Stress-induced Phosphorylation of Thr\textsuperscript{486} in c-Myb by p38 Mitogen-activated Protein Kinases Attenuates Conjugation of SUMO-2/3*

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Background: Regulation of c-Myb transcriptional activity and protein level is essential for hematopoietic homeostasis.

Results: p38 MAPKs phosphorylate c-Myb on Thr\textsuperscript{486}, resulting in repression of SUMOylation in stressed cells.

Conclusion: There is negative cross-talk between the p38 MAPK phosphorylation pathway and the SUMO-2/3 conjugation pathway that regulates c-Myb activity in stressed cells.

Significance: The novel mechanism that modulates activity of c-Myb in stressed cells was identified.

Oncogenic forms of c-Myb, in animal model systems, are frequently associated with truncations of either amino, carboxyl, or both termini (14, 15). These truncations result in removal of structural regions with a negative impact on proteolytic stability and transactivation capacity (16, 17). In contrast to many animal leukemic models, increased expression of unaltered c-Myb is commonly detected in human leukemia (18, 19). More recently rearrangements of the MYB locus were also reported (20–25). However, these alterations result mostly in increased expression of the full-length, unaltered c-Myb protein (21–23). These examples are similar to an activation of c-myb in murine leukemia, where a retrovirus integrates several kilobases upstream of the c-myb transcription start site and deregulates its expression (26, 27). Nevertheless, these leukemic cells seem to be dependent on the elevated levels of c-Myb, because knocking down the gene expression is detrimental for those cells, whereas normal untransformed progenitors survive similar treatment (28, 29). Thus, these results clearly show that strict regulation of the protein level of c-Myb and/or activity is

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essential for balanced hematopoietic cell homeostasis, and its deregulation can be leukemogenic.

Post-translational modifications of c-Myb, such as phosphorylation, acetylation, ubiquitination, and SUMOylation are crucial for regulation of the transcriptional activity and steady-state levels of c-Myb (16). c-Myb is a short-lived protein that is rapidly turned over by the ubiquitin/26S proteasome proteolytic system (30). In contrast to ubiquitination, conjugation of the SUMO2 proteins to the lysine residues, located in the negative regulatory domain of c-Myb, results in decreased transcriptional activity and increased proteolytic stability (31–33). Although SUMO-1 conjugates to c-Myb predominantly under normal physiological growth conditions, cellular stress induces rapid inactivation of c-Myb through covalent conjugation of SUMO-2/3 paralogs (34). SUMOylation of target proteins, like conjugation of polyubiquitin chains, can be regulated by phosphorylation (35, 36). Because cellular stresses activate several protein kinases that phosphorylate many downstream targets (37), we decided to investigate whether phosphorylation of c-Myb changes in cells subjected to cellular stress and whether these changes in phosphorylation of c-Myb influence its stress-induced SUMOylation.

Here, we report that the phosphorylation of Thr486 in the negative regulatory domain of c-Myb by p38 MAPKs occurs in cells subjected to several metabolic stresses. Furthermore, we show that phosphorylation of Thr486 negatively regulates conjugation of SUMO-2/3 to c-Myb and leads to modulation of its transcriptional activity in stressed cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Constructs encoding wild-type c-Myb (c-MybWT) and a p5xMRE-A-luc construct were described previously (31). The mutant T486A of c-Myb (cMybT486A) was generated by the QuikChange site-directed mutagenesis kit (Stratagene) and subcloned into pcDNA3.1+ (Invitrogen) vector. Plasmid encoding GST-cMyb(464–544aa) was prepared by subcloning of c-Myb(464–544aa), in frame with GST, into the pGEX-KG bacterial expression vector. The base pair substitution of c-Myb(464–544aa), in frame with GST, into the retroviral vector pMSCV-IRES-GFP (MIG), kindly provided by R. Hay (University of St. Andrews, St. Andrews, United Kingdom).

Cell Cultures and Viruses—COS-7 cells (SV40-transformed monkey kidney) and RAW264.7 macrophage cells (both from American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 2 mm L-glutamine (Invitrogen), and penicillin/streptomycin (100 µg/ml each) (Invitrogen). Murine M1 myeloid cells (30) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (Invitrogen). M1 cells were transduced with MSCV-IRES-GFP-based retroviruses expressing cMybWT or cMybT486A or pool of lentiviruses expressing shRNAs targeting mouse p38 MAPKα (Sigma-Aldrich) in the presence of Polybrene (10 µg/ml; Sigma-Aldrich). Twenty-four hours after retrovirus infection, green fluorescent protein-positive (GFP+) cells were sorted using a FACS. Knockdown of p38 MAPKα was evaluated 72 h after infection by immunoblotting.

Transient Transfection, Immunoprecipitation, and Immunoblotting—COS-7 cells were transiently transfected with Effectene™ transfection reagent (Qiagen) as described previously (34). Protein expression was analyzed by immunoblotting of total cell lysates or immunoprecipitates fractionated by SDS-PAGE with the following antibodies: mouse monoclonal antibody anti-cMyb (34), rabbit polyclonal anti-c-Myb (34), rabbit polyclonal anti-P-T486pept, anti-T486pept (Rockland Immunocchemicals, Inc.), anti-SUMO-2/3 (34), anti-FLAG, anti-HA (Sigma-Aldrich), anti-p38 MAPKα, anti-p38 MAPKβ, anti-p38 MAPKγ, anti-p38 MAPKδ, anti-actin, anti-tubulin, anti-cleaved PARP, anti-Bcl-2, anti-Bcl-xL, and anti-Mcl-1 antibody (Cell Signaling).

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described previously (38). COS7 cells, transiently expressing c-MybWT, were metabolically labeled in phosphate-free DMEM with 0.5 mCi/ml of carrier-free 32P orthophosphate (MP Biomedicals) for 4 h. c-Myb was immunoprecipitated, separated by SDS-PAGE, and transferred to an Immobilon-P membrane (Millipore). The membrane immobilized c-Myb was excised, hydrolyzed in 200 µl of 6 N HCl (constant boiling) (Sigma) at 110 °C for 2 h, lyophilized in a SpeedVac (Savant), and resuspended in 10 µl of water containing 0.1 mg/ml of each phosphoamino acid standard, phosphoserine, phosphothreonine, and phosphotyrosine (Sigma). The samples were spotted onto thin layer cellulose plates (Sigma) and separated by electrophoresis (38). The migration of phosphoamino acid standards was detected by staining with ninhydrin (Sigma), and 32P-labeled phosphoamino acids were visualized by direct autoradiography.

LC-MS/MS Analyses to Detect Phosphorylation of c-Myb—c-Myb protein immunoprecipitated from stressed or unstressed cells with anti-c-Myb antibody was separated by SDS-PAGE and stained with a PageBlue protein staining solution (Fermantas). The gel bands were destained, reduced, alkylated, and digested with trypsin in the presence of ProteaseMAX™ Surfactant (Promega) at 50 °C for 1 h according to the manufacturer’s protocol. MS was performed in the Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., NCI (Frederick, MD). The tryptic peptides were analyzed by nanobore reversed phase LC-MS/MS, nanobore reversed phase LC-MS/MS was performed using an Agilent 1100 nanoflow liquid chromatography system coupled online with a linear ion trap-Fourier transform mass spectrometer (ThermoElectron). The linear ion trap-Fourier transform mass spectrometer was operated in a data-dependent mode where each full MS scan was...
followed by seven tandem MS scans, and the seven most abundant molecular ions were dynamically selected for collision-induced dissociation using a normalized collision energy of 35%. Tandem mass spectra were searched against a mouse database using SEQUEST software (ThermoElectron).

Luciferase Assay—RAW264.7 cells (2 × 10^6 cells/ml) were washed in PBS and resuspended in 100 μl of Nucleofector kit V (Lonza) supplemented with 0.5 μg of a cMyb-dependent firefly luciferase reporter construct (p5xMRE-A-Luc), 30 ng of Renilla luciferase pCMV-RL (Promega), a control construct used for normalization, and 1.5 μg of constructs encoding either cMybWT, cMybT486A, or cMybSU. Cells were transfected by electroporation using Nucleofector II (Lonza) in accordance with the manufacturer’s instructions. Luciferase activity was assessed using a dual-luciferase reporter system (Promega) and the TurnerTD-20e luminometer (Turner Designs).

RESULTS

Hyperthermic Stress Induces Rapid Changes in Phosphorylation of c-Myb—We have shown previously that cellular stresses induce rapid conjugation of SUMO-2/3 to two lysines (Lys499 and Lys353) located in NRD of c-Myb. Several protein kinases, including p38 MAPK and JNK families, are also rapidly activated in response to cellular stresses and phosphorylate many targets (37). Therefore, we decided to investigate whether cellular stresses affect phosphorylation of c-Myb and whether these changes in c-Myb phosphorylation may regulate stress-induced SUMOylation of c-Myb. To compare the c-Myb phosphorylation status in cells treated (43 °C) or not treated (37 °C) with hyperthermia, we first labeled metabolically cellular proteins with [32P]orthophosphate in COS7 cells transiently transfected with hyperthermia using Nucleofector II (Lonza) in accordance with the manufacturer’s instructions. Luciferase activity was assessed using a dual-luciferase reporter system (Promega) and the TurnerTD-20e luminometer (Turner Designs).

Identification of Phosphorylated Residues in c-Myb by Mass Spectrometry—Reversed phase liquid chromatography-tandem mass spectrometry was used to identify phosphorylated residues in c-Myb isolated from stressed or unstressed cells. As shown in Fig. 2A, the c-Myb-specific peptide coverage that was detected ranged from 42–44%. Phosphorylation of Ser528, described previously (39, 40), and Thr462 and Thr464 were detected in nonSUMOylated c-Myb isolated from both stressed and unstressed cells. Phosphorylation of Thr208, Ser443, and Thr486 was detected in cMyb isolated from stressed cells, but not in c-Myb isolated from cells growing under physiological conditions (Fig. 2A). Because Thr462 is located closely to Lys499, which is an acceptor of the SUMO-2 molecule and conjugation of SUMO-2 to Lys499 is strongly modified in response to stress (34), we decided to investigate whether phosphorylation of Thr486 may regulate stress-induced SUMOylation of c-Myb. High conservation of the NRD region of c-Myb surrounding Thr486 among species (Fig. 2B) also suggests an important function. Interestingly, however, phosphorylation of Thr486 was detected only in the nonSUMOylated form of c-Myb isolated from cells treated with hyperthermia (Fig. 2A). This observation is also in line with the phosphoamino acid experiment, where decreased Thr phosphorylation was detected in the SUMO-2-modified form of c-Myb (Fig. 1D).

Phosphorylation of Thr486 Negatively Regulates Stress-induced Conjugation of SUMO-2/3—To further confirm the results from mass spectrometry, we decided to prepare an antibody that specifically recognizes the phosphorylated Thr486 residue in murine c-Myb. In collaboration with Rockland Immunochemicals Inc., rabbit polyclonal antibody that recognizes either phosphorylated Thr486 (α-P-T486pept) or nonphosphorylated region in murine c-Myb (amino acid residues 480–494, α-T486pept) were prepared and tested in COS7 cells transfected with c-Myb. Strong induction of Thr486 phosphorylation in c-Myb (Fig. 3A, P-T486-cMyb) was detected in cells treated with hyperthermia using Thr(P)486-specific antibody, whereas no difference in the c-Myb level was detected using antibody raised against the nonphosphorylated peptide (Fig. 3A). To further investigate phosphorylation of Thr486 in SUMOylated and nonSUMOylated forms of c-Myb, we transfected COS-7 cells with constructs encoding c-MybWT,
HA-SUMO-2 and treated them with hyperthermia for 15 min. Strong conjugation of SUMO-2 protein was detected by immunoblotting using anti-c-Myb monoclonal antibody (Fig. 3B). c-Myb was immunoprecipitated from cell lysates using rabbit antibodies that recognize specifically either phosphorylated (α-P-T486pept) or nonphosphorylated Thr486 (α-T486pept) in murine c-Myb and analyzed by immunoblotting. Anti-HA antibody detected SUMO-2 conjugated to c-Myb, whereas anti-c-Myb monoclonal antibody recognized both SUMOylated and nonSUMOylated forms of c-Myb. Antibodies specific for phosphorylated Thr486 (α-P-T486pept) precipitated largely nonSUMOylated c-Myb, whereas antibody specific for nonphosphorylated c-Myb (α-T486pept) immunoprecipitated efficiently both SUMOylated (S21-c-Myb and S22-c-Myb) and nonSUMOylated (c-Myb) protein (Fig. 3C). These results confirmed mass spectrometry data and suggested that phosphorylation of Thr486 may negatively regulate stress-induced conjugation of SUMO-2 to c-Myb. To further investigate this possibility, we mutated Thr486 to Ala (cMybT486A) and assessed its stress-induced SUMOylation. Immunoblotting analyses of whole cell lysates and c-Myb immunoprecipitates showed that mutation of T486A resulted in enhanced stress-induced SUMOylation of c-Myb as compared with wild-type protein specifically in stressed cells (Fig. 3D).
Activation of p38 MAPKs Negatively Regulates SUMOylation of c-Myb—Several stress-induced protein kinases are rapidly activated in response to different types of cellular stresses (37). Hyperthermia and other types of metabolic stresses rapidly induce activation of p38 MAPKs in M1 myeloid cells (34) (Fig. 4, A and B). It was previously reported that p38 MAPKs regulate steady-state levels of c-Myb (41). Furthermore, several Ser and Thr residues in the NRD, including Thr486, were implicated in p38 MAPK-dependent destabilization of c-Myb (41). However, the authors did not investigate whether any of these residues were indeed phosphorylated by p38 MAPKs. To examine the possibility that the p38 MAPKs phosphorylate Thr486 in...
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**A**

| Time [min] | Anisomycin | Heat stress |
|-----------|-------------|-------------|
| 0         | +           | +           |
| 5         | +           | +           |
| 10        | +           | +           |

**B**

| Time [min] | Heat stress |
|-----------|-------------|
| 0         | +           |
| 5         | +           |
| 10        | +           |

**C**

| Time [min] | Heat stress |
|-----------|-------------|
| 0         | +           |
| 5         | +           |
| 10        | +           |

**D**

| Condition | p38MAPK | HA-SUMO2 | cMybWT | Anisomycin | Heat stress |
|-----------|---------|----------|--------|------------|-------------|
| +         | +       | +        | +      | +          |             |
| +         | +       | +        | +      | +          | +           |

**FIGURE 4. Activation of p38 MAPKs represses stress-induced SUMOylation of c-Myb.** A and B, M1 cells treated with hyperthermia (43°C) for the indicated times or anisomycin (5 μg/ml) or with other metabolic stresses (0.7 M NaCl, 100 μM H2O2, 7% EtOH) for 15 min (B). Total cell lysates were analyzed by immunoblotting with phospho-specific p38 MAPK and total p38 MAPK antibodies. Levels of activated (P-p38MAPK) and total p38 MAPK are shown. C, activated forms of p38 MAPK immunoprecipitated from M1 cells treated with hyperthermia and used in an *in vitro* p38 MAPK kinase assay following the manufacturer’s instructions (Cell Signaling) with GST-cMyb(464–544aa) protein as a substrate. Phosphorylation of Thr486 in GST-cMyb(464–544aa) was evaluated by immunoblotting with anti-P-T486cMyb phospho-specific antibody. Increased phosphorylation of Thr486 and conjugation of SUMO-2/3 to c-Myb was assessed by immunoblotting with anti-c-Myb-specific antibody. Levels of activated (P-p38MAPK) and total p38 MAPK are shown. D, activated forms of p38 MAPK immunoprecipitated from M1 cells treated with hyperthermia and used in an *in vitro* p38 MAPK kinase assay following the manufacturer’s instructions (Cell Signaling) with GST-cMyb(464–544aa) protein as a substrate. Phosphorylation of Thr486 in GST-cMyb(464–544aa) was evaluated by immunoblotting with anti-P-T486cMyb phospho-specific antibody. Increased phosphorylation of Thr486 and conjugation of SUMO-2/3 to c-Myb was assessed by immunoblotting with anti-c-Myb-specific antibody.

**p38 MAPKs Negatively Regulate SUMOylation of Endogenous c-Myb in Myeloid Cells**—Immunoprecipitation of c-Myb from anisomycin-treated myeloid M1 cells with anti-Thr(P)486-cMyb-specific serum confirmed phosphorylation of Thr486 in c-Myb at the endogenous level (Fig. 5A). Similar to our previous experiments in COS7 cells ectopically expressing c-Myb, pre-activation of p38 MAPKs in myeloid M1 cells resulted in a substantial decrease of stress-induced conjugation of SUMO-2/3 to c-Myb at the endogenous level (Fig. 5B). In contrast, treatment of M1 cells with an inhibitor of p38 MAPK activity SB203580 resulted in an increase of SUMOylated c-Myb (Fig. 5C). The identity of slower migrating c-Myb-immunoreactive bands as SUMO-2/3-modified forms of c-Myb was confirmed by immunoprecipitation with anti-c-Myb-specific antibody and immunoblotting with anti-SUMO-2/3 antibody (Fig. 5D). Immunoblotting analyses revealed that M1 cells express high levels of the p38 MAPKα isoform and low levels of the p38 MAPKγ isoform (Fig. 5E). However, expression of p38 MAPKγ is rapidly increased upon IL-6-induced monocytic differentiation of M1 cells. Interestingly, induction of p38 MAPK expression is accompanied by a rapid reduction of c-Myb in M1 cells (Fig. 5E) that can be reversed by treatment with MG132, an inhibitor of 26S proteasome (Fig. 5F). Knockdown of p38 MAPKα by shRNA (Fig. 5G) resulted in accumulation of total and SUMOylated forms of c-Myb in M1 cells (Fig. 5G). These results, along with experiments employing the T486A mutant, show that there is negative cross-talk between stress-induced phosphorylation of Thr486 and conjugation of SUMO-2/3 to c-Myb in stressed cells.

**Mutation of T486A Modulates Proteolytic Stability and Transcriptional Activity of c-Myb**—A previous report showed that activation of p38 MAPKs may regulate the proteolytic stability of murine c-Myb. Through mutational analyses, the authors implicated several Ser and Thr phosphorylation sites located in the NRD of c-Myb in destabilization of the protein (41). We also reported previously that conjugation of SUMO to c-Myb increases its half-life (31). Because T486A mutation results in an enhanced SUMOylation under stress (Fig. 3D), we compared protein turnovers of c-MybWT and cMybT486A in COS7 cells. There was no significant difference in the proteolytic processing of wild-type and mutant c-Myb in cells without activation of p38 MAPKs (data not shown). However, activation of p38 MAPKs by anisomycin treatment resulted in a slower protein turnover of cMybT486A as compared with cMybWT (Fig. 6, A and B). We hypothesized that the observed partial resistance of the cMybT486A mutant to proteolytic degradation in cells with chronic activation of p38 MAPKs can be explained, at least in part, by increased conjugation of SUMO-2/3 proteins to c-MybT486A, as compared with wild-type protein. To test this, we constructed the c-MybSU protein, resistant to a cleavage by SUMO-specific proteases, by in frame fusion of SUMO (2–93 amino acids) to the NRD of c-Myb (1–519aa). A significantly increased proteolytic stability of c-MybSU, as compared with both cMybWT and cMybT486A, in cells treated with anisomycin (Fig. 6, A, B, and C) provided additional evidence that SUMOylation of c-Myb increases its resistance to proteolytic degradation. Interestingly, we detected phosphorylation of Thr486 in c-MybSU after treatment of cells with anisomycin (data not shown).
shown), suggesting that conjugation of SUMO stabilizes the c-Myb transcription factor independently of Thr486 phosphorylation.

To investigate the transactivation activity of c-MybWT and cMybT486A, we used macrophage cell line RAW264.7 that does not express endogenous c-Myb (data not shown), but it expresses many cofactors that modulate the transcriptional activity of c-Myb. Transactivation activity of the wild-type and the T486A mutant was evaluated in cells transfected with a c-Myb-responsive firefly luciferase reporter construct (p5xMRE-A-luc) and constructs encoding either cMybWT or cMybT486A as indicated. We did not detect any significant difference in transcriptional activity between c-MybWT and c-MybT486A in unstressed cells. However, pretreatment of cells with anisomycin (5 μg/ml) and cycloheximide (CHX, 10 μg/ml) for the indicated times and analyzed by immunoblotting with anti-c-Myb and anti-actin antibody. The migrations of molecular mass markers are shown on the left. B, scanned images were quantitated using ImageJ software. The relative amount of c-Myb protein during the cycloheximide treatment was normalized to actin, and the level of c-Myb at 0 min was assigned to value 1 (normalized intensity). Each point on the graph represents an average ± S.E. (error bars) from two independent experiments. C, bar graph presents half-lives of cMybWT, cMybT486A, and cMybSU. Data are shown as averages ± S.E. (error bars) from three independent experiments, calculated as described previously (30). *, p < 0.05; **, p < 0.01. D, Raw264.7 cells were transfected by electroporation in triplicate with c-Myb-responsive reporter construct p5xMRE-A-luc (5xMRE), and constructs encoding cMybWT, cMybT486A, and cMybSU as indicated. Plasmid pRL-TK (encoding Renilla luciferase gene) was also transfected to control for transfection efficiency. Twenty-four hours after transfection, cells were left untreated (gray bars) or pretreated with anisomycin (5 μg/ml) for 30 min, followed by hyperthermic stress for another 60 min (black bars). Firefly luciferase activity was normalized using Renilla luciferase values. The results show the mean ± S.D. (error bars) for two independent experiments performed in triplicate. The unpaired two-tailed Student’s t test was used to calculate p values. *, p < 0.05; RLU, relative light units.
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For 30 min followed by heat stress for 60 min resulted in stronger suppression of transcriptional activity by cMybT486A than cMybWT (Fig. 6D). Additionally, transcriptional activity of the cMybSU protein was very low and confirmed a strong suppressive activity of SUMO conjugation to c-Myb (Fig. 6D).

**Ectopic Expression of cMybT486A Results in Accelerated Apoptosis of Myeloid Cells**—To evaluate the physiological consequences of c-Myb Thr^{486} phosphorylation under more normal conditions, we prepared myeloid M1 cell lines that ectopically express either wild-type (M1/cMybWT) or T486A mutant of c-Myb (M1/cMybT486A). Both M1/cMybWT and M1/cMybT486A cells proliferated with a similar doubling time as parental M1 cells (data not shown). Activation of p38 MAPKs by anisomycin treatment resulted in a rapid decrease in cell viability with virtually no viable cells in parental M1 cells or cell lines ectopically expressing either cMybWT or cMybT486A after 24 h of treatment (data not shown). Interestingly, however, at early time points, acceleration of cell death was detected in a cell line ectopically expressing the mutant form of c-Myb (cMybT486A) (Fig. 7A). Enhanced apoptotic cell death in M1/c-MybT486A was confirmed by higher levels of the cleaved PARP protein in cells expressing mutant protein (Fig. 7B) and by increased degradation of genomic DNA (Fig. 7C). Decreased transcriptional activity of cMybT486A detected in stressed cells suggests that some of the transcriptional targets of c-Myb that are involved in regulation of apoptosis may be misregulated. Therefore, we analyzed expression of two important anti-apoptotic regulators, Bcl-2 and Bcl-xL, that were shown to be transcriptionally activated by c-Myb (42–44). Indeed, we detected lower levels of Bcl-2 and Bcl-xL in stressed M1/cMybT486A as compared with M1/cMybWT, whereas the level of another anti-apoptotic protein Mcl-1 was not considerably changed (Fig. 7, D and E). Together, these results suggest that phosphorylation of Thr^{486} modulates the transcriptional activity of c-Myb through suppression of SUMOylation in cells under stress and can lead to stress-triggered apoptotic cell death.

**DISCUSSION**

In the present work, we have shown for the first time that environmental stresses, in addition to rapid covalent conjugation of SUMO-2/3 proteins (34), induce changes in the phosphorylation of c-Myb. Although stress-induced phosphorylation of Thr^{208} and Ser^{444} was identified in both SUMOylated and nonSUMOylated forms of the phosphorylation of Thr^{486}, located in close proximity to SUMOylation site Lys^{499} of c-Myb, was detected preferentially in the nonSUMOylated form. Furthermore, we have shown that a T486A mutation results in increased SUMOylation of c-Myb in cells treated with hyperthermia, suggesting a repressive effect Thr^{486} phosphorylation on stress-induced conjugation of SUMO-2/3 to c-Myb. p38 MAPKs were identified as protein kinase candidates that regulate the activity of c-Myb through phosphorylation-dependent repression of SUMOylation in stressed cells. Thus, we have uncovered a novel regulatory interplay between phosphorylation and SUMOylation of c-Myb that regulates its activity in stressed cells.

**SUMOylation dramatically modulates transcriptional activity, as well as proteolytic turnover of c-Myb (31–33). Phosphorylation-dependent stimulation of SUMOylation of target proteins is frequently detected when modified Ser or Thr residues are located within the specific bipartite phosphorylation-dependent SUMOylation motif composed of a SUMO consensus site and an adjacent proline-directed phosphorylation site (\(S/T/P\)) (35). Although the major SUMOylation site Lys^{223} and constitutively phosphorylated Ser^{528} (39) are both located within the phosphorylation-dependent SUMOylation motif in c-Myb, no significant regulatory effect of Ser^{528} phosphorylation on SUMOylation of c-Myb has been detected (31,
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34). It has been reported that suppression of SUMOylation through phosphorylation can also occur when phosphorylated residues are located outside of the phosphorylation-dependent SUMOylation motif in target proteins (45). In this regard, we have identified phosphorylation of Thr\(^{486}\) in c-Myb isolated from stressed cells but largely in the nonSUMOylated form of c-Myb, suggesting a negative role for Thr\(^{486}\) phosphorylation in stress-induced SUMOylation of c-Myb. Accordingly, we observed a significant increase in SUMOylation of a cMybT486A mutant as compared with wild-type c-Myb (Fig. 3). Although a detailed mechanism through which phosphorylation of Thr\(^{486}\) suppresses SUMOylation of c-Myb is not known, we can speculate that phosphorylation changes the conformation of the NRD of c-Myb, and these changes result in decreased affinity of phosphorylated c-Myb for SUMO-conjugating enzymes necessary for efficient SUMOylation. Indeed, conformational changes in the NRD of c-Myb in cells treated with okadaic acid, an inhibitor of Ser/Thr protein phosphatases that induces strong induction of Thr phosphorylation, were described previously (38, 46). Alternatively, activation of p38 MAPKs can also stimulate specific SUMO-2/3 proteases that in turn affect SUMOylation and stability of c-Myb in stressed cells.

Our results also indicate that the cMybT486A mutant, in stressed cells, has a decreased proteolytic turnover as compared with wild-type c-Myb. Several protein kinases and phosphatases were reported to destabilize c-Myb through increased phosphorylation of its NRD, but identification of a critical residue previously remained largely elusive (38, 41, 47, 48). More recently, Kitagawa et al. (49) reported that phosphorylation of Thr\(^{572}\) by GSK3 protein kinase in murine c-Myb is recognized by Fbw7b, a protein component of SCF-ubiquitin ligase complex. Fbw7b binds to phosphorylated Thr\(^{572}\) and catalyzes polyubiquitin chain conjugation that triggers degradation of c-Myb by the 26S proteasome. Regulation of c-Myb turnover through phosphorylation of Thr\(^{572}\) seems to be specific for murine c-Myb, because this residue is not evolutionary conserved. Furthermore, both positive and negative roles for GSK3 protein kinase in regulation of human c-Myb were reported for different cell lines (50, 51). Here, we show that phosphorylation of Thr\(^{486}\) by p38 MAPKs in stressed cells destabilizes c-Myb. Our results are in agreement with a previous report where the authors showed increased proteolytic processing of c-Myb in cells upon activation of p38 MAPKs by anisomycin. This report also suggested that several Ser and Thr residues located in the NRD of c-Myb, including Thr\(^{486}\), can be phosphorylated by p38 MAPKs and involved in proteolytic degradation of c-Myb (41). We confirmed this observation, and by using a newly developed anti-phospho-Thr\(^{486}\) cMyb-specific antibody, we confirmed that Thr\(^{486}\) in murine c-Myb is phosphorylated by activated p38 MAPKs (Fig. 4C). Furthermore, we showed that overexpression of all four different isoforms (α, β, γ, and δ) attenuated stress-induced SUMOylation of c-Myb, with the strongest effect observed for p38 MAPK\(\gamma\) and p38 MAPK\(\delta\) (Fig. 4D).

Whether phosphorylated Thr\(^{486}\) is recognized by a specific ubiquitin ligase that catalyzes conjugation of polyubiquitin chains that targets c-Myb for degradation or whether decreased SUMOylation in phosphorylated protein increases proteolytic turnover of c-Myb is not clear at present. However, increased proteolytic stability of the cMybT486A mutant can be at least partially explained by augmented conjugation of SUMO to c-Myb in stressed cells. We have previously reported that conjugation of SUMO increases the half-life of modified c-Myb (31). Here we provide additional evidence based upon the observation that a c-Myb-SUMO fusion protein (cMybSU), resistant to SUMO-specific proteases, has dramatically increased proteolytic stability in cells (Fig. 6, A–C).

Stress-induced SUMOylation of targets proteins can have diverse effects on their activities. Although numerous proteins involved in DNA repair processes are strongly activated by stress-induced SUMOylation (52), transcription factors with a positive role in regulation of cellular proliferation are repressed by conjugation of SUMO proteins (53). Rapid inactivation of positive regulators of cell proliferation may represent a mechanism through which cells adapt to stress by ceasing proliferation, thus providing time for the enzymatic repair machinery to assess and restore damages imposed to cells by stress. c-Myb regulates transcription of diverse genes directly involved in broad set of cellular processes such as proliferation, differentiation, and programmed cells death. Regulated expression of different target genes by a differentially modified transcriptional regulator may represent a mechanism responsible for a proper cellular adaptation response to different growth conditions. Accordingly, attenuation of stress-induced SUMOylation of c-Myb through phosphorylation of Thr\(^{486}\) may maintain expression of anti-apoptotic genes, resulting in modulation of apoptotic processes in stressed cells. Indeed, in myeloid M1 cells ectopically expressing the T486A mutant (M1/cMybT486A), we detected lower levels of two anti-apoptotic proteins Bcl-2 and Bcl-xL that are encoded by genes directly regulated by c-Myb (42–44). Consequently, M1/cMybT486A cells displayed accelerated induction of programmed cell death in response to stress (Fig. 7). We speculate that a fraction of c-Myb protein that regulates genes directly involved in cellular proliferation is rapidly inactivated by conjugation of SUMO in stressed cells. Inactivation of the second fraction of c-Myb by SUMOylation can be attenuated through phosphorylation of Thr\(^{486}\) and possibly other residues and may still positively regulate a different set of genes important for survival and adaptation to stress. Whether differently modified populations of c-Myb exist in cells and regulate diverse target genes is not clear at present. However, there is experimental evidence that in a single cell, several differently modified histone molecules serve diverse functions in regulation of gene transcription (54).

In summary, we have identified phosphorylation of Thr\(^{486}\) in c-Myb as a new stress-induced post-translational modification that regulates c-Myb function. Moreover, discovery of negative cross-talk between the stress-activated p38 MAPKs and SUMOylation pathways add further complexity to post-translational regulation of the NRD of c-Myb, which affects transactivation activity and proteolytic stability of c-Myb. Strict regulation of c-Myb during hematopoiesis is critical for hematopoietic homeostasis, and deregulation of even wild-type c-Myb protein can be leukemogenic (21–23). Therefore, it is essential to identify novel post-translational modifications and signal transduction pathways that mutually cross-talk and modulate c-Myb activity because they may represent important targets for pharma-
logical intervention in diseases where deregulated activity of c-Myb plays important roles.

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