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SHORT REPORT

Autophagic feedback-mediated degradation of IKKα requires CHK1- and p300/CBP-dependent acetylation of p53

Xiuduan Xu[1,2], Chongchong Zhang[1,3], Huan Xu[1,2], Lin Wu[1], Meiru Hu[1] and Lun Song[1,2,*]

ABSTRACT

In our previous report, we demonstrated that one of the catalytic subunits of the IκB kinase (IKK) complex, IKKα (encoded by *CHUK*), performs an NF-κB-independent cytoprotective role in human hepatoma cells under the treatment of the anti-tumor therapeutic reagent arsenite. IKKα triggers its own degradation, as a feedback loop, by activating p53-dependent autophagy, and therefore contributes substantially to hepatoma cell apoptosis induced by arsenite. Interestingly, IKKα is unable to interact with p53 directly but plays a critical role in mediating p53 phosphorylation (at Ser15) by promoting CHK1 activation and CHK1–p53 complex formation. In the current study, we found that p53 acetylation (at Lys373 and/or Lys382) was also critical for the induction of autophagy and the autophagic degradation of IKKα during the arsenite response. Furthermore, IKKα was involved in p53 acetylation through interaction with the acetyltransferases for p53, p300 (also known as EP300) and CBP (also known as CREBBP) (collectively p300/CBP), inducing CHK1-dependent p300/CBP activation and promoting p300–p53 or CBP–p53 complex formation. Therefore, taken together with the previous report, we conclude that both IKKα- and CHK1-dependent p53 phosphorylation and acetylation contribute to mediating selective autophagy feedback degradation of IKKα during the arsenite-induced proapoptotic responses.

KEY WORDS: IKKα, p53 acetylation, p300/CBP, Apoptosis, Autophagy

INTRODUCTION

p53 (also known as TP53) is a transcription factor that functions as a critical regulator in cell fate determination under multiple stress conditions. Normally, p53 is maintained at low levels by constitutive degradation via binding to its E3 ubiquitin ligases (such as MDM2, Pirh2, COP1 or ARF-BP1) (Meek, 2015; Hafner et al., 2019; Sullivan et al., 2018). Various stress stimuli initiate signaling pathways to increase the protein stability and transcriptional activity of p53, which subsequently induces the expression of a large number of p53 downstream target genes to exert multiple biological effects, including growth arrest, DNA repair, senescence, apoptosis, autophagy, metabolism, angiogenesis and cell migration, depending on the cell types and the nature of the stimuli (Meek, 2015; Hafner et al., 2019; Sullivan et al., 2018).

[1]Institute of Military Cognitive and Brain Sciences, 27 Taiping Road, Beijing 100850, P. R. China. [2]Department of Basic Medical Sciences, Anhui Medical University, 81 Meishan Road, Hefei 230032, P. R. China. [3]Henan University Joint National Laboratory for Antibody Drug Engineering, Kaifeng 475004, P. R. China.

*Author for correspondence (lunsong0752@163.com)

X.X., 0000-0002-7161-9906; C.Z., 0000-0002-8117-252X; H.X., 0000-0001-9272-840X; L.W., 0000-0001-9358-0814; M.H., 0000-0003-4039-0173; L.S., 0000-0001-7314-2649

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Posttranslational modifications (PTMs), including ubiquitylation, phosphorylation, acetylation, sumoylation, methylation and neddylation, perform critical roles in regulating p53 protein stability and/or transcriptional activity under physiological and stress conditions (Sullivan et al., 2018; Niazi et al., 2018; Yogosawa and Yoshida, 2018; Reed and Quelle, 2014). Acetylation of p53 has been found to occur at several lysine residues within the C-terminal regulatory domain, the DNA-binding domain and the connecting region between these two domains (K120, K164, K305, K320, K370, K372, K373, K381 and K382) under various stress stimuli, which is carried out by histone acetyltransferases (HATs), such as p300 (also known as EP300) and CREB-binding protein (CBP; also known as CREBBP) (collectively known as p300/CBP), p300/CBP-associated factor (PCAF), Tip60, MOZ and MOF (Meek, 2015; Hafner et al., 2019; Sullivan et al., 2018; Reed and Quelle, 2014). In most cases, p300/CBP-mediated p53 acetylation at the lysine residues within the C-terminal domain of p53 is thought to divert this protein from ubiquitylation and degradation, therefore stabilizing p53 or promoting its transcriptional activity by increasing the DNA-binding ability of p53 or facilitating the recruitment of its coactivators to the promoter regions of p53-responsive genes (Li et al., 2002; Reed and Quelle, 2014; Luo et al., 2004). However, there is also a controversial report demonstrating that p300-dependent p53 acetylation at K381 and/or K382 (K381/382) in neuronal cells specifically inhibits p53 binding to the *PUMA* promoter, preventing PUMA-dependent DNA damage and cell death (Brochier et al., 2013). The complexity of the outcomes of p53 acetylation suggests that more research needs to be performed on the regulatory mechanisms involved in p53 acetylation. To date, several reports, including our own, have identified regulators involved in enhancing or suppressing p53 acetylation and transactivity, most of which exert their function by targeting p300/CBP (such as p85α, WTX, Bat3, SOX4, ING2, ING4 and ING5, HIPK2, ASAP, RPS26, HBZ and Skp2) (Reed and Quelle, 2014; Pan et al., 2009; Basbous et al., 2012; Cui et al., 2014; Song et al., 2011; Kim et al., 2012; Grishina et al., 2012; Aikawa et al., 2006; Kitagawa et al., 2008; Gronroos et al., 2004; Graczyk et al., 2013; Wright et al., 2016; Schuldner et al., 2019).

IKKα (encoded by *CHUK*) is one of the catalytic subunits of the IκB kinase (IKK) complex, and it shares structural similarity with another catalytic subunit, IKKβ (encoded by *IKBKB*) but triggers NF-κB activation by different mechanisms (Chariot, 2009; Huang and Hung, 2013). Furthermore, IKKα also possesses some unique functions that are independent of NF-κB activity and are mediated by NF-κB-unrelated substrates, such as Aurora A, Maspin, 14-3-3σ, SMRT, p53, SRC3, c-Fos, p85α, mTOR and ATG16L1 (Huang and Hung, 2013; Dong et al., 2012; Song et al., 2010; Tan et al., 2020). Therefore, it is believed that IKKα can act as a multifunctional signaling protein with roles going far beyond its well-known action in NF-κB pathway regulation.

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In our previous reports, we demonstrated an NF-κB-independent role for IKKα in inducing cytoprotective autophagy by triggering CHK1-dependent p53 phosphorylation, which mediates the subsequent degradation of IKKα (Tan et al., 2020). In the current study, we further revealed the role of IKKα in coactivating CHK1- and p300/CBP-dependent p53 acetylation, which is also involved in autophagy induction and the feedback control of IKKα expression, and, thereby, mediating arsenite cytotoxicity.

RESULTS AND DISCUSSION

Arsenite induces p300/CBP-dependent p53 acetylation in HepG2 cells

In a previous report, we demonstrated that IKKα triggers its own feedback degradation by activating p53-dependent autophagy in HepG2 hepatoma cells under arsenite exposure. Interestingly, although IKKα is unable to interact with p53 directly, it plays a critical role in mediating p53 phosphorylation (at S15) by promoting CHK1 activation and CHK1–p53 complex formation (Tan et al., 2020). Since acetylation at lysine residues (such as K373 and/or K382; K373/382) also contributes substantially to p53 transactivation under stress conditions (Hafner et al., 2019), we wondered whether p53 acetylation was also involved in autophagic feedback degradation of IKKα during the arsenite response. As shown in Fig. 1A, the activation and degradation of IKKα under arsenite exposure, was accompanied by a time-dependent acetylation of p53 at K373/382. Under the same conditions, strong activation of p300 and CBP was also detected, as evidenced by the induced acetylation of these two enzymes at K1535 and K1499 (K1535/1499). When p300 and CBP siRNAs were

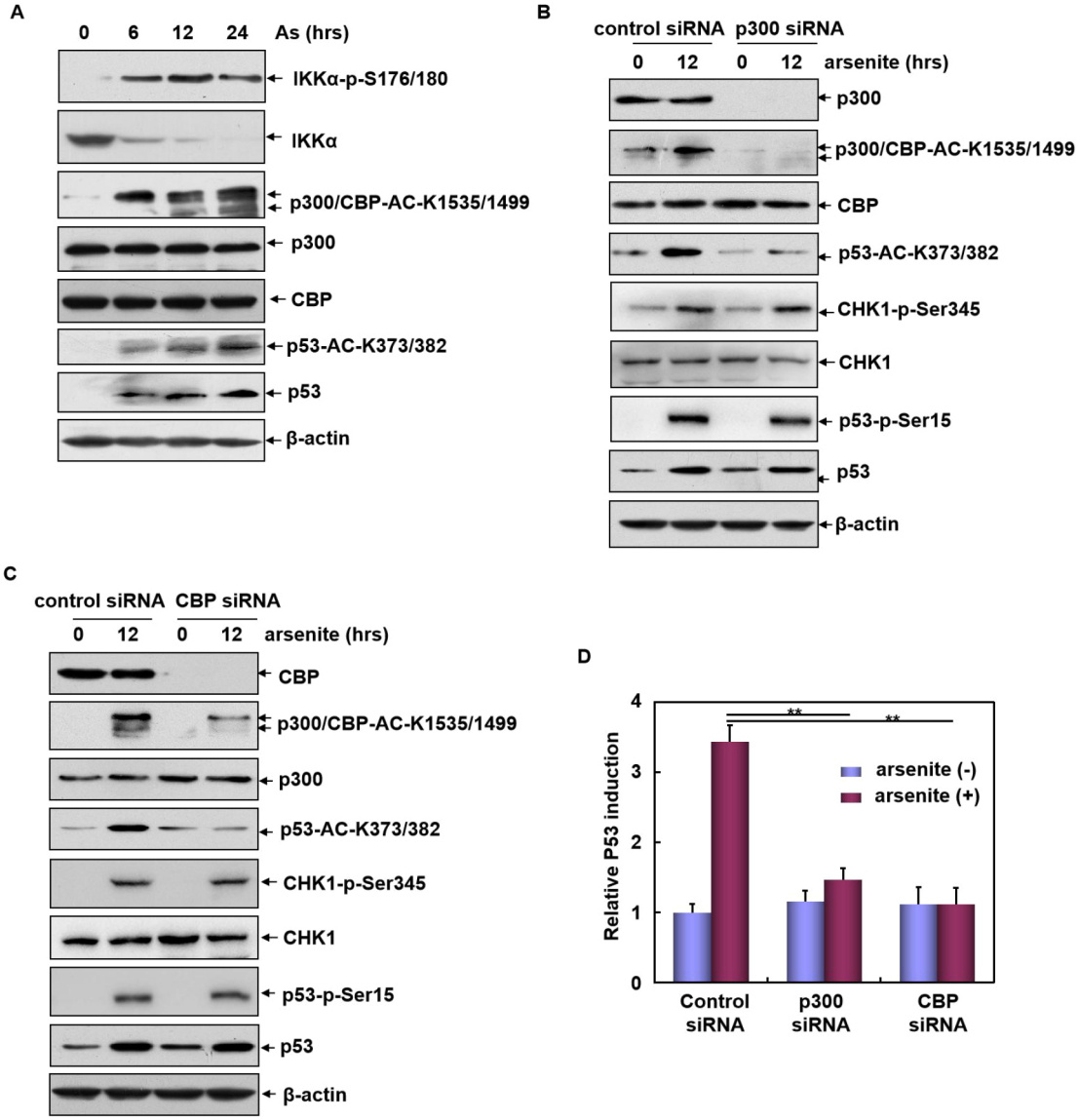

Fig. 1. Arsenite induces p300/CBP-dependent p53 acetylation in HepG2 cells. (A) HepG2 cells were treated with arsenite (20 μM) for the indicated time periods, and then the expression/activation of IKKα and the expression and acetylation (AC) of p53, p300 and CBP were examined. (B,C) HepG2 cells were transfected with p300 siRNA, CBP siRNA or their control siRNAs followed by exposure to arsenite (20 μM) at 36 h after transfection. The expression and acetylation of p53, p300 and CBP and the phosphorylation of p53 and CHK1 were examined 12 h after arsenite exposure. (D) HepG2 cells were transfected with a p53-dependent luciferase reporter, and stable transfectants were established. Then, p300 siRNA, CBP siRNA or their control siRNAs were introduced into the stable transfectants followed by exposure to arsenite (20 μM), and the induction of p53-dependent luciferase activity was examined. Results are mean±s.d. (n=3) **P<0.01 (factorial design, ANOVA).

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separately transfected into HepG2 cells followed by arsenite exposure, we observed a significant suppression of p53 acetylation with the impairment of either p300 or CBP expression, while the accumulation of p53 and CHK1-dependent p53 phosphorylation did not change obviously under the same conditions (Fig. 1B,C). These results indicate that p300/CBP activation is essential for inducing p53 acetylation at K373/382 in response to arsenite.

Next, we examined whether p53 transactivation was also regulated by p300/CBP-dependent p53 acetylation. To this end, p300 and CBP siRNAs were separately introduced into HepG2 cells stably transfected with a p53 luciferase reporter (Tan et al., 2020). Here, we found that upregulation of p53-dependent luciferase activity was also significantly inhibited by knocking down p300 or CBP expression (Fig. 1D). These data suggest that p300/CBP-dependent p53 acetylation contributes to p53 transactivation during the arsenite response.

p300/CBP mediates the induction of DRAM1-dependent autophagy and IKKα degradation during the arsenite response

After uncovering the role of p300/CBP in regulating p53 acetylation and transactivation, we next investigated whether p300/CBP was also responsible for DRAM1-dependent autophagy induction and IKKα degradation under arsenite exposure. When using the commercial Cyto-ID Autophagy Detection Reagent, we observed an increase in the green Cyto-ID fluorescence signals, specifically indicating autophagy induction after arsenite exposure. However, these signals dramatically decreased after knocking down p300 or CBP expression (Fig. 2A). These data suggest the potential contribution of p300 and CBP to the induction of autophagy during the arsenite responses.

We also observed the inhibition of autophagic hallmark gene expression [DRAM1, LC3B (also known as MAP1LC3B) and Beclin1 accumulation; and p62 (also known as SQSTM1) degradation] upon knocking down p300 or CBP expression, which was accompanied by a rescue of IKKα degradation in arsenite-treated HepG2 cells. However, phosphorylation and activation of IKKα did not change obviously under the same conditions (Fig. 2B,C). Collectively, these data indicate that p300 and CBP are not involved in the initial step of IKKα activation, but are required for inducing p53- and DRAM1-dependent autophagy, and IKKα degradation in response to arsenite stimulation.

IKKα plays a critical role in inducing p300/CBP-dependent p53 acetylation after arsenite exposure

In a previous report, we demonstrated that IKKα activation is essential for p53 phosphorylation during the arsenite-induced response (Tan et al., 2020). Therefore, here, we examined whether IKKα is also involved in p300/CBP-dependent p53 acetylation. Here, we found that knockdown of IKKα expression resulted in the total block of p300, CBP and p53 acetylation in arsenite-treated HepG2 cells. However, p53 accumulation levels remained unchanged with or without IKKα expression (Fig. 3A). In contrast, overexpression of IKKα in HepG2 cells enhanced p300, CBP and p53 acetylation induced by arsenite. Most importantly, when the IKKα kinase mutant, IKKα-KM, was transfected into HepG2 cells, the responses of p53 acetylation and p300/CBP activation were totally lost under arsenite exposure conditions (Fig. 3B). Taken together, these data indicate that IKKα functions as an upstream activator for mediating p300/CBP-dependent p53 acetylation in response to arsenite stimulation, and this function of IKKα requires its kinase activity.

IKKα interacted with p300/CBP and determines the binding ability of p53 with p300/CBP

Next, we want to address the functional link between IKKα and p300/CBP in arsenite responses. Here, we found that p53 was induced to interact with the activated form of its acetyltransferase, p300/CBP, under arsenite stimulation (Fig. 4A), suggesting that the induced interaction of p300/CBP with p53 is a critical step for p53 acetylation under arsenite exposure. We also observed that CBP constitutively interacted with IKKα, while the p300–IKKα interaction was only detected in HepG2 cells after arsenite exposure. Most importantly, IKKα could interact with the activated form of p300/CBP under arsenite treatment (Fig. 4B). These findings indicate that IKKα performs its regulatory function

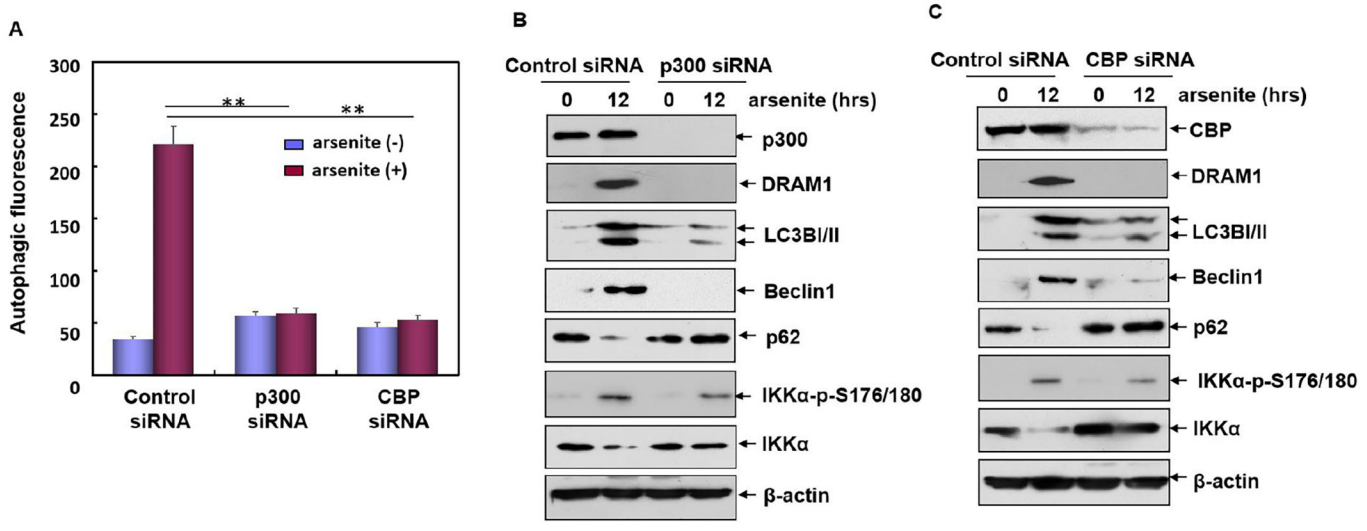

Fig. 2. p300/CBP mediated DRAM1-dependent autophagy and IKKα degradation during the arsenite response. (A) HepG2 cells were transfected as described in Fig. 1B,C. Then, the cells were stained with Cyto-ID Green Autophagy Detection Reagent and subjected to flow cytometric analysis to quantitatively measure the autophagic fluorescence intensity inside the cells. Results are mean±s.d. (n=3) $^{**}P$<0.01 (factorial design, ANOVA). (B,C) HepG2 cells were transfected as described in Fig. 1B,C. The expression and activation levels of DRAM1, IKKα, and autophagic hallmarks were examined 12 h after arsenite exposure. LC3BI and LC3BII represent the non-lipidated and lipdated forms of LCB3, respectively.

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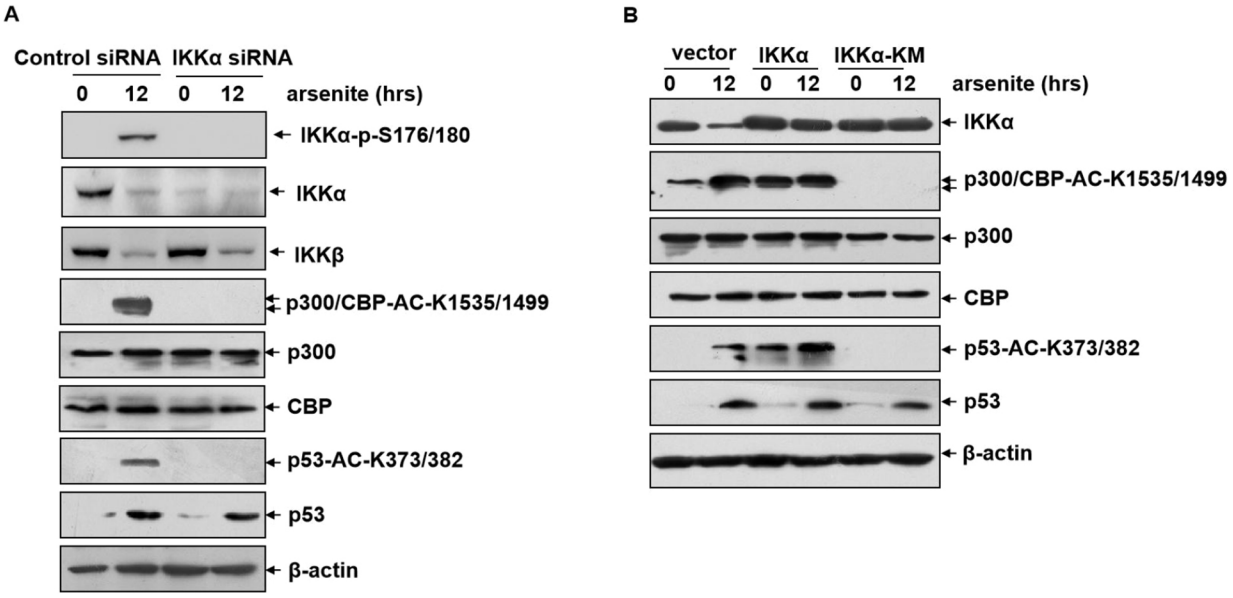

Fig. 3. IKKα plays a critical role in inducing p300/CBP-dependent p53 acetylation under arsenite exposure. (A) HepG2 cells were transfected with siRNA specifically targeting IKKα or the control siRNA, followed by treatment with arsenite (20 μM). Then, the expression and acetylation (AC) of p53, p300 and CBP were examined 12 h after arsenite exposure. (B) HepG2 cells were transfected with the expression plasmids encoding FLAG–IKKα, FLAG–IKKα-KM or a control vector, followed by treatment with arsenite (20 μM). Then, detection was performed as described in A.

in arsenite-induced p53 acetylation by interacting with and triggering the activation of p300 and CBP.

IKKα is a Ser/Thr kinase, while p300/CBP activation depends on acetylation at K1535/1499. Therefore, IKKα might not be directly involved in the acetylation of p300/CBP. Interestingly, we found that complex formation between p300/CBP and p53 was completely blocked upon the impairment of IKKα expression (Fig. 4C), indicating that IKKα determines the induced binding ability of p53 with its acetyltransferases during the arsenite responses.

CHK1 contributes to p53 acetylation by modulating p300/CBP activity under arsenite exposure

Crosstalk between phosphorylation and acetylation of p53 is critical for regulating its transcriptional activity. Since knockdown of p300/CBP expression reduced p53 acetylation but did not affect N-terminal phosphorylation of p53 (Fig. 1B,C), we next addressed whether phosphorylation of p53 at S15 affected its C-terminal acetylation. To this end, CHK1 siRNA was transfected into HepG2 cells to block the phosphorylation of p53 at S15. Under this condition, acetylation of p53 at K373/382 was also significantly reduced after arsenite exposure. However, in contrast to previous reports (Ferreon et al., 2009; Teufel et al., 2009; Lee et al., 2010; Puca et al., 2009), the induced interaction of p53 with p300/CBP did not change, while p300/CBP activation was obviously inhibited with the impairment of CHK1 expression, leading to a reduction in the amount of the activated form of p300/CBP interacting with p53 (Fig. 4D). These data indicate that crosstalk between phosphorylation and acetylation of p53 seems to occur at the upstream level for these two PTMs, which are mediated by CHK1-dependent p300/CBP activation during arsenite responses.

Taken together, we conclude that arsenite exposure induces the activation of IKKα and CHK1, which cooperatively induces p53 phosphorylation within its N-terminus, as well as p300/CBP-dependent p53 acetylation within its C-terminus. The synergistic effects of these two events lead to the activation of p53 and subsequent autophagic degradation of IKKα (Fig. 4E).

PTMs of p53 play an important role in regulating its activities. The coordinated phosphorylation and acetylation events on p53 have been explored in several previous studies. Most reports support that changes in p53 phosphorylation within the N-terminus facilitate its acetylation within the C-terminus, which is mediated by the increased recruitment of p300/CBP to p53. (Ferreon et al., 2009; Teufel et al., 2009; Lee et al., 2010; Puca et al., 2009). However, in contrast to previous reports, we did not observe any changes in the p300/CBP–p53 complex formation with or without phosphorylation at the N-terminal S15. However, the regulation of p53 acetyltransferase (p300/CBP) activities by protein kinases responsible for p53 phosphorylation (IKKα and CHK1) was readily observed after arsenite exposure. Therefore, crosstalk between the upstream signaling events, before p53 phosphorylation and acetylation, seems to be critical for the synergistic induction of p53 activation under arsenite stimulation.

p300 and/or CBP have been identified as targets for most p53 acetylation regulators. The mechanism through which these regulators are involved in p53 acetylation include (1) forming complexes with p300 and p53, and promoting p300-dependent p53 acetylation (Pan et al., 2009; Basbous et al., 2012; Cui et al., 2014); (2) interacting with p300 and then facilitating p300–p53 complex formation and p300-dependent p53 acetylation (Song et al., 2011); (3) regulating p300/CBP stability and promoting or suppressing p300/CBP-dependent p53 acetylation (Kim et al., 2012; Grishina et al., 2012); (4) directly phosphorylating p300 and stimulating its HAT activity for p53, as well as other substrates (Aikawa et al., 2006); and (5) competing with p300 for binding with p53 and disrupting the p300–p53 association (Kitagawa et al., 2008; Gronroos et al., 2004; Graczyk et al., 2013). According to the data in this study, IKKα contributes to arsenite-induced p53 acetylation by promoting the interaction of IKKα with p300/CBP and enhancing the p300/CBP HAT activities. CHK1 only modulated p300/CBP HAT activities instead of affecting the formation of complexes of p53 and its acetyltransferases. Since both IKKα and CHK1 are Ser/Thr kinases and their activities are indispensable for mediating p300/CBP activation, we speculate that

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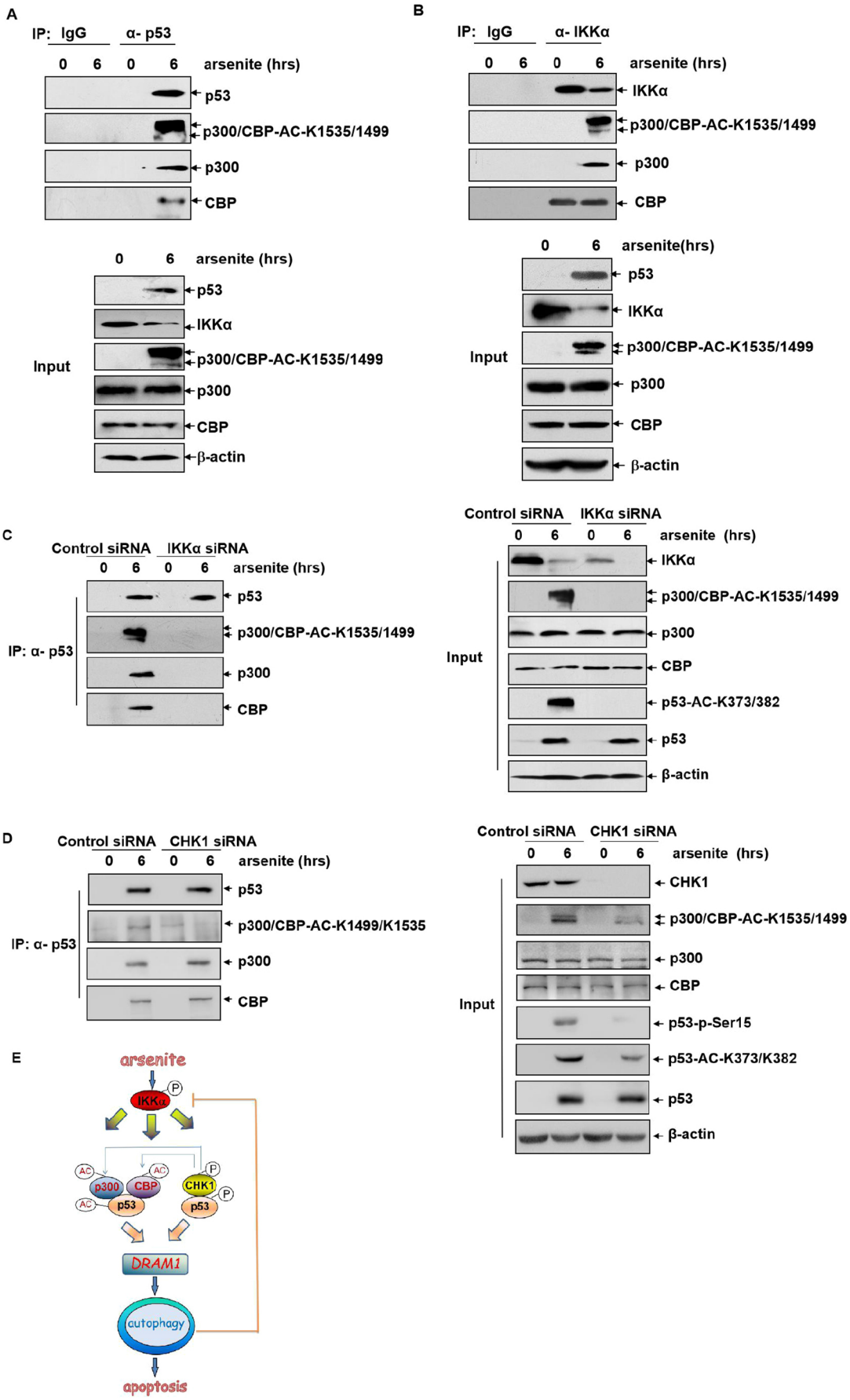

Fig. 4. See next page for legend.

Fig. 4. IKKα and CHK1 cooperatively regulate p300/CBP activity but act differently in the induced interaction between p53 and p300/CBP. (A,B) HepG2 cells were left untreated or were treated with arsenite (20 μM) for 6 h. Cell lysates were immunoprecipitated (IP) with anti-p53 antibody (A) or anti-IKKα antibody (B) and the control IgG. Then, the immunoprecipitants were probed with the antibodies as indicated. Inputs are shown underneath. AC, acetylation. (C,D) HepG2 cells were transfected with siRNA specifically targeting IKKα (C) or CHK1 (D) and their respective control siRNAs. Then the cells were subjected to arsenite (20 μM) exposure and cell lysates were immunoprecipitated with an anti-p53 antibody or a control IgG. The immunoprecipitants were probed with the antibodies as indicated. Inputs are shown to the right. (E) Working model of our results. IKKα controls both CHK1-dependent p53 phosphorylation and p300/CBP-dependent p53 acetylation pathway activation, which leads to p53/DRAM1-dependent autophagy and feedback degradation of IKKα in the proapoptotic responses induced by arsenite. CHK1 is also involved in regulating p300/CBP activities and p53 acetylation, which constitutes a novel mechanism explaining the cross-talk between p53 phosphorylation and acetylation.

IKKα and/or CHK1 might induce p300/CBP phosphorylation at unidentified sites, which subsequently promote p300/CBP acetylation at K1535/1499 and increase their HAT activities. In fact, a previous report demonstrated that CBP phosphorylation at S1382/1386 by IKKα upregulates HAT activity in response to TNFα stimulation. In addition, this effect is CBP specific, and no involvement of p300 is seen (Huang et al., 2007). In contrast, here we found that IKKα and CHK1 can regulate the activities of both p300 and CBP during arsenite-induced responses. Therefore, identification of the common target sequences through which IKKα and/or CHK1 acting on p300 and CBP is critical for further revealing the crosstalk between protein kinases and HATs involved in p53 activation.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

The p53-dependent luciferase reporter plasmids and the expression constructs FLAG–IKKα and FLAG–IKKα-KM were described in our previous reports (Tan et al., 2020). Primary antibodies against the following proteins were purchased from Cell Signaling Technology (Beverly, MA, USA): Beclin1 (1:1000; 3495), LC3B (1:1000; 3868), p62 (1:1000; 5114), acetyl-p300/CBP-K1535/1499 (1:1000; 4771), p53 (1:1000; 2524), phospho-p53-Ser15 (1:1000; 9284), acetyl-p53-K373/382 (1:1000; 2525), IKKα (1:1000; 11930), phospho-CHK1-Ser345 (1:1000; 2348), CHK1 (1:1000; 2360) and β-actin (1:1000; 4970). Primary antibodies against the following proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): DRAM1 (1:200; 98654), p300 (1:200; 584) and CBP (1:1000; 583). Anti-phospho-IKKα-S176/180 antibody (1:1000; 44-714) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). p300 siRNA and CBP siRNA were purchased from Riobo Technology (Guangzhou, China). Arsenite was purchased from Sigma (St Louis, MO, USA).

Cell culture and transfection

HepG2 cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum supplemented with antibiotic/antimycotic. No contamination was observed. Transfections were performed with Lipofectamine 2000 or LipofectAMINE™ RNAi MAX (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation and immunoblot assay

HepG2 cells were left untreated or were treated with arsenite for 6 h, and then reciprocal immunoprecipitation (IP) was performed to detect the endogenous IKKα–p300 and IKKα–CBP, IKKα–p53 or p300–p53 and CBP–p53 interaction. To determine whether IKKα and CHK1 are required for p300–p53 or CBP–p53 interaction during the arsenite response, HepG2 cells were transfected with IKKα or CHK1 siRNA or their respective control siRNAs, and then IPs were performed to detect the changes in p300–p53 or CBP–p53 interaction with or without IKKα or CHK1 expression. Cellular protein preparation and immunoblot assays were performed as previously described (Tan et al., 2020).

Luciferase reporter assay

Cells were co-transfected with p53-dependent luciferase reporter and the control *Renilla* luciferase reporter, and then stable transfectants were established. Luciferase activity was tested at 12 h after arsenite exposure using the Firefly–Renilla Dual-Luciferase Reporter Assay System (Promega). The results were presented as previously described (Tan et al., 2020).

Autophagy assay

A cellular autophagy was monitored by using western blot assay to detect the levels of the autophagic hallmark proteins (LC3B, Beclin1 and p62) or by using the Cyto-ID Autophagy Detection Kit (Enzo Life Sciences) to quantitatively measure the autophagic fluorescence intensity by flow cytometric analysis as previously described (Tan et al., 2020).

Statistics

To determine the effect of a single treatment within a group, a Student's *t*-test was used to test the significance of the data. To determine the effects of treatment and group interactions, factorial design (ANOVA) was employed to test the significance of the data. At least three independent experiments were performed. The results are presented as the mean±s.d. The level of significance was set at $P<0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: X.X., H.X.; Formal analysis: L.S.; Investigation: X.X., C.Z., H.X.; Resources: L.W., M.H.; Data curation: X.X., C.Z., H.X.; Writing - original draft: L.S.; Writing - review & editing: L.S.; Visualization: L.S.; Supervision: L.S.; Project administration: L.S.; Funding acquisition: L.S.

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