO3 (CN-SUMO3) and its C-terminal half to BMAL1 (CC-BMAL1), whereas for ubiquitinated BMAL1, we used the same plasmid constructs as used in Fig. 6. Coexpression of all of the fusion proteins in COS-7 cells revealed two different fluorescence signals in the same nuclear foci (Fig. 7A, upper panels). To define the two BiFC signals, we in addition expressed the ubiquitin protease UBP41 (3) or the SUMO2/3 protease SUSP1. As expected, UBP41 specifically inhibited the BiFC signal between ubiquitin and BMAL1 and even increased the number of nuclear foci sequestering SUMO3/BMAL1 complexes (Fig. 7A, middle panels). However, SUSP1 eliminated the BiFC signals generated by ubiquitination, as well as sumoylation, even though immunostaining revealed robust expression of YN-Ub (Fig. 7A, lower panels). Using a similar experimental paradigm to the BiFC assay, we quantified BMAL1 by immunoprecipitation (Fig. 7B). Coexpression of ubiquitin and SUMO3 combined with proteasome inhibition with MG132 caused extensive modification of BMAL1 by ubiquitin and SUMO3. In agreement with the results of the BiFC assay, SUSP1 abolished the accumulation of BMAL1 modified by either ubiquitin or SUMO3, while UBP41 only inhibited modification by ubiquitin and led to striking accumulation of the polysumoylated form. These observations demonstrate that BMAL1 is modified sequentially by SUMO and ubiquitin in the NB and further suggest that sumoylation of BMAL1 is a prerequisite for its ubiquitination.

Role of sequential modifications of BMAL1 in circadian physiology. To investigate the relevance of the sequential modifications of BMAL1 by SUMO and ubiquitin to clock gene expression, we analyzed the effects of SUMO and ubiquitin on *Per1* promoter activity mediated by the CLOCK/BMAL1 heterodimer. Introduction of either exogenous SUMO3 or ubiquitin into NIH 3T3 cells boosted CLOCK/BMAL1-mediated reporter activity moderately, and the expression of both modifiers had a synergistic effect, which was abrogated by treatment with MG132 (Fig. 8A). To further dissect the role of the sequential modifications in the circadian regulation of clock gene transcription, we analyzed the protein profile of BMAL1 modified by SUMO and ubiquitin in the phases of maximal and minimal transcriptional activation. For this experiment, we measured unstable luciferase activity (half-life < 0.5 h) driven by the *Per2* promoter in living cells synchronized by Dex treatment and defined the time points exhibiting maximal and min-

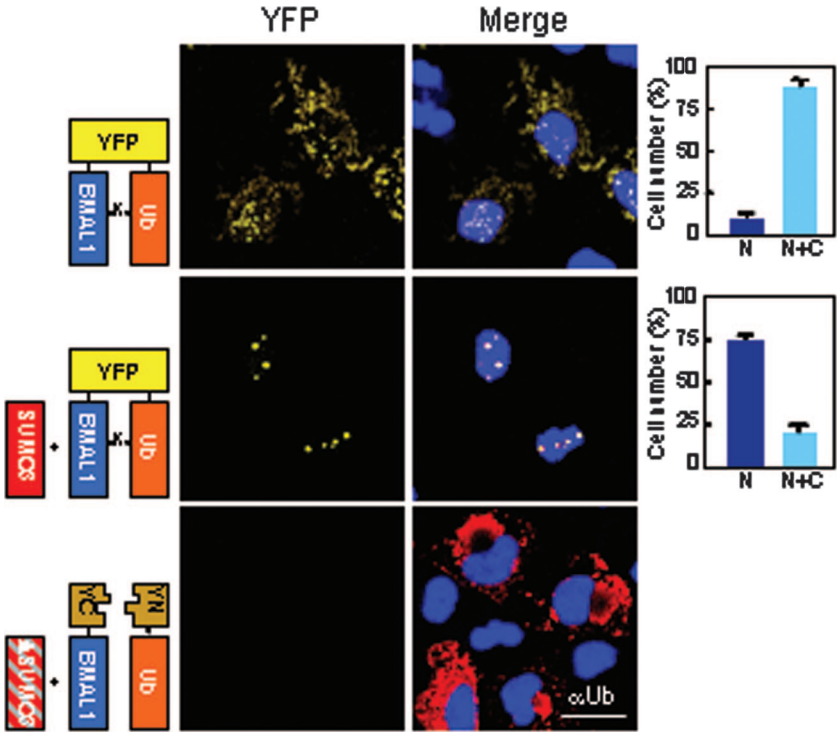

FIG. 6. SUMO3 enhances ubiquitinated BMAL1 in the NB. YN-Ub and YC-BMAL1 were transiently expressed with wild-type SUMO3 or ΔSUMO3 in COS-7 cells and observed by confocal laser scanning microscopy. Quantitative analysis was performed by calculating the ratio of cells showing a nuclear (N) versus both a nuclear and cytoplasmic (N+C) pattern of BiFC signals in each sample. The data are expressed as mean ± the SEM ($n = 3$). Scale bar, 10 μm.

imal transcriptional enhancement in the circadian cycle (15 and 24 h, respectively, after Dex treatment) (Fig. 8B). In parallel with the real-time luciferase assays, we measured the amounts of BMAL1 modified by SUMO and by ubiquitin in cytoplasm and nucleus (Fig. 8C). The levels of both the sumoylated protein and the ubiquitinated version were markedly higher in the transcriptionally active phase than in the inactive phase. These effects were also more prominent in the nucleus than in the cytoplasm. We conclude that the sequential modification of BMAL1 by SUMO and ubiquitin occurs in the nucleus and that it facilitates circadian gene expression driven by the CLOCK/BMAL1 complex.

DISCUSSION

Posttranslational protein modification by SUMO and ubiquitin is an essential regulatory mechanism implicated in diverse cellular processes. The two modification systems often communicate and differentially affect the properties of common substrate proteins, in some cases by being targeted to the same lysine residue (45, 51). Although their cross talk leads to very different consequences depending on the protein, studies of the common substrates of SUMO and ubiquitin suggest that there is antagonism between the two modification systems or that at least they have different functions (8, 19, 43). In the present study, we provide the first evidence that simultaneous modification by SUMO2/3 and ubiquitin is directed to a common result. More specifically, polysumoylation of BMAL1 with the SUMO2/3 moiety serves as a targeting signal for its ubiquitination in the NB, and this ultimately leads to transactivation and proteasomal degradation of BMAL1, as described in the schematic model (Fig. 9).

However, the present findings appear not to be an exception to the general consensus. In yeast cells, mutation of the E2 ubiquitin-conjugating enzyme *ubc4*/*ubc5* resulted in accumulation of polysumoylated proteins (46), and similar results were obtained in a mutant that lacks the E3 ubiquitin ligase Hex3/Slx8 complex (50). More importantly, Hex3 directly interacts with SUMO in this E3 complex, while Slx8 has ubiquitin ligase activity, thereby facilitating the ubiquitination of SUMO conjugates. These findings provide an insight into the reason why the SUMO2/3-specific protease SUSP1 simultaneously abrogates BMAL1 modification by SUMO3 and ubiquitin, whereas the ubiquitin protease UBP41 enhances the accumulation of polysumoylated BMAL1 while strongly inhibiting BMAL1 ubiquitination (Fig. 7B). Moreover, inhibition of proteasome with MG132 in yeast greatly increased levels of high-molecular-weight proteins that are conjugated to both SUMO and ubiquitin (46). In human cells under the same conditions, such SUMO conjugates were not detected by anti-SUMO1 antibodies but by anti-SUMO2/3 antibodies, indicating that ubiquitination of SUMO conjugates also occurs in mammals, but in a SUMO2/3-specific manner; the target proteins have not been identified. These observations are consistent with our present finding that MG132 treatment leads to dramatic accumulation in the nuclear fraction of BMAL1 polysumoylated by SUMO2/3, as well as of the polyubiquitinated form (Fig. 8C).

Sumoylation of BMAL1 itself has been reported by Cardone

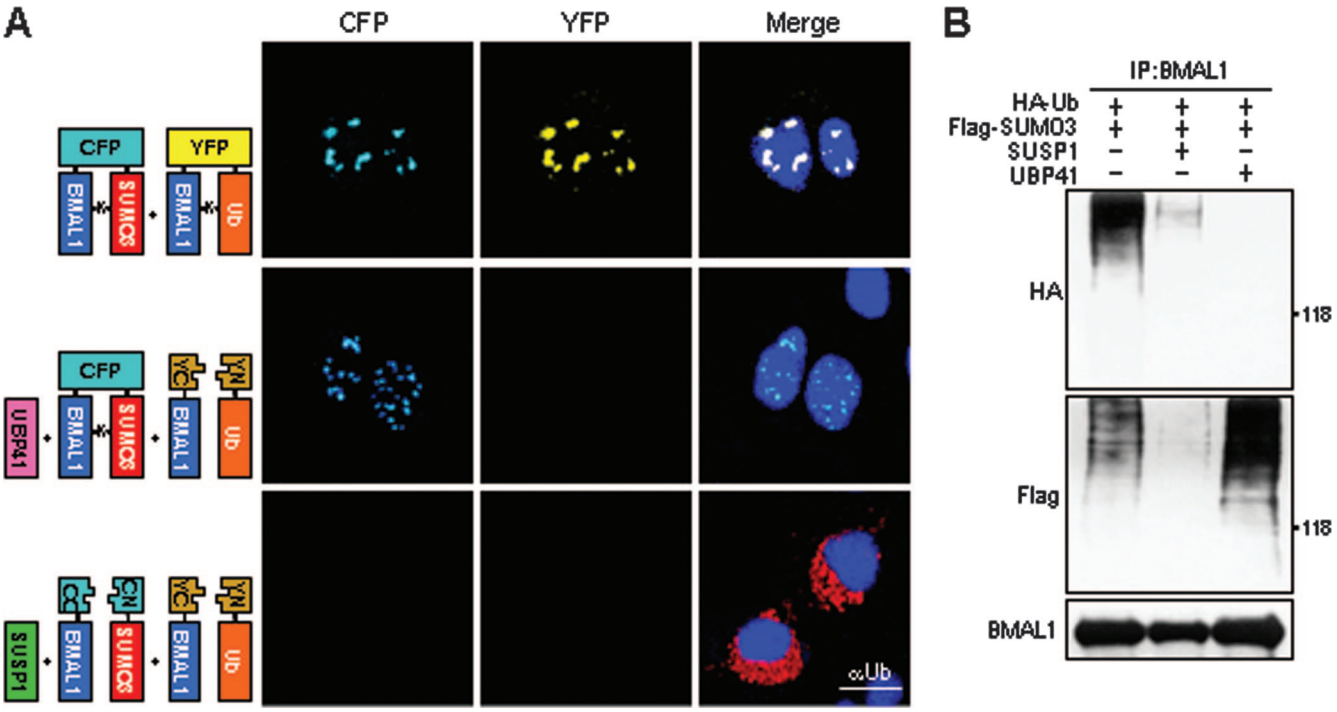

FIG. 7. Sumoylation is essential for ubiquitination of BMAL1. (A) Representative fluorescence images demonstrating that ubiquitination of BMAL1 is abolished by inhibition of BMAL1 sumoylation. Constructs encoding SUMO3, Ub, and BMAL1 fused to the indicated fluorescent protein fragments were coexpressed with UBP41 or SUSP1 in COS-7 cells. Fluorescence images were visualized by confocal laser scanning microscopy using CFP- and YFP-selective filters. Scale bar, 10 μm. (B) NIH 3T3 cells were transfected with Flag-SUMO3 and HA-Ub in the presence of SUSP1 or UBP41. Cells were incubated with MG132 at 50 μM for 5 h, immunoprecipitated with anti-BMAL1, and analyzed by immunoblotting with anti-HA and anti-Flag antibodies. UBP41, ubiquitin-specific protease.

et al. (6). In their study, BMAL1 modification by SUMO1 was emphasized in contrast to our data revealing that under physiological conditions BMAL1 sumoylation preferentially involved SUMO2/3 rather than SUMO1 (Fig. 1C, 2A, 5B, and 8C). These contradictory results, however, appear to be reconcilable since overexpression of a given SUMO paralogue may overcome the normal paralogue preference of target proteins (Fig. 1A and B) (2). In better agreement with the present findings, Cardone et al. (6) also showed that BMAL1 sumoylation is implicated in regulating the stability of BMAL1 and in

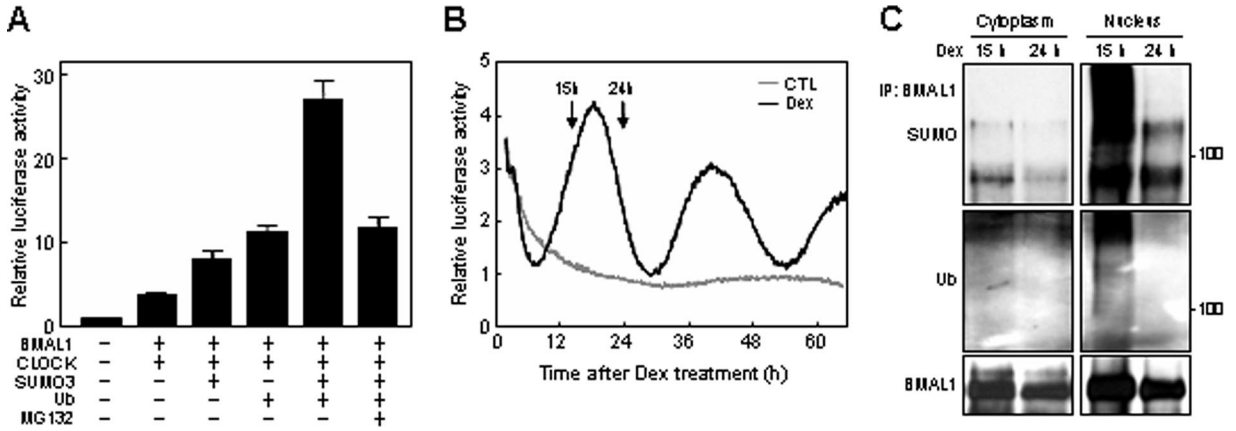

FIG. 8. Modifications of BMAL1 by SUMO and ubiquitin regulate the circadian clock. (A) CLOCK/BMAL1-dependent transcriptional activity is increased by SUMO3 and Ub. NIH 3T3 cells were transiently transfected with the *Per1*-Luc reporter construct and the indicated factors. MG132 (50 μM) was present for 8 h. The data are shown as means ± the SEM of three independent experiments. (B) Representative circadian rhythms of bioluminescence generated from the *Per2* promoter fused to a dsLuc reporter gene. Cells were synchronized by treatment with 1 μM Dex or vehicle (CTL) for 2 h, and their activities were monitored using a real-time measuring system (AB-2550 Kronos-Dio; ATTO). (C) Changes in the abundance of modified BMAL1 by SUMO2/3 and Ub. The levels of modified BMAL1 in cytoplasmic and nuclear fractions were analyzed by immunoprecipitation and immunoblotting at 15 h and 24 h after Dex treatment. Cells were treated with MG132 (50 μM) for 1 h before the indicated times.

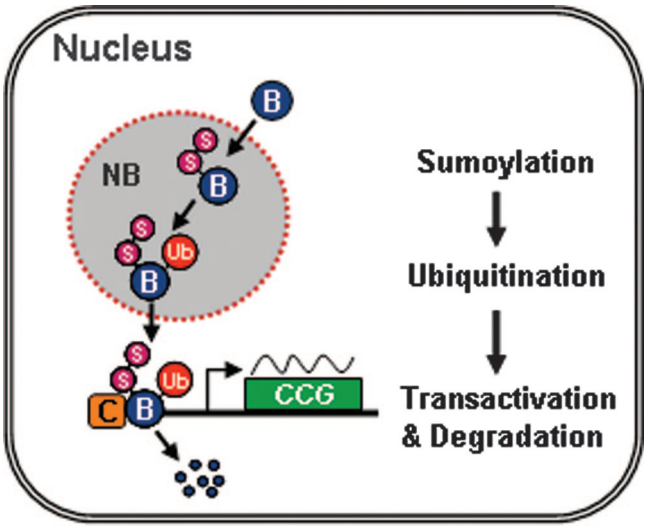

FIG. 9. Model of the circadian regulation of BMAL1 by sumoylation and ubiquitination. Sequential conjugation of BMAL1 by poly-SUMO2/3 and ubiquitin in the NBs accelerates transcriptional activation of the CLOCK/BMAL1 heterodimer and its rapid degradation. C, CLOCK; B, BMAL1; S, SUMO2/3; Ub, ubiquitin; CCG, clock-controlled gene.

circadian activation of target gene expression. This dual function is most likely accounted for by the sequential modification by SUMO2/3 and ubiquitin of BMAL1, which transactivates it, at the same time promoting its proteolysis via the ubiquitin proteasome pathway (Fig. 7 and 8).

Another important biological consequence of BMAL1 sumoylation is the targeting of the modified protein to the PML NB, a discrete nuclear domain enriched with PML that can also be modified by both SUMO1 and SUMO2/3. Sumoylation of PML-especially, polysumoylation by SUMO3 (11), appears to be required not only for its own localization in the NB but also for recruitment of other NB-associated proteins, including SP100 and numerous transcription regulators such as p53, Daxx, HDAC1, HSFs, CBP, and Lef-1 (4, 41). All of these proteins can also be sumoylated, but whether sumoylation is a requirement for their NB association remains unclear since it is difficult to discriminate between the SUMO modifications of different target proteins associated with the NB by microscopic colocalization analysis. In this context, it is noteworthy that the BiFC assay, a recently developed molecular imaging strategy based on complementation between two fragments of fluorescent proteins, allows one to visualize a given protein specifically conjugated with SUMO or ubiquitin in living cells (10, 20). Indeed, several different configurations of the BiFC assay have clearly demonstrated that SUMO3 conjugation localizes BMAL1 to the NB and also that this is followed by ubiquitination in the same location (Fig. 4, 6, and 7A). Moreover, this dual modification of BMAL1 is evident only when it is transcriptionally active (Fig. 8) (30) and recruitment of the transcriptional cofactor CBP to BMAL1 exclusively occurs in the NB (Y. Lee, K. H. Lee, and K. Kim, unpublished data). Thus, these observations raise a possibility that NBs may serve as active sites of the BMAL1-dependent transcription. This notion is further supported by recent data suggesting that NBs are extensively associated with chromatin fibers that are transcriptionally active, although their physiological roles are still controversial (9, 49).

Circadian timekeeping is ubiquitous throughout the human body and involves periodic oscillation of clock gene transcription. This rhythmic gene expression is driven primarily by a negative-feedback loop composed of the CLOCK/BMAL1 heterodimeric transcription factor and repressors that it itself induces. To generate circadian rhythmicity, a time delay between transcription of the repressor genes and negative feedback by their own protein products is essential. A growing body of evidence indicates that posttranslational modifications of the core clock components are pivotal for achieving this time delay. In lower animals, such as *Cyanobacteria* and *Neurospora*, cyclic phosphorylation of clock molecules has been proposed as the basic timekeeping principle (21, 38, 40). In mammals, recent studies on the Fbxl3 ubiquitin E3 ligase have demonstrated that ubiquitin-dependent proteasomal degradation of CRYs, the key repressors of the CLOCK/BMAL1 complex, is important for fine-tuning the circadian rhythm of both gene expression and behavior (5, 14). More recently, it has been shown that BMAL1 can be acetylated during the transcriptional inhibition phase and that this modification facilitates recruitment of CRY1 to the CLOCK/BMAL1 complex (18). Thus, acetylation of BMAL1 appears to contribute to negative regulation of the heterodimeric transcription factor, in contrast to the dual modification by SUMO and ubiquitin that boosts CLOCK/BMAL1-dependent transcription during the transcriptionally active phase.

In conclusion, the antagonistic effect of the antiphasic modifications of BMAL1 by SUMO/ubiquitin and acetylation may serve as a key posttranslational regulatory mechanism that times activation of the CLOCK/BMAL1 complex and also potentiates circadian oscillation of the molecular clock. The increased understanding of the posttranslational control of BMAL1 may provide novel insights into how one might manipulate the timekeeping system.

ACKNOWLEDGMENTS

We thank Tom Kerppola (University of Michigan Medical School) for generously providing the BiFC plasmids.

This study was supported by the Brain Research Center of the 21st Century Frontier Program in Neuroscience and the Biotool R&D Project for Cell Research from the Korean Ministry of Science and Technology. K.H.L. was supported by the BK 21 program from the Korea Ministry of Education. J.L. was supported by a Seoul Science Fellowship.

REFERENCES

1. **Ayaydin, F., and M. Dasso.** 2004. Distinct in vivo dynamics of vertebrate SUMO paralogues. Mol. Biol. Cell **15:**5208–5218.
2. **Azuma, Y., A. Arnaoutov, and M. Dasso.** 2003. SUMO-2/3 regulates topoisomerase II in mitosis. J. Cell Biol. **163:**477–487.
3. **Baek, S. H., K. S. Choi, Y. J. Yoo, J. M. Cho, R. T. Baker, K. Tanaka, and C. H. Chung.** 1997. Molecular cloning of a novel ubiquitin-specific protease, UBP41, with isopeptidase activity in chick skeletal muscle. J. Biol. Chem. **272:**25560–25565.
4. **Bernardi, R., and P. P. Pandolfi.** 2007. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nat. Rev. Mol. Cell. Biol. **8:**1006–10016.
5. **Busino, L., F. Bassermann, A. Maiolica, C. Lee, P. M. Nolan, S. I. Godinho, G. F. Draetta, and M. Pagano.** 2007. SCF^{Fbxl3} controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. Science **316:**900–904.
6. **Cardone, L., J. Hirayama, F. Giordano, T. Tamaru, J. J. Palvimo, and P.**

Sassone-Corsi. 2005. Circadian clock control by SUMOylation of BMAL1. Science **309:**1390–1394.
7. **Cheng, J., X. Kang, S. Zhang, and E. T. Yeh.** 2007. SUMO-specific protease 1 is essential for stabilization of HIF1α during hypoxia. Cell **131:**584–595.
8. **Desterro, J. M., M. S. Rodriguez, and R. T. Hay.** 1998. SUMO-1 modification of IκBα inhibits NF-κB activation. Mol. Cell **2:**233–239.
9. **Eskiw, C. H., G. Dellaire, and D. P. Bazett-Jones.** 2004. Chromatin contributes to structural integrity of promyelocytic leukemia bodies through a SUMO-1-independent mechanism. J. Biol. Chem. **279:**9577–9585.
10. **Fang, D., and T. K. Kerppola.** 2004. Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. Proc. Natl. Acad. Sci. USA **101:**14782–14787.
11. **Fu, C., K. Ahmed, H. Ding, X. Ding, J. Lan, Z. Yang, Y. Miao, Y. Zhu, Y. Shi, J. Zhu, H. Huang, and X. Yao.** 2005. Stabilization of PML nuclear localization by conjugation and oligomerization of SUMO-3. Oncogene **24:**5401–5413.
12. **Gallego, M., and D. M. Virshup.** 2007. Post-translational modifications regulate the ticking of the circadian clock. Nat. Rev. Mol. Cell. Biol. **8:**139–148.
13. **Geiss-Friedlander, R., and F. Melchior.** 2007. Concepts in sumoylation: a decade on. Nat. Rev. Mol. Cell. Biol. **8:**947–956.
14. **Godinho, S. I., E. S. Maywood, L. Shaw, V. Tucci, A. R. Barnard, L. Busino, M. Pagano, R. Kendall, M. M. Quwailid, M. R. Romero, J. O'neill, J. E. Chesham, D. Brooker, Z. Lalanne, M. H. Hastings, and P. M. Nolan.** 2007. The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. Science **316:**897–900.
15. **Griffin, E. A., Jr., D. Staknis, and C. J. Weitz.** 1999. Light-independent role of CRY1 and CRY2 in the mammalian circadian clock. Science **286:**768–771.
16. **Harms, E., S. Kivimäe, M. W. Young, and L. Saez.** 2004. Posttranscriptional and posttranslational regulation of clock genes. J. Biol. Rhythms. **19:**361–373.
17. **Hay, R. T.** 2005. SUMO: a history of modification. Mol. Cell **18:**1–12.
18. **Hirayama, J., S. Sahar, B. Grimaldi, T. Tamaru, K. Takamatsu, Y. Nakahata, and P. Sassone-Corsi.** 2007. CLOCK-mediated acetylation of BMAL1 controls circadian function. Nature **450:**1086–1090.
19. **Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis, and S. Jentsch.** 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature **419:**135–141.
20. **Hu, C. D., and T. K. Kerppola.** 2003. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. Nat. Biotechnol. **21:**539–545.
21. **Huang, G., S. Chen, S. Li, J. Cha, C. Long, L. Li, Q. He, and Y. Liu.** 2007. Protein kinase A and casein kinases mediate sequential phosphorylation events in the circadian negative feedback loop. Genes. Dev. **21:**3283–3295.
22. **Janssen, K., T. G. Hofmann, D. A. Jans, R. T. Hay, K. Schulze-Osthoff, and U. Fischer.** 2007. Apoptin is modified by SUMO conjugation and targeted to promyelocytic leukemia protein nuclear bodies. Oncogene **26:**1557–1566.
23. **Johnson, E. S.** 2004. Protein modification by SUMO. Annu. Rev. Biochem. **73:**355–382.
24. **Kim, K. I., S. H. Baek, Y. J. Jeon, S. Nishimori, T. Suzuki, S. Uchida, N. Shimbara, H. Saitoh, K. Tanaka, and C. H. Chung.** 2000. A new SUMO-1-specific protease, SUSP1, that is highly expressed in reproductive organs. J. Biol. Chem. **275:**14102–14106.
25. **Kim, K. I., S. H. Baek, and C. H. Chung.** 2002. Versatile protein tag, SUMO: its enzymology and biological function. J. Cell Physiol. **191:**257–268.
26. **Kiyohara, Y. B., S. Tagao, F. Tamanini, A. Morita, Y. Sugisawa, M. Yasuda, I. Yamanaka, H. R. Ueda, G. T. van der Horst, T. Kondo, and K. Yagita.** 2006. The BMAL1 C terminus regulates the circadian transcription feedback loop. Proc. Natl. Acad. Sci. USA **103:**10074–10079.
27. **Ko, C. H., and J. S. Takahashi.** 2006. Molecular components of the mammalian circadian clock. Hum. Mol. Genet. **2:**R271–R277.
28. **Kondratov, R. V., M. V. Chernov, A. A. Kondratova, V. Y. Gorbacheva, A. V. Gudkov, and M. P. Antoch.** 2003. BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. Genes. Dev. **17:**1921–1932.
29. **Kume, K., M. J. Zylka, S. Sriram, L. P. Shearman, D. R. Weaver, X. Jin, E. S. Maywood, M. H. Hastings, and S. M. Reppert.** 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell **98:**193–205.
30. **Kwon, I., J. Lee, S. H. Chang, N. C. Jung, B. J. Lee, G. H. Son, K. Kim, and K. H. Lee.** 2006. BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. Mol. Cell. Biol. **26:**7318–7330.
31. **Lee, C., J. P. Etchegaray, F. R. Cagampang, A. S. Loudon, and S. M. Reppert.** 2001. Posttranslational mechanisms regulate the mammalian circadian clock. Cell **107:**855–867.
32. **Mohan, R. D., A. Rao, J. Gagliardi, and M. Tini.** 2007. SUMO-1-dependent allosteric regulation of thymine DNA glycosylase alters subnuclear localization and CBP/p300 recruitment. Mol. Cell. Biol. **27:**229–243.
33. **Mukhopadhyay, D., F. Ayaydin, N. Kolli, S. H. Tan, T. Anan, A. Kametaka, Y. Azuma, K. D. Wilkinson, and M. Dasso.** 2006. SUSP1 antagonizes formation of highly SUMO2/3-conjugated species. J. Cell Biol. **174:**939–949.
34. **Muratani, M., and W. P. Tansey.** 2003. How the ubiquitin-proteasome system controls transcription. Nat. Rev. Mol. Cell. Biol. **4:**192–201.
35. **Okamura, H., S. Miyake, Y. Sumi, S. Yamaguchi, A. Yasui, M. Muijtjens, J. H. Hoeijmakers, and G. T. van der Horst.** 1999. Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. Science **286:**2531–2534.
36. **Reppert, S. M., and D. R. Weaver.** 2002. Coordination of circadian timing in mammals. Nature **418:**935–941.
37. **Roscic, A., A. Möller, M. A. Calzado, F. Renner, V. C. Wimmer, E. Gresko, K. S. Lüdi, and M. L. Schmitz.** 2006. Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. Mol. Cell **24:**77–89.
38. **Rust, M. J., J. S. Markson, W. S. Lane, D. S. Fisher, and E. K. O'Shea.** 2007. Ordered phosphorylation governs oscillation of a three-protein circadian clock. Science **318:**809–812.
39. **Saitoh, H., and J. Hinchey.** 2000. Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J. Biol. Chem. **275:**6252–6258.
40. **Schibler, U.** 2006. Circadian time keeping: the daily ups and downs of genes, cells, and organisms. Prog. Brain Res. **153:**271–282.
41. **Seeler. J. S., and A. Dejean.** 2003. Nuclear and unclear functions of SUMO. Nat. Rev. Mol. Cell. Biol. **4:**690–699.
42. **Shim, H. S., H. Kim, J. Lee, G. H. Son, S. Cho, T. H. Oh, S. H. Kang, D. S. Seen, K. H. Lee, and K. Kim.** 2007. Rapid activation of CLOCK by Ca^{2+}-dependent protein kinase C mediates resetting of the mammalian circadian clock. EMBO Rep. **8:**366–371.
43. **Steffan, J. S., N. Agrawal, J. Pallos, E. Rockabrand, L. C. Trotman, N. Slepko, K. Illes, T. Lukacsovich, Y. Z. Zhu, E. Cattaneo, P. P. Pandolfi, L. M. Thompson, and J. L. Marsh.** 2004. SUMO modification of Huntingtin and Huntington's disease pathology. Science **304:**100–104.
44. **Tatham, M. H., E. Jaffray, O. A. Vaughan, J. M. Desterro, C. H. Botting, J. H. Naismith, and R. T. Hay.** 2001. Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. J. Biol. Chem. **276:**35368–35374.
45. **Ulrich, H. D.** 2005. Mutual interactions between the SUMO and ubiquitin systems: a plea of no contest. Trends Cell Biol. **15:**525–532.
46. **Uzunova, K., K. Göttsche, M. Miteva, S. R. Weisshaar, C. Glanemann, M. Schnellhardt, M. Niessen, H. Scheel, K. Hofmann, E. S. Johnson, G. J. Praefcke, and R. J. Dohmen.** 2007. Ubiquitin-dependent proteolytic control of SUMO conjugates. J. Biol. Chem. **282:**34167–34175.
47. **Verger, A., J. Perdomo, and M. Crossley.** 2003. Modification with SUMO. A role in transcriptional regulation. EMBO Rep. **4:**137–142.
48. **Vertegaal, A. C., J. S. Andersen, S. C. Ogg, R. T. Hay, M. Mann, and A. I. Lamond.** 2006. Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. Mol. Cell. Proteomics **5:**2298–2310.
49. **Wang, J., C. Shiels, P. Sasieni, P. J. Wu, S. A. Islam, P. S. Freemont, and D. Sheer.** 2004. Promyelocytic leukemia nuclear bodies associate with transcriptionally active genomic regions. J. Cell Biol. **164:**515–526.
50. **Xie, Y., O. Kerscher, M. B. Kroetz, H. F. McConchie, P. Sung, and M. Hochstrasser.** 2007. The yeast Hex3 · Slx8 heterodimer is a ubiquitin ligase stimulated by substrate sumoylation. J. Biol. Chem. **282:**34176–34184.
51. **Zhang, P. J., J. Zhao, H. Y. Li, J. H. Man, K. He, T. Zhou, X. Pan, A. L. Li, W. L. Gong, B. F. Jin, Q. Xia, M. Yu, B. F. Shen, and X. M. Zhang.** 2007. CUE domain containing 2 regulates degradation of progesterone receptor by ubiquitin-proteasome. EMBO J. **26:**1831–1842.
52. **Zhao, J.** 2007. Sumoylation regulates diverse biological processes. Cell. Mol. Life Sci. **64:**3017–3033.