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c-Jun NH_2-terminal Kinase Promotes Apoptosis by Down-regulating the Transcriptional Co-repressor CtBP*

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Genetic knock out of the transcriptional co-repressor carboxyl-terminal-binding protein (CtBP) in mouse embryonic fibroblasts results in up-regulation of several genes involved in apoptosis. We predicted, therefore, that a propensity toward apoptosis might be regulated through changes in cellular CtBP levels. Previously, we have identified the homeodomain-interacting protein kinase 2 as such a regulator and demonstrated that HIPK2 activation causes Ser-422 phosphorylation and degradation of CtBP. In this study, we found that c-Jun NH_2-terminal kinase 1 activation triggered CtBP phosphorylation on Ser-422 and subsequent degradation, inducing p53-independent apoptosis in human lung cancer cells. JNK1 has previously been linked to UV-directed apoptosis. Expression of MKK7-JNK1 or exposure to UV irradiation reduced cellular levels of CtBP via a proteasome-mediated pathway. This effect was prevented by JNK1 deficiency. In addition, sustained activation of the JNK1 pathway by cisplatin similarly triggered CtBP degradation. These findings provide a novel target for chemotherapy in cancers lacking p53.

Carboxyl-terminal-binding protein (CtBP)[2] was originally identified by virtue of its ability to interact with the carboxyl terminus of the adenoviral protein E1A (1). Like other E1A-binding proteins, CtBP also interacts with a wide variety of cellular factors, many of which have been characterized as DNA binding transcriptional repressors (2, 3). Thus, CtBP has been categorized as a co-repressor. Genetic and biochemical studies in *Drosophila* have shown that CtBP is required for the functions of several developmentally important transcription factors, including snail, kruppel, knirps, and Tramtrack69 (4–6). In mammals, CtBP has two isoforms, CtBP1 and CtBP2, which have been shown to participate in multiple developmental pathways as well (2). A human disease, holoprosencephaly, results from a mutation in the CtBP-interacting domain of TGIF, a three amino acid loop extension (TALE) homeodomain protein (7). The embryonic lethality of CtBP1 and two genetic knock-outs in mice supports the idea that these factors are essential for mammalian development (8). Additionally, Evi-1, an oncoprotein responsible for acute myelogenous leukemia, requires interaction with CtBP to mediate transformation (9, 10).

Initially, CtBP was thought to negatively modulate the oncogenic transformation activity of the E1A protein (1, 11). Later, Grooteclaes and Frisch (12) demonstrated that this occurs by sequestering CtBP from its cellular targets. Genes regulated by CtBP have been identified serendipitously, for the most part, by identifying certain CtBP binding transcription factor binding sites in gene promoters. In this manner, for example, Grooteclaes and Frisch (12) determined that the cell adhesion gene E-cadherin was regulated by CtBP through the transcriptional repressor ZEB. Subsequent experiments showed that reducing cellular CtBP levels via small interference RNA up-regulates E-cadherin transcription (13). A more comprehensive analysis of possible CtBP targets was achieved using microarray assays of mouse embryonic fibroblasts (MEFs) derived from animals engineered to contain mutations in the two mammalian CtBP isoforms (14). These studies supported the idea that CtBP is critically involved in gene regulation in mammalian systems. Of note, a group of genes up-regulated in the knock-out MEFs have been linked to apoptosis, including PERP (p53-effector related to pmp-22), p21, Noxa, and Bax. Interestingly, despite the fact that these genes are known p53 targets, CtBP and p53 appear to act through distinct mechanisms. Similar to cells in which p53 has been activated, the CtBP-null MEFs had an increased sensitivity to pro-apoptotic stimuli (14). Thus, CtBP can be considered to be an anti-apoptotic factor. Disruption of the two CtBP isoforms in mice causes lethality at day 8 of development due to a variety of defects (8).

Relatively little is known about the regulation of transcriptional co-activators and co-repressors by intracellular signaling pathways. As opposed to modifications of specific DNA binding factors, phosphorylation of co-regulators has the potential to influence a large number of transcriptional programs. Such pathways are just beginning to be characterized. The understanding of CtBP regulation, however, is rudimentary. Kagey *et al.* (15) reported that CtBP was sumoylated on Lys-428 via the actions of PC2, a polycomb group protein, and this modification could contribute to the regulation of CtBP localization. The fact that CtBP is well known to be a phosphoprotein points to another possible mode of regulation. Our studies demonstrated that the nuclear serine/threonine kinase termed homeodomain-interacting protein kinase-2 (HIPK2) phosphorylates

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[2] The abbreviations used are: CtBP, carboxyl-terminal-binding protein; MEF, mouse embryonic fibroblast; HIPK2, homeodomain-interacting protein kinase-2; JNK1, c-Jun NH_2-terminal kinase 1; TNFα, tumor necrosis factor α.

CtBP at Ser-422 (16). HIPK2 had previously been shown to mediate the phosphorylation of p53 in response to UV irradiation, thereby activating p53 function and promoting apoptosis (17, 18). Our studies showed that HIPK2 also promotes apoptosis through its effects on CtBP. Phosphorylation by HIPK2 targets CtBP to a proteasomal degradation pathway. The resultant decrease in CtBP levels, like the HIPK2 activation of p53, sensitizes cells to apoptosis. UV irradiation also appears to activate the CtBP degradation pathway and similarly promotes apoptosis in cells (including most tumor cells) that lack p53 (16).

The HIPK2 phosphorylation site on CtBP might also be recognized by other kinases. We have raised a phospho-specific antibody to follow CtBP modification at Ser-422 in response to UV irradiation (19) and other stimuli. This reagent allowed us to screen additional signaling pathways that stimulate Ser-422 phosphorylation and regulate CtBP stability. The mitogen-activated protein kinase family member c-Jun NH_2-terminal kinase 1 (JNK1) plays important roles in triggering apoptosis in response to cellular stresses such as UV irradiation and cytokines (20–23). In this study, we found that JNK1 can phosphorylate Ser-422 of CtBP and triggers CtBP degradation. Both UV irradiation and cisplatin induced sustained JNK activation and decreased CtBP levels. This pathway promotes p53-independent apoptosis in tumor cells, suggesting a novel possibility for cancer therapy by targeting JNK1-mediated CtBP degradation.

EXPERIMENTAL PROCEDURES

Plasmids—pcDNA3-MKK7-JNK1 was from R. Davis (University of Massachusetts, Worcester, MA). FLAG-CtBP and PET24bCtBP were described previously (16). The CtBP S422A mutation was created by QuikChange™ mutagenesis.

Proteins—Bacterially purified JNK1 and p38 were activated by their specific upstream kinases (immobilized for easy separation). His-CtBP (both wild type and S422A mutant) was expressed in strain BL21(DE3) and purified by nickel-nitrilotriacetic acid affinity chromatography (Qiagen).

Cell Culture and Transfection—Cos 7, H1299, wild-type, and JNK1-null MEFs were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and antibiotics. The spectral characterization of the UV-B source and the mode of the irradiation of cells with UV-B have been described previously (24). Cisplatin, TNFα, and proteasome inhibitor MG-132 were from Sigma, R&D Systems, and Calbiochem, respectively. Cells were transfected by Lipofectamine 2000 (Invitrogen). Cell morphology and the condensed nuclei by Hoechst staining were examined by light and fluorescence microscopy, respectively.

In Vitro Kinase Assay—Recombinant CtBP and activated JNK1 and p38 were incubated in kinase buffer (20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.5 mM dithiothreitol, and 0.1 μCi of [γ-P^{32}]ATP) for 20 min at 30 °C. Reactions were stopped by adding 15 μl of 5× sample buffer, and the samples were resolved by 10% SDS-PAGE. The phosphorylated CtBP was analyzed from dried gels with a PhosphorImager (GE Healthcare).

Western Blotting—The CtBP levels were assayed by Western blotting with a CtBP antibody. The CtBP signal was quantified by densitometry at the linear range of the standard curve and expressed as arbitrary units. Results from three independent experiments were presented as mean ± S.D. The anti-phospho Ser-422 antibody was described before (19). The apoptotic response was assayed by Hoechst dye to monitor apoptosis-associated chromatin condensation or by Western blotting using antibodies to the active forms of caspase-3 (Cell Signaling). Antibodies to α-tubulin and to phospho-JNK and phospho-c-Jun were from Sigma and Cell Signaling, respectively.

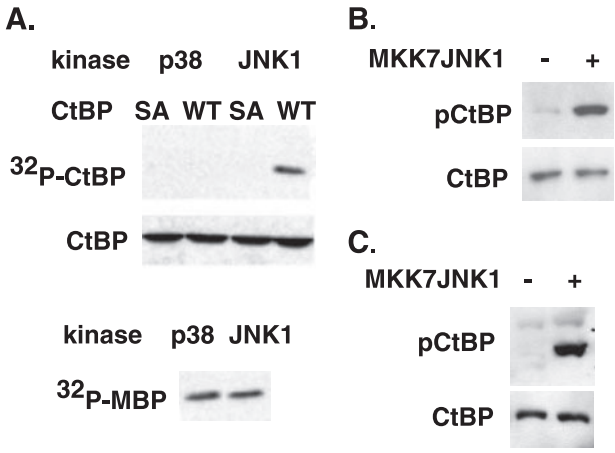

FIGURE 1. **JNK1 phosphorylates CtBP on Ser-422.** *A*, CtBP is phosphorylated by JNK1 *in vitro*. JNK1 and p38 were activated by MKK7 and MKK3 kinases and incubated with [^{32}P]ATP and recombinant CtBP, either the wild type (*WT*) or the S422A mutant form (*SA*). *B*, constitutively active JNK1 (*MKK7JNK1*) caused CtBP phosphorylation on Ser-422 in Cos 7 cells. MKK7-JNK1 and FLAG-CtBP were transfected into Cos 7 cells, and 16 h later CtBP was assayed by Western blot using a CtBP antibody (*CtBP*) or an antibody recognizing Ser-422-phosphorylated CtBP (*pCtBP*). *C*, CtBP in human non-small cell lung cancer H1299 cells was phosphorylated on Ser-422 following MKK7-JNK1 expression.

RESULTS

Previously, we have demonstrated that HIPK2 activation causes Ser-422 phosphorylation and degradation of CtBP (16). To elucidate the role of other stress-induced signal pathways in CtBP regulation, we screened stress-activated MAP kinases JNK1 and p38 using an *in vitro* phosphorylation assay. Bacterially purified JNK1 and p38 were activated by their specific upstream kinases (MKK7 and MKK3, respectively) and incubated with [^{32}P]ATP in the presence of recombinant CtBP protein. Maltose-binding protein was used as positive control for the activities of these kinases. CtBP is specifically phosphorylated by JNK1 but not p38 (Fig. 1*A*).

JNK1 prefers Ser/Thr residues surrounded by prolines. Ser-422 of CtBP is flanked by two prolines. To test whether JNK1-mediated CtBP phosphorylation is specific to Ser-422, recombinant CtBP mutant (S422A) was tested by the *in vitro* phosphorylation assay. No phosphorylation signal was detected with S422A mutant (Fig. 1*A*), suggesting Ser-422 is the specific target of JNK1.

To test whether JNK1 activation contributes to CtBP Ser-422 phosphorylation *in vivo*, we co-expressed the constitutively activated JNK1 and CtBP in Cos 7 cells. The active JNK is a fusion of JNK1 and MKK7 and causes constitutive JNK1 activation in cells without stimulation (25). Our previous study showed that Ser-422 phosphorylation is a transient event preceding CtBP clearance (19). Therefore, we assayed CtBP phosphorylation on Ser-422 16 h after the transfection. MKK7-JNK1 expression caused robust Ser-422 phosphorylation on

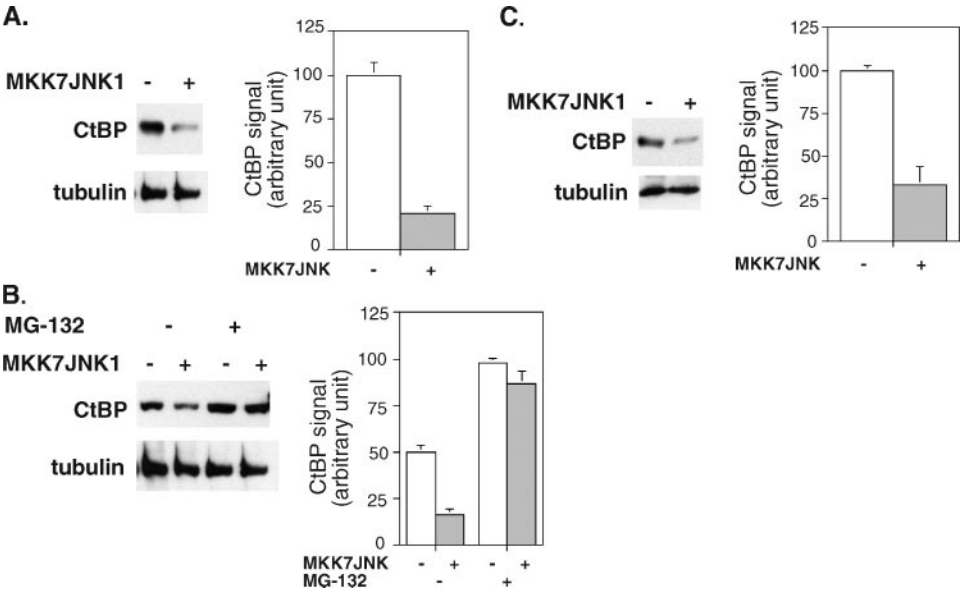

FIGURE 2. **JNK1 activation directs CtBP toward proteasomal degradation.** *A*, CtBP levels in Cos 7 cells expressing constitutively active JNK1 (*MKK7JNK1*) were assayed by Western blot using a CtBP antibody (*CtBP*). Tubulin served as a loading control. CtBP signal was quantified from three independent experiments and expressed as arbitrary units in the *bar graph*. *B*, Cos 7 cells expressing MKK7-JNK1 were treated with the proteasome inhibitor MG-132 at 2.5 μM (+) or the carrier Me_2SO (−). The level of CtBP was assayed by Western blot using an anti-CtBP antibody. *C*, MKK7-JNK1 decreased endogenous CtBP in H1299 lung cancer cells. MKK7-JNK1 was transfected into H1299 cells, and 24 h later CtBP was assayed by Western blot using a CtBP antibody (*CtBP*).

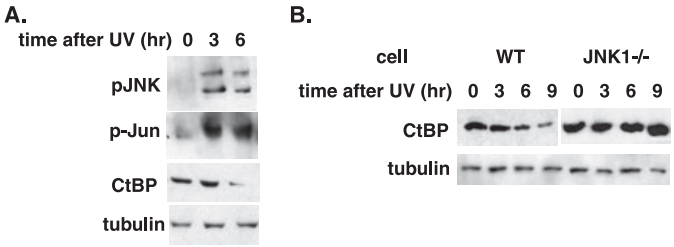

FIGURE 3. **JNK1 is responsible for UV-triggered CtBP degradation.** *A*, UV irradiation induced sustained JNK activation and decreased levels of CtBP. H1299 cells were irradiated by UV-B (2000 J/m^2) and assayed for the phosphorylation of JNK (*pJNK*) and c-Jun (*p-Jun*) by Western blotting at 3- and 6-h post-irradiation. CtBP levels were also measured to correlate with JNK activation. *B*, JNK1 knock out prevented the CtBP degradation induced by UV irradiation. Wild-type (*WT*) or JNK1-null (*JNK1*$^{-/-}$) MEFs were exposed to UV-B. CtBP levels were assayed at different times after UV irradiation using an anti-CtBP antibody. Cellular levels of α-tubulin remained constant.

CtBP in Cos 7 cells (Fig. 1*B*). Furthermore, the expression of the constitutively active JNK1 also strongly stimulated Ser-422 phosphorylation on CtBP in human lung cancer cells lacking p53 (Fig. 1*C*), suggesting a potential link between cellular stress signals and CtBP regulation in human cancer cells.

Ser-422 phosphorylation of CtBP has been demonstrated to trigger the proteasomal degradation (19); thus, we asked whether activation of the JNK1 pathway induces CtBP degradation. MKK7-JNK1 expression in Cos 7 cells decreased FLAG-CtBP levels (Fig. 2*A*). Next, we asked whether the decrease in CtBP levels can be inhibited by proteasomal blockage. MKK7-JNK1-transfected cells were treated overnight with 2.5 μM MG-132, a specific proteasome inhibitor, and their CtBP levels were assayed by Western blotting (Fig. 2*B*). The basal CtBP level was higher in the presence of MG-132, consistent with our previous finding that CtBP is actively turned over even at non-stimulated conditions (19). The MG-132 treatment largely attenuated the CtBP decrease induced by MKK7-JNK1 (Fig. 2*B*), suggesting that JNK1 activation triggers CtBP degradation through the proteasomal pathway. The expression of the active JNK1 in human lung cancer H1299 cells also decreased endogenous levels of CtBP (Fig. 2*C*). The evidence from both Cos 7 cell and H1299 cells suggests that JNK1 activation triggers CtBP degradation.

JNK is a stress-induced kinase responding to stimuli, including UV irradiation and chemotherapeutic drugs. We monitored JNK activation over time in H1299 cells and found that UV-B (2000 J/m^2) caused sustained JNK activation for up to 6 h (Fig. 3*A*). UV irradiation concomitantly decreased CtBP levels. To test whether the JNK1 pathway is involved in the UV-induced CtBP response, we made use of the JNK1-null MEFs immortalized with human papilloma viral E6 and E7 proteins (26). CtBP level decreased following UV irradiation in the wild-type MEFs, whereas JNK1 knock out prevented UV-triggered CtBP degradation (Fig. 3*B*), suggesting that JNK1 is an important mediator for CtBP degradation in response to UV irradiation.

In addition to UV irradiation, JNK has been shown to be activated by either TNFα or chemotherapeutic drugs such as cisplatin (22). To test whether CtBP is responsive to these stimuli, H1299 cells were stimulated with 10 ng/ml TNFα or 1 μg/ml cisplatin. TNFα caused a transient activation of the JNK signal pathway, whereas a prolonged activation of JNK was observed after cisplatin treatment (up to 6 h) (Fig. 4*A*). CtBP degradation was only observed in cells with sustained JNK activation by cisplatin but not transient JNK activation by TNFα. This effect was partially blocked by JNK1 knock out (Fig. 4*B*), suggesting that sustained JNK activation is required for CtBP degradation.

Previously, we have shown that CtBP degradation triggers apoptosis (16). We asked whether JNK1 activation in tumor cells would lead to cell death as well. H1299 cells were transfected with MKK7-JNK1, and 2 days later the cells were stained with Hoechst dye to monitor apoptosis-associated chromatin condensation (Fig. 5*A*). MKK7-JNK1 increased apoptosis in p53-null H1299 cells. One hundred cells from three random fields were counted. The numbers of condensed nuclei are 6.3 ± 1.0 for the control *versus* 39.3 ± 4.0 for the MKK7-JNK1-transfected cells.

To further evaluate the involvement of CtBP degradation in JNK1-induced apoptosis, we introduced either the wild-type CtBP or the non-phosphorylatable S422A mutant of CtBP to H1299 cells and checked whether it would prevent the apoptosis triggered by JNK1 activation. MKK7-JNK1-

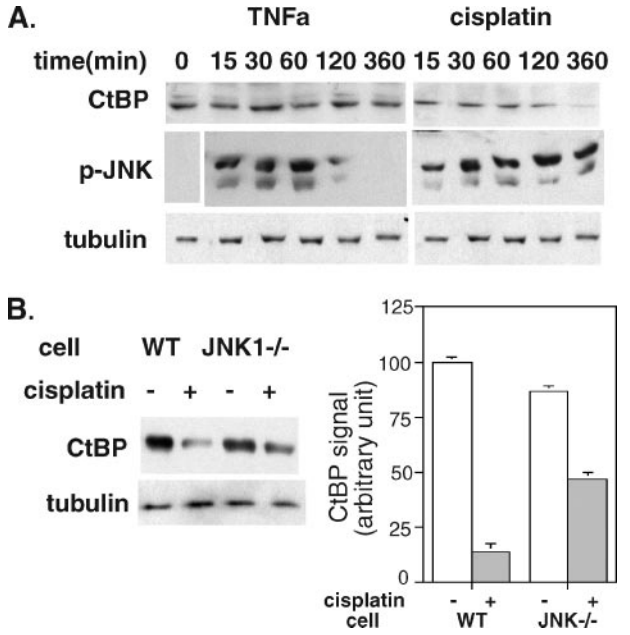

FIGURE 4. **Cisplatin induces CtBP clearance.** *A*, H1299 cells were stimulated with 10 ng/ml TNFα or 1 μg/ml cisplatin. JNK phosphorylation (*pJNK*) and CtBP levels were assayed over time. *B*, JNK1 knock out attenuated the CtBP degradation induced by cisplatin. Wild-type (*WT*) or JNK1-null (*JNK1*$^{-/-}$) MEFs were treated with 1 μg/ml cisplatin. CtBP levels were assayed 6 h later using an anti-CtBP antibody.

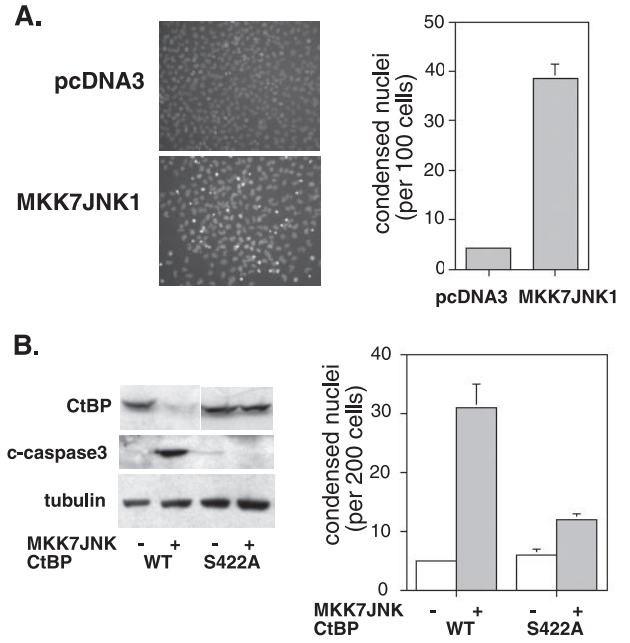

FIGURE 5. **CtBP decrease by JNK1 activation triggers apoptosis of human tumor cells.** *A*, MKK7-JNK1 induced apoptosis in p53-null H1299 cells. H1299 cells were transfected with MKK7-JNK1, and 2 days later the cells were stained with Hoechst dye to monitor apoptosis-associated chromatin condensation. *Left panel* shows the representative image of the Hoechst staining of H1299 cells expressing MKK7-JNK1 (*MKK7JNK1*) compared with the vector control (*pcDNA3*). One hundred cells from three random fields were counted, and the numbers of condensed nuclei were expressed as mean $\pm$ S.D. *B*, the non-phosphorylatable CtBP mutant attenuated the apoptotic response of H1299 cells transfected with MKK7-JNK1. Wild type or S422A mutant (*S422A*) of CtBP were co-transfected with (+) or without (−) MKK7-JNK1 (*MKK7JNK1*) into H1299 cells. 24 h later, cellular apoptosis was quantified by the appearance of condensed nuclei (*right panel*). CtBP content and the apoptotic marker, cleaved caspase-3 (*c-caspase3*), were assayed by Western blotting (*left panel*).

induced apoptosis of H1299 cells was measured by either the cleavage of caspase 3 (Fig. 5*B*, *left panel*) or the appearance of the condensed nuclei (Fig. 5*B*, *right panel*). As expected, the non-phosphorylatable CtBP mutant is more resistant to degradation induced by MKK7-JNK1 (Fig. 5*B*, *left panel*). Co-transfecting the H1299 cells with S422A CtBP mutant significantly rescued apoptosis triggered by JNK1 activation (Fig. 5*B*), highlighting the apoptotic function of the JNK1-CtBP pathway in human cancer cells lacking p53.

DISCUSSION

Multiple pro- and anti-apoptotic molecules act in concert to regulate commitment to apoptosis in cells; the balance between survival and death can be tipped by the effects of single molecules such as the stress-induced p53 protein. It has been proposed that agents targeted toward p53 may be useful therapeutically for cancer. While half of the human cancers have lost the functional p53 apoptotic pathway, the search for p53-independent tumor cell death has attracted major efforts in the cancer research field. Isolation of the cellular binding partners of the viral transforming oncoprotein E1A has identified CtBP as a key regulator that coordinately regulates the expression of multiple apoptosis control genes independent of p53 (27). The finding that CtBP levels can be down-regulated by JNK1 could have implications for many transcriptional pathways that lead to cell death.

Despite the clear indications that CtBP is critical for embryonic development and cellular transformation, little is known about CtBP regulation. Kagey *et al.* (15) recently reported that CtBP was sumoylated on lysine 428 via the actions of PC2, a polycomb group protein. Sumoylation is believed to be important for targeting transcription factors to specific subnuclear domains, and it is possible that this modification could contribute to the regulation of CtBP function.

The fact that CtBP is a phosphoprotein points to another possible mode of regulation. Our previous studies indicate that the nuclear serine/threonine kinase HIPK2 phosphorylates CtBP at Ser-422 and directs CtBP toward degradation, inducing cell death through a p53-independent mechanism (16, 19). Here, we present evidence that activation of JNK1 triggers a similar mode of CtBP regulation. JNK1 is a well known stress-induced kinase that participates in the apoptotic response. Tumor cells are frequently deficient in functional p53 but can still undergo apoptosis in response to chemotherapeutic drugs. Our results suggest that CtBP depletion mediated through JNK1 could contribute to apoptosis in this setting.

JNK1 plays important roles in triggering apoptosis in response to cellular stresses such as UV irradiation and cytokines. JNK1 is activated through phosphorylation by MKK4 and MKK7, which convey upstream stress signals. Simultaneous disruption of the *Mkk4* and *Mkk7* genes in mice is required to block JNK activation caused by exposure of cells to environmental stress (28). Although it has been well established that JNK activation is involved in stress-triggered apoptosis, the mechanism by which JNK activation triggers apoptosis remains largely unknown. Our data demonstrate that JNK1 activation directs the anti-apoptotic co-

repressor CtBP to clearance, thus contributing to cell death in tumor cells.

The loss of JNK expression results in substantial increases in the number and size of tumor nodules *in vivo* (29). Complementation assays demonstrated that this phenotype is caused by JNK deficiency. Therefore, JNK may suppress tumor development by contributing to the apoptotic elimination of transformed cells (29). This conclusion is consistent with the presence in human tumors of loss-of-function mutations in the JNK pathway (30). Mkk4 is mutated in human pancreatic, lung, breast, colorectal, and prostate cancer (31) (32–34). Loss-of-function mutations in Mkk4 markedly reduce JNK activation (28, 35–37) and correlate with aggressive tumor development and metastasis (31, 34, 38–40). It will be interesting to monitor CtBP levels in human tumors associated with loss-of-function mutations in the JNK pathway.

The clearance of CtBP induced by MKK7-JNK1 is blocked by the proteasomal inhibitor MG-132, suggesting that JNK1 directs the co-repressor CtBP to the proteasome machinery. We demonstrated that this process depends upon JNK1 phosphorylation of CtBP at Ser-422. This residue is flanked by prolines. Ser-422 phosphorylation of CtBP seems to mark CtBP for proteasomal degradation. Both JNK1 and HIPK2 activation induced Ser-422 phosphorylation and CtBP degradation. Previously, HIPK2 has been shown to be activated by UV irradiation (17, 18) and thus lead to CtBP regulation (16). Interestingly, JNK1 knock out inhibited UV-triggered CtBP degradation, pointing to the potential link between these two kinases. In fact, HIPK2 has been demonstrated to activate the JNK pathway in hepatoma cells (41). Further study is needed to address the possible cross-talk between HIPK2 and JNK1 in CtBP regulation and define the molecular nature of their functional connection. Whether these kinases directly phosphorylate Ser-422 *in vivo* and whether phosphorylation at Ser-422 affects other modifications of CtBP remains unknown.

The ability of JNK1 to regulate CtBP levels is consistent with the finding that CtBP levels are reduced after UV exposure or cisplatin treatment. Involvement of JNK1 in these responses was supported by studies using the JNK1-null cells. Our study shows that CtBP degradation is triggered by sustained JNK activation by either UV irradiation or cisplatin, whereas transient JNK activation by TNFα failed to decrease CtBP levels. This is interesting given that tumor cell derivatives that lack sustained JNK activation are resistant to chemotherapeutic drugs (42).

Relatively little is known about the regulation of transcriptional co-activators and co-repressors by intracellular signaling pathways. As opposed to modifications of specific DNA binding factors, phosphorylation of co-regulators has the potential to influence a large number of transcriptional programs. Our study demonstrated that Ser-422 on CtBP can be recognized by the stress-induced kinases. Conceivably, activation of one of these signaling pathways could be utilized therapeutically to down-regulate CtBP and block the ability of factors such as Evi-1 to mediate cell transformation. Alternatively, because E-cadherin and other cell adhesion genes are up-regulated by CtBP ablation, it is possible that manipulation of CtBP levels could be used to decrease the propensity of certain tumors to metastasize. Further characterization of the signal pathways of CtBP degradation and the downstream targets of CtBP is needed to enhance our understanding of transcription regulation in response to cellular stress. It may also provide therapeutic strategies and targets in cancer therapy, particularly in treating cancer with mutant p53.

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